Retention in Plaque and Remineralization of Enamel Lesions by Various Forms of Calcium in a Mouthrinse or Sugar-free Chewing Gum

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ABSTRACT

Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) nanocomplexes incorporated into sugar-free chewing gum have been shown to remineralize enamel subsurface lesions *in situ*. The aim of this study was to compare the ability of CPP-ACP, with that of other forms of calcium, to be retained in supragingival plaque and remineralize enamel subsurface lesions *in situ* when delivered in a mouthrinse or sugar-free gum in randomized, double-blind trials. In the mouthrinse study, only the CPP-ACP-containing mouthrinse significantly increased plaque calcium and inorganic phosphate levels, and the CPP were immunolocalized to the surfaces of bacterial cells as well as the intercellular matrix. In the chewing gum studies, the gum containing the CPP-ACP, although not containing the most calcium *per* piece of gum, produced the highest level of enamel remineralization independent of gum-chewing frequency and duration. The CPP could be detected in plaque extracts 3 hrs after subjects chewed the CPP-ACP-containing gum. The results showed that CPP-ACP were superior to other forms of calcium in remineralizing enamel subsurface lesions.

KEY WORDS: casein phosphopeptide-amorphous calcium phosphate, dental plaque, incorporation, mouthrinse, sugar-free chewing gum, enamel remineralization.

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INTRODUCTION

Casein phosphopeptides (CPP) containing the sequence Ser(*P*)-Ser(*P*)-Ser-(*P*)-Glu-Glu stabilize nanoclusters of amorphous calcium phosphate (ACP) in metastable solution (Reynolds, 1998; Reynolds *et al*., 1999). The multiple phosphoseryl residues of the CPP bind to forming nanoclusters of ACP in supersaturated solutions, preventing growth to the critical size required for phase transformations. Casein phosphopeptide-amorphous calcium phosphate nanocomplexes (CPP-ACP) have been demonstrated to have anticariogenic potential in laboratory, animal, and human *in situ* experiments (Reynolds *et al*., 1995, 1999; Reynolds, 1997; Rose, 2000a,b; Shen *et al*., 2001). The CPP-ACP reduced caries activity in specific-pathogen-free rats orally infected with *Streptococcus sobrinus* when applied to the animals' molar teeth as a solution twice daily (Reynolds *et al*., 1995). Recently, Shen *et al*. (2001) have shown that 18.8 mg and 56.4 mg of CPP-ACP in a sugar-free chewing gum enhanced remineralization of enamel subsurface lesions *in situ* by 101% and 151%, respectively, when compared with the control sugar-free gum not containing CPP-ACP. The proposed anticariogenic mechanism of CPP-ACP is the incorporation of the nanocomplexes into plaque and onto the tooth surface. The localized CPP-ACP nanocomplexes purportedly then act to buffer the free calcium and phosphate ion activities, thereby maintaining a state of supersaturation with respect to tooth enamel, preventing enamel demineralization and promoting remineralization. The aim of this study therefore was to compare the ability of the CPP-ACP with that of other forms of calcium to be retained in dental plaque and to remineralize enamel subsurface lesions *in situ* when delivered in a mouthrinse or sugar-free gum.

MATERIALS & METHODS

Subject Recruitment

Thirty healthy adult subjects ranging in age from 22 to 44 yrs were recruited from the staff and students of the School of Dental Science, the University of Melbourne. Approval for the studies was obtained from the University of Melbourne Human Research Ethics Committee, and all subjects provided informed consent. All subjects had at least 22 natural teeth with no current caries activity, periodontal disease, or other oral pathology. None of the subjects was using antibiotics or medications. The stimulated whole salivary flow rate of each subject was more than 1.0 mL/min as stimulated by the chewing of sugar-free gum. The unstimulated salivary flow rate of each subject was in excess of 0.2 mL/min.

Mouthrinse Study

The mouthrinse study was a randomized, double-blind cross-over design involving 4 coded mouthrinses. The purpose of this study was to determine whether short-term (5 days') use of a CPP-ACP-containing mouthrinse could substantially increase the level of calcium and inorganic phosphate in

supragingival plaque compared with an unstabilized calcium phosphate mouthrinse. Each subject crossed over to use each mouthrinse with at least a four-week washout period between treatments. Two of the mouthrinses contained CPP-ACP (Recaldent™) as supplied by Bonlac Foods Ltd. (Melbourne, Australia). One rinse contained 2% w/v Recaldent™ and the other 6% w/v Recaldent™ in de-ionized water. The mouthrinses were adjusted to pH 7.0 with 1 M HCl. The 2% Recaldent™ solution contained 1% CPP, 60 mM Ca^{2+}, and 40 mM inorganic phosphate as a colloidal solution of CPP-stabilized ACP nanoclusters. The 6% w/v Recaldent™ solution contained 3% CPP, 180 mM Ca^{2+}, and 120 mM inorganic phosphate as a stabilized colloidal solution. The third mouthrinse was an unstabilized slurry of 60 mM $CaCl_2$ and 40 mM sodium phosphate (pH 7.0) that was prepared immediately before use by the mixing of an equal volume at 120 mM $CaCl_2$ with 80 mM sodium phosphate (pH 7.0). The fourth mouthrinse was de-ionized water. Subjects were supplied with the coded rinses in opaque plastic tubes and used 15 mL of each rinse for 30 sec three times a day (after breakfast, after lunch, and at night before retiring), for 4 days and kept a diary of mouthrinse use. On the fifth day, the rinse was used after breakfast, and supragingival plaque was collected 2-3 hrs later. Subjects refrained from all oral hygiene procedures while using the rinses. Supragingival plaque was collected by means of a Gracey 7/8 curette from the buccal and lingual surfaces of all teeth except the mandibular anterior teeth from the permanent lower left canine to the permanent lower right canine. Plaque was collected into a pre-weighed microcentrifuge tube, re-weighed, and then stored at -70°C.

Sugar-free Chewing Gum Studies

Two randomized, double-blind, cross-over remineralization studies were conducted with 3 pellet and 3 slab sugar-free gums containing different forms of calcium, according to an *in situ* model previously described (Shen *et al*., 2001). The purpose of these studies was to determine whether CPP-ACP was superior to other forms of calcium, when delivered in sugar-free gum, in remineralizing enamel subsurface lesions in an *in situ* model. Palatal appliances containing 4 human enamel half-slabs with subsurface demineralized lesions were prepared as described by Shