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 ARTICLE TITLE: Effect of addition of citric acid and casein phosphopeptide-amorphous calcium phosphate to a sugar-free chewing gum on enamel remineralization in situ.  
 ARTICLE AUTHOR: Cai,  
 VOLUME: 41  
 ISSUE: 5  
 MONTH:  
 YEAR: 2007  
 PAGES: 377-383  
 ISSN: 0008-6568  
 OCLC #:  
 CROSS REFERENCE ID: [TN:1850603][ODYSSEY:216.54.119.76/GZM]  
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## Effect of Addition of Citric Acid and Casein Phosphopeptide-Amorphous Calcium Phosphate to a Sugar-Free Chewing Gum on Enamel Remineralization in situ

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### Key Words

Calcium chelation · Chewing gum · Citric acid · CPP-ACP™ · Enamel · pH · Remineralization

### Abstract

Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) has been shown to remineralize enamel subsurface lesions in situ. The aim of this study was to investigate the effects of CPP-ACP in a fruit-flavoured sugar-free chewing gum containing citric acid on enamel remineralization, and acid resistance of the remineralized enamel, using an in situ remineralization model. The study utilized a double-blind, randomized, crossover design with three treatments: (i) sugar-free gum (2 pellets) containing 20 mg citric acid and 18.8 mg CPP-ACP, (ii) sugar-free gum containing 20 mg citric acid alone, (iii) sugar-free gum not containing CPP-ACP or citric acid. Ten subjects were instructed to wear removable palatal appliances, with 4 half-slab insets of human enamel containing demineralized subsurface lesions and to chew gum (2 pellets) for 20 min 4 times per day for 14 days. At the completion of each treatment the enamel half-slabs were removed and half of the remineralized lesion treated with demineralization buffer for 16 h in vitro. The enamel slabs (remineralized, acid-challenged and control) were then em-

bedded, sectioned and subjected to microradiography to determine the level of remineralization. Chewing with gum containing citric acid and CPP-ACP resulted in significantly higher remineralization ( $13.0 \pm 2.2\%$ ) than chewing with either gum containing no CPP-ACP or citric acid ( $9.4 \pm 1.2\%$ ) or gum containing citric acid alone ( $2.6 \pm 1.3\%$ ). The acid challenge of the remineralized lesions showed that the level of mineral after acid challenge was significantly greater for the lesions exposed to the gum containing CPP-ACP.

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Casein phosphopeptides (CPP) containing the sequence -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- have the ability to stabilize amorphous calcium phosphate (ACP) in metastable solution [Reynolds, 1998]. CPP-ACP nano-complexes (CASRN 691364-49-5) have been demonstrated to have anticariogenic activity in laboratory, animal and human in situ experiments [Reynolds, 1998; Reynolds et al., 1999, 2003]. This activity has been attributed to the ability of the complexes to provide calcium ions and phosphate ions at the tooth surface, decreasing the net loss of mineral from acid-challenged enamel and promoting remineralization [Shen et al., 2001; Reynolds et al., 2003; Iijima et al., 2004].

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0008-6568/07/0415-0377\$23.50/0

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Saliva stimulated by chewing sugar-free gum has an increased calcium concentration [Dawes and Dong, 1995] and has been shown to remineralize enamel subsurface lesions in situ [Shen et al., 2001]. Recently, there has been a substantial increase in the marketing of sugar-free gums and confectionery containing fruit flavours and food acids, particularly citric acid to enhance flavour. Citric acid has been shown to stimulate a higher saliva flow rate, through a gustatory effect, than stimulation via the action of mechanical chewing [Watanabe and Dawes, 1988]. Therefore sugar-free gum containing citric acid may increase salivary flow rate by gustatory, as well as mechanical stimulation. However, addition of citric acid to sugar-free gum may also have a negative impact on saliva's ability to remineralize enamel by lowering the pH of saliva. Citric acid has three pKa values (pK<sub>1</sub>, 2.89; pK<sub>2</sub>, 4.34; pK<sub>3</sub>, 5.83), with two below what is considered to be the critical pH for enamel dissolution [Pearce, 1980; Larsen and Pearce, 2003]. Therefore on release of citric acid from the gum it will donate H<sup>+</sup> ions possibly lowering saliva's pH depending on the concentration of the citric acid released and the buffering capacity of the saliva. Citrate anions also bind calcium ions [Pearce, 1980] such that as the tri-carboxylic acid is progressively dissociated by saliva, the anions produced will bind calcium with higher affinity, with the fully dissociated citrate anion exhibiting the strongest calcium binding [ $\log K(\text{CaH}_2\text{Cit}^+)$ , 1.04;  $\log K(\text{CaHCit}^0)$ , 2.03;  $\log K(\text{CaCit}^-)$ , 3.64]. The binding of salivary calcium ions by citrate will lower the calcium ion activity and therefore its remineralization potential. The objective of this in situ study was to determine if the inclusion of citric acid in a sugar-free gum would decrease the gum's ability to promote enamel remineralization. A further objective was to determine if inclusion of CPP-ACP nanocomplexes with the citric acid would restore the gum's ability to promote enamel remineralization with acid-resistant mineral.

## Materials and Methods

### Subject Recruitment

Approval for the study was obtained from The University of Melbourne Human Research Ethics Committee and the Royal Dental Hospital of Melbourne Ethics in Clinical Research Committee. Ten healthy adult subjects (7 males and 3 females) were recruited from the staff and postgraduate students (age 23–46 years) of the School of Dental Science. All subjects had at least 22 natural teeth with no current caries activity, periodontal disease or other oral pathology. Antibiotics or medications, which could have affected salivary flow rate, were not being used by any subject.

### Stimulated Salivary Flow Rates, Salivary pH, Calcium and Phosphate Measurements and Calculations of Calcium Ion Activity and Degree of Saturation with Respect to Hydroxyapatite

Subjects' saliva was collected on 3 consecutive days at 10.00 a.m. Three types of chewing gum were coded and distributed to the subjects for each session. In each session subjects were asked to chew two pieces of gum and collect stimulated saliva for 20 min into pre-weighed test tubes. Stimulated saliva from each chewing period between 0 and 2, 2 and 5, 5 and 10, 10 and 15 and 15 and 20 min was collected and volume determined by weight measurement. The pH, total and filtrate calcium concentrations and inorganic phosphate concentrations, as well as ionic calcium concentrations, of the collected saliva samples were determined immediately after collection. Less than 10% of each saliva sample was collected as a filtrate by centrifugation at 1,000 g for 15 min using a Centrifree MPS-1 Micropartition cell (Amicon Corp., Danvers, Mass., USA) equipped with a YM-1 (1,000 molecular weight exclusion limit) membrane. Calcium ion concentrations of acidified saliva and filtrate samples were measured at 422.7 nm by atomic absorption spectroscopy (Model AA240, Varian, Inc., Mulgrave, Vic., Australia) with the addition of 1% LaCl<sub>3</sub> to prevent phosphate interference [Cross et al., 2005]. Inorganic phosphate levels of saliva and filtrate samples were determined colorimetrically [Itaya and Ui, 1966]. Ionic calcium concentrations of the collected saliva samples were determined using a calcium ion-selective electrode (ISE25Ca Radiometer Analytical SAS, France) according to the manufacturer's instructions.

Calcium ion activity and the ion activity product for hydroxyapatite were determined from the filtrate calcium and phosphate concentrations and the sample pH using an iterative computational procedure that calculates the ion activity fugacities using the expanded Debye-Hückel equation. The procedure, similar to that used in earlier works [Smales, 1972; Marsh, 1989], takes into account ion pairs CaHPO<sub>4</sub><sup>0</sup>, CaH<sub>2</sub>PO<sub>4</sub><sup>+</sup>, CaPO<sub>4</sub><sup>-</sup>, and CaOH<sup>+</sup>, the dissociation of H<sub>3</sub>PO<sub>4</sub> and H<sub>2</sub>O, and the ionic strength. In contrast to the method of Marsh [1989], it was not assumed that the activity of the CaOH<sup>+</sup> ion could be neglected. The procedure uses an iterative approach to determine the ion fugacities (*f*) according to the Davies equation:

$$\log(f) = -Az^2 \left( \frac{\sqrt{I}}{1+\sqrt{I}} - 0.3I \right)$$

The Davies equation provides reasonably reliable estimates of fugacities for solutions having ionic strengths (*I*) of up to 0.5. The fugacity of neutral species was estimated using the Setchenow equation:

$$\log(f) = 0.0434 \times I$$

This equation takes into account the phenomenon of 'salting out' of neutral species. The ionic strength of saliva was initially used at 0.05 [Larsen and Pearce, 2003], allowing initial estimates of the fugacities according to the various ion charges (*z*). Once the activities of all the species were known, an improved estimate of the ionic strength of the solution was made and the method iterated until convergence was achieved. A Tcl script was written to perform these calculations, the script included checks to confirm that the initial conditions were satisfied by the calculated solutions.

Dissociation constants at 37°C were used from the following sources:  $\text{H}_2\text{PO}_4^-$  [Bates and Acree, 1943];  $\text{HPO}_4^{2-}$  [Bjerrum, 1929];  $\text{CaH}_2\text{PO}_4^+$  and  $\text{CaHPO}_4^0$  [Gregory, 1970];  $\text{CaPO}_4$  [Chughtai et al., 1968]. The constants for the citrate/calcium citrate system were taken from Pearce [1980] and converted to 37°C using standard enthalpy ( $\Delta H$ ) changes from Martell and Smith [1977]. Values for low ionic strength were used if thermodynamic values (for zero ionic strength) were not available. The source of the solubility product ( $K_{sp}$ ) at 37°C for hydroxyapatite was McDowell et al. [1977].

Previous studies [Shen et al., 2001] have shown that flavours and the CPP-ACP are released from sugar-free gum within 8 min of chewing, with 66% released in the first 2 min, 19% released in the next 3 min and the last 15% released in the next 3 min of chewing. For the citric gum and the citric plus CPP-ACP gum it was assumed that the 20 mg of citric acid was released in a similar manner. Using the measured volume of saliva collected for each time period and the estimated amount of citric acid released its concentration in the saliva samples was calculated. This citric acid concentration and the acidity constants and calcium citrate formation constants were then used in the computational procedure to calculate the calcium ion activity and ion activity product for hydroxyapatite in the citric gum saliva samples.

#### *Preparation of Intraoral Appliances and Enamel Subsurface Lesions*

Removable mid-palatal acrylic appliances were fabricated for each subject as described by Shen et al. [2001]. Enamel slabs were prepared from human third molars and subsurface lesions created using the 96-hour carbopol procedure exactly as described by Reynolds [1997]. The enamel slabs were sectioned into a  $3 \times 4$  and  $5 \times 4$  mm pair of blocks and the smaller block ( $3 \times 4$  mm) was retained as the demineralization control and stored in a labelled 1.5-ml microcentrifuge tube together with a drop of distilled/deionized water to create a humidified environment. The other enamel block of the pair was inset into one of the bilateral troughs in the intraoral appliance using dental wax as described by Iijima et al. [2004] to produce a 1-mm trough above the enamel surface to allow plaque to establish and be retained. Four enamel slabs were inset into each appliance, two on each side in the bilateral troughs [Shen et al., 2001].

#### *Study Protocol*

This study utilized a double-blind, randomized, crossover design with three treatments: (i) sugar-free gum (2 pellets) containing 20 mg citric acid and 18.8 mg CPP-ACP (Recaldent™, CASRN 691364-49-5), (ii) gum containing 20 mg citric acid alone, (iii) sugar-free gum not containing citric acid or CPP-ACP. The test chewing gum products were provided by the Clinical and Consumer Packaging Group, Cadbury Schweppes Science and Technology. The chewing gum products were coded in sealed packages and stored at room temperature. The code was released after all data had been acquired and statistical analysis performed.

One of the chewing gum products was randomly assigned to each of the subjects for each of three 2-week treatment periods. Four times a day at specified times (10.00 a.m., 11.30 a.m., 2.00 p.m. and 3.30 p.m.), with appliances inserted, subjects chewed with two pellets of gum for 20 min. This was repeated for 14 consecutive days (treatment period). Subjects were instructed to remove the intraoral appliances when they ate or drank and when

they conducted their normal oral hygiene procedures. However, subjects were asked not to remove the appliances until 40 min after gum chewing ceased. The times of wearing the appliance and gum chewing were recorded in a diary, collected after each treatment period. On removing the appliances, subjects were instructed to rinse them briefly with distilled/deionized water and then to store them in sealed, humidified containers at 37°C until they were reinserted. At the end of each treatment period, subjects rested for 7 days, then were crossed over to another gum product until each subject used the three products. After completion of each treatment period, the enamel slabs were removed from the appliances, rinsed with distilled/deionized water and stored moist in labelled microcentrifuge tubes. Subjects were instructed to clean the appliances, avoiding the enamel slabs, with a toothbrush and fluoride-free denture paste provided by the sponsor. Subjects maintained normal oral hygiene and dietary habits throughout the study. At the commencement of the study, 1 week prior to starting treatment, all subjects were provided with standard fluoride toothpaste (1,000 ppm F) to use morning and night for the duration of the study.

#### *Acid Challenge*

At the completion of each treatment period, half of each remineralized block was acid-challenged in the carbopol demineralization solution for 16 h at 37°C as described by Iijima et al. [2004]. Half of each remineralized block was covered with acid-resistant nail varnish exposing only half of the remineralized (occlusal and gingival) mesiodistal windows (approximately  $1 \times 1$  mm) separated from each other by approximately 1 mm.

#### *Sectioning, Microradiography and Microdensitometry*

Each remineralized enamel block combined with its acid-challenged and demineralized control block were embedded, sectioned and subjected to microradiography exactly as described by Iijima et al. [2004]. The microdensitometric analysis of the radiographic images was also conducted using the procedure described by Iijima et al. [2004]. The image of the median strip of sound enamel between the two lesions (gingival and occlusal lesions of each section) was scanned 6 times and averaged to give a control densitometric profile of sound enamel [Iijima et al., 2004]. The lesion images (remineralization windows and demineralization control windows) to the gingival and occlusal side of the median strip of sound enamel were similarly scanned, as close as possible to the median strip avoiding any irregularities commonly found at the lesion edges. Lesion depth (LD) of each scanned lesion was measured in micrometres and recorded.  $\text{LD}_D$  represents the lesion depth of the initial demineralized lesion before remineralization and  $\text{LD}_{AC}$  represents the lesion depth of the remineralized lesion after acid challenge.

#### *Data Analysis*

The vol% mineral versus micrometre for each enamel block's demineralized and remineralized lesion (including remineralized/acid-challenged lesion) were compared with the median sound mineral profile of the same section. The difference between the areas under the densitometric profile of the demineralized lesion and the median sound enamel, calculated by trapezoidal integration, is represented by  $\Delta Z_d$ . The difference between the areas under the densitometric profile of the remineralized lesion and the median sound enamel, calculated by trapezoidal integration,

is represented by  $\Delta Z_r$ . These parameters were then converted to percentage change values after remineralization with respect to each control untreated, demineralized lesion. As such, percentage remineralization (%R) represents the percentage change in  $\Delta Z$  values:

$$\%R = \frac{\Delta Z_d - \Delta Z_r}{\Delta Z_d} \times 100$$

The change in %R after acid challenge is represented by  $\Delta\%R$ .

#### Statistical Analyses

Homogeneity of variance was tested using Levene's test and normality of the data was examined using normal probability plots and the Kolmogorov-Smirnov test [Sokal and Rohlf, 1969; Norusis, 1993]. All experimental data (saliva and remineralization data) were statistically analysed using a within-groups Analysis of Variance with a Bonferroni correction for post hoc comparisons [Sokal and Rohlf, 1969; Norusis, 1993]. All statistical analyses were performed using SPSS Version 13.0 software [Norusis, 1993].

### Results

Salivary flow rates were not significantly different for the three gum types (table 1). The pH of saliva stimulated by chewing the neutral gum during the first 2 min was significantly higher ( $p < 0.05$ ) than the pH of the saliva stimulated chewing either the citric gum or the citric plus CPP-ACP gum (table 2). However, the pH of saliva stimulated by the citric-containing gums did not fall below 6.5 (table 2). The salivary pH values after 2 min of chewing (e.g. 2 to 20 min) were not significantly different for the three gum types (table 2). Chewing the citric gum significantly lowered ionic calcium concentrations, both measured and calculated, in the saliva collected for the first 5 min of chewing (table 3). This decrease in ionic calcium concentration resulted in a significant reduction in the degree of saturation for hydroxyapatite (table 3). Chewing the citric plus CPP-ACP gum significantly increased the total and filtrate calcium and inorganic phosphate levels in saliva for the first 10 min of chewing but did not increase ionic calcium concentrations nor degree of saturation for hydroxyapatite relative to the neutral gum but did increase these values relative to the citric gum (table 3).

The level of remineralization produced by chewing the neutral gum was significantly higher than that produced by chewing the citric gum but significantly lower than that produced by chewing the citric plus CPP-ACP gum (table 4). The 16-hour *in vitro* acid challenge of the remineralized lesions resulted in significantly less loss of mineral from the lesions remineralized by chewing the citric plus CPP-ACP gum when compared with the other two gum types (table 4).

**Table 1.** Salivary flow data (mean  $\pm$  SD)

Time	Salivary flow, ml/min		
	neutral gum	citric gum	citric plus CPP-ACP gum
0-2 min	3.9 $\pm$ 1.0	4.4 $\pm$ 0.9	4.2 $\pm$ 0.9
2-5 min	2.8 $\pm$ 0.9	2.9 $\pm$ 1.8	3.1 $\pm$ 0.8
5-10 min	2.0 $\pm$ 0.6	1.8 $\pm$ 0.5	2.0 $\pm$ 0.6
10-15 min	1.9 $\pm$ 0.7	1.7 $\pm$ 0.5	1.8 $\pm$ 0.5
15-20 min	1.9 $\pm$ 0.7	1.8 $\pm$ 0.6	1.7 $\pm$ 0.7
Average	2.3 $\pm$ 0.7	2.3 $\pm$ 0.6	2.3 $\pm$ 0.6

**Table 2.** Salivary pH data (mean  $\pm$  SD)

Time	Salivary pH		
	neutral gum	citric gum	citric plus CPP-ACP gum
0-2 min	7.1 $\pm$ 0.2	6.5 $\pm$ 0.3 <sup>a</sup>	6.5 $\pm$ 0.5 <sup>a</sup>
2-5 min	7.3 $\pm$ 0.2	7.0 $\pm$ 0.4	7.0 $\pm$ 0.4
5-10 min	7.2 $\pm$ 0.2	7.1 $\pm$ 0.3	7.1 $\pm$ 0.3
10-15 min	7.3 $\pm$ 0.3	7.2 $\pm$ 0.3	7.2 $\pm$ 0.4
15-20 min	7.2 $\pm$ 0.3	7.2 $\pm$ 0.4	7.3 $\pm$ 0.3

<sup>a</sup> Significantly lower ( $p < 0.05$ ) than pH produced by the neutral gum over the same time period.

### Discussion

Chewing sugar-free gum has been shown previously to stimulate salivary flow rate and to increase the concentration of calcium in the stimulated saliva [Dawes and Dong, 1995]. The remineralization of the subsurface enamel lesions by the neutral sugar-free gum demonstrated in this study therefore can be explained by the availability of calcium and phosphate ions in stimulated saliva. Inclusion of citric acid in the gum was expected to have an effect on salivary flow rate with the citric gum producing a higher stimulated salivary flow rate than the neutral gum. However, in this study, the stimulated salivary flow rates were not significantly different for the three gum types (table 1). The results of the study showed that inclusion of citric acid significantly lowered the remineralizing ability of the sugar-free chewing gum (table 4). When comparing the pH of saliva collected in the first 2 min (table 2), saliva stimulated by the neutral gum did show a significantly higher pH value than that stimulated by the other two gums containing citric acid ( $p <$

**Table 3.** Salivary calcium and phosphate concentrations

Time	Salivary calcium, mM											
	neutral gum				citric gum				citric plus CPP-ACP gum			
	total	filtrate <sup>e</sup>	ionic <sup>d</sup>	(Ca <sup>2+</sup> ) <sup>c</sup>	total	filtrate	ionic	(Ca <sup>2+</sup> )	total	filtrate	ionic	(Ca <sup>2+</sup> )
0-2 min	2.08 ± 0.22	1.37 ± 0.09	0.51 ± 0.05	0.41 ± 0.02	2.12 ± 0.34	1.90 ± 0.25	0.27 ± 0.07 <sup>b</sup>	0.07 ± 0.01 <sup>b</sup>	4.19 ± 0.66 <sup>a</sup>	3.15 ± 0.36 <sup>a</sup>	0.55 ± 0.07	0.15 ± 0.03
2-5 min	1.69 ± 0.16	1.21 ± 0.27	0.53 ± 0.08	0.36 ± 0.08	1.80 ± 0.29	1.30 ± 0.12	0.34 ± 0.10 <sup>b</sup>	0.12 ± 0.02 <sup>b</sup>	3.86 ± 0.64 <sup>a</sup>	2.41 ± 0.46 <sup>a</sup>	0.66 ± 0.04	0.32 ± 0.10
5-10 min	1.42 ± 0.30	1.04 ± 0.07	0.54 ± 0.07	0.31 ± 0.02	1.36 ± 0.33	1.06 ± 0.24	0.42 ± 0.09	0.31 ± 0.07	2.29 ± 0.53 <sup>a</sup>	1.51 ± 0.40 <sup>a</sup>	0.59 ± 0.06	0.43 ± 0.11
10-15 min	1.33 ± 0.13	0.98 ± 0.18	0.51 ± 0.05	0.29 ± 0.05	1.37 ± 0.15	1.04 ± 0.28	0.43 ± 0.07	0.30 ± 0.08	1.53 ± 0.22	1.02 ± 0.21	0.54 ± 0.03	0.29 ± 0.07
15-20 min	1.00 ± 0.16	0.89 ± 0.14	0.48 ± 0.05	0.26 ± 0.03	1.21 ± 0.16	0.86 ± 0.24	0.42 ± 0.07	0.25 ± 0.07	1.43 ± 0.17	1.16 ± 0.45	0.49 ± 0.07	0.33 ± 0.14

  

Time	Salivary phosphate, mM								
	neutral gum			citric gum			citric plus CPP-ACP gum		
	total	filtrate <sup>e</sup>	DS <sup>f</sup>	total	filtrate	DS	total	filtrate	DS
0-2 min	3.70 ± 0.94	3.09 ± 0.61	15.86 ± 3.36	4.15 ± 1.02	3.53 ± 1.17	3.28 ± 0.60 <sup>b</sup>	5.83 ± 1.59 <sup>a</sup>	5.58 ± 1.18 <sup>a</sup>	5.59 ± 0.87
2-5 min	3.60 ± 0.69	2.75 ± 0.57	17.27 ± 4.68	3.99 ± 1.38	3.24 ± 1.39	8.50 ± 1.61 <sup>b</sup>	5.57 ± 1.41 <sup>a</sup>	4.57 ± 1.09 <sup>a</sup>	15.91 ± 1.38
5-10 min	3.89 ± 0.88	3.24 ± 0.70	16.25 ± 4.03	3.93 ± 1.53	3.56 ± 1.16	18.65 ± 3.34	5.10 ± 1.25 <sup>a</sup>	4.33 ± 1.37 <sup>a</sup>	19.90 ± 2.98
10-15 min	4.14 ± 1.07	3.29 ± 0.52	15.83 ± 5.02	4.86 ± 1.15	3.70 ± 1.17	17.90 ± 4.21	4.91 ± 1.06	3.94 ± 0.87	18.77 ± 1.51
15-20 min	4.52 ± 1.07	3.59 ± 0.44	16.68 ± 5.51	4.81 ± 1.14	3.87 ± 1.01	16.79 ± 4.63	5.17 ± 1.16	4.13 ± 0.89	19.48 ± 3.89

<sup>a</sup> Significantly higher ( $p < 0.05$ ) than neutral gum or citric gum value at same time period.

<sup>b</sup> Significantly lower ( $p < 0.01$ ) than neutral gum or citric plus CPP-ACP gum value at same time period.

<sup>c</sup> Calcium ion activity calculated from filtrate calcium level as described in 'Materials and Methods'.

<sup>d</sup> Ionic calcium as measured using a calcium ion-selective electrode as described in 'Materials and Methods'.

<sup>e</sup> Calcium or inorganic phosphate concentration in saliva filtrate as described in 'Materials and Methods'.

<sup>f</sup> Degree of saturation for hydroxyapatite =  $(IAP_{HA}/K_{spHA})^{1/9}$ .

**Table 4.** Remineralization data

Gum type	$\Delta Zd$ vol%min· $\mu m$	LD <sub>D</sub> , $\mu m$	Before acid challenge		After acid challenge			$\Delta\%R$
			$\Delta Zd - \Delta Zr$ vol%min· $\mu m$	%R	$\Delta Zd - \Delta Zr$ vol%min· $\mu m$	LD <sub>AC</sub> <sup>b</sup> $\mu m$	%R	
Neutral	3,274 ± 521 <sup>a</sup>	94.0 ± 6.7 <sup>b</sup>	307.9 ± 61.1 <sup>c</sup>	9.4 ± 1.2 <sup>d</sup>	-91.7 ± 84.3 <sup>d</sup>	95.6 ± 5.6 <sup>b</sup>	-2.8 ± 1.9 <sup>d</sup>	-12.2 ± 1.5 <sup>c</sup>
Citric	3,215 ± 415	93.9 ± 6.4	82.4 ± 73.8 <sup>c</sup>	2.6 ± 1.3 <sup>d</sup>	-376.7 ± 142.8 <sup>d</sup>	98.4 ± 7.5	-11.7 ± 1.6 <sup>d</sup>	-14.3 ± 2.0 <sup>c</sup>
Citric plus CPP-ACP	3,288 ± 529	92.6 ± 7.6	427.8 ± 84.1 <sup>c</sup>	13.0 ± 2.2 <sup>d</sup>	95.5 ± 56.3 <sup>d</sup>	93.4 ± 6.1	2.9 ± 1.8 <sup>d</sup>	-10.1 ± 1.2 <sup>c</sup>

<sup>a</sup> No significant difference between  $\Delta Zd$  values in column for the three gum types.

<sup>b</sup> No significant difference between lesion depth values in column for the three gum types.

<sup>c</sup> All values in column significantly different ( $p < 0.05$ ) from each other.

<sup>d</sup> All values in column significantly different ( $p < 0.01$ ) from each other.

0.05). The saliva pH did not fall below 6.5 for the citric gums and for the rest of the chewing time (2 to 20 min) the saliva pH values were similar for the three gum types, suggesting that the buffering capacity of the saliva prevented the released citric acid from lowering the pH of the bulk saliva below 6.5. However, there may have been localized areas of low pH caused by release of the citric acid which would not be observed by measuring pH of the bulk saliva. Released citric acid from the sugar-free chewing gum would have been rapidly neutralized to citrate by the stimulated saliva and then would have rapidly bound free calcium ions (table 3). Measurement of ionic

calcium in saliva collected during chewing of the citric gum confirmed that the released citric acid decreased the ionic calcium concentration for the first 5 min of chewing (table 3). This decrease in ionic calcium concentration can be attributed to the binding of calcium ions by citrate [Pearce, 1980]. Binding of free calcium ions by citrate would lower the activity of the calcium ions (table 3) and thereby reduce the ability of the saliva to remineralize enamel subsurface lesions. This mechanism, therefore, is the most likely explanation for the decreased remineralization observed with the citric gum compared with the neutral gum.

CPP containing the sequence -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- have been shown not only to stabilize ACP but also to deliver and localize ACP at the tooth surface [Reynolds, 1998; Reynolds et al., 1999]. A recent study [Reynolds et al., 2003] has demonstrated that CPP could still be detected on the tooth surface 3 h after chewing sugar-free gum containing CPP-ACP. The CPP-ACP/citric gum contained 18.8 mg of CPP-ACP and as the CPP-ACP is completely released from the gum in 8 min [Shen et al., 2001]; this should have increased the salivary calcium concentration in the first 5–10 min of chewing. The measurement of total and filtrate calcium and inorganic phosphate in saliva samples for the first 5–10 min of chewing confirmed that the CPP-ACP was released from the chewing gum (table 3). However, as the CPP-ACP binds to surfaces in the mouth [Reynolds et al., 2003] a significant amount of this released CPP-ACP will be localized on the surface of teeth and not found in the bulk saliva. In fact, the difference between the total salivary calcium produced in the first 10 min of chewing the citric plus CPP-ACP gum and the neutral gum suggested that a substantial amount of the released CPP-ACP had already bound to oral surfaces as shown previously for CPP-ACP released from sugar-free gum [Reynolds et al., 2003]. The CPP localizing calcium, phosphate and hydroxide ions as nanoclusters on the enamel surface of the lesions would explain why the CPP-ACP nanocomplexes overcame the negative impact of the citric acid binding calcium in the bulk saliva. The bound CPP-ACP, unaffected by the citrate in the bulk saliva, could then provide calcium ions, phosphate ions and hydroxide ions to diffuse into the enamel subsurface lesion. The binding and localization of the CPP-ACP onto the enamel surface would also help explain why the saliva produced by the citric plus CPP-ACP gum resulted in significantly greater remineralization than the saliva produced by the neutral gum (table 4), although it did not contain a higher ionic calcium concentration nor degree of saturation with respect to hydroxyapatite when compared with the neutral gum saliva (table 3).

An *in vitro* acid challenge of the remineralized lesions was included in the study to evaluate the ability of the deposited mineral to resist acid challenge [Iijima et al., 2004]. Lesions remineralized by chewing the gum containing citric acid showed the least resistance to acid challenge while lesions remineralized by chewing the gum containing citric acid plus CPP-ACP were more resistant to acid challenge than the lesions remineralized by chewing the other two gum types (table 4). This may be attributable to a difference in the activity of calcium ions, phos-

phate ions and hydroxide ions within the lesion fluid during the remineralization process, leading to differences in crystallinity of the deposited mineral [Nancollas, 1989]. It has been shown previously that CPP-ACP nanocomplexes carrying bioavailable, basic ACP as ions in the ratio  $\text{Ca}_{3.0877}(\text{PO}_4)_2(\text{OH})_{0.1754}$  [Cross et al., 2005] were superior to other forms of calcium phosphate in remineralizing enamel subsurface lesions *in situ* [Reynolds et al., 2003]. A higher activity of calcium ions, phosphate ions and hydroxide ions localized on the enamel surface and in the lesion fluid, produced by chewing the sugar-free gum containing CPP-ACP, may have promoted a more homogeneous hydroxyapatite crystallization, thereby producing a mineral more resistant to acid challenge when compared with the other two gum types. The relative resistance of the CPP-ACP-produced mineral to acid challenge may also relate to the presence of the CPP-ACP on the enamel surface, which has been shown to inhibit the demineralization process [Reynolds, 1998].

In conclusion, the addition of citric acid to sugar-free chewing gum significantly reduced the remineralization effects of saliva. The addition of CPP-ACP to sugar-free gum containing citric acid negated the effect of the citric acid and produced a remineralizing effect greater than the neutral sugar-free gum without citric acid. The CPP-ACP nanocomplexes induced mineral which was more acid-resistant than saliva-alone-induced mineral.

### Acknowledgement

The support of Cadbury Schweppes Science and Technology is gratefully acknowledged.

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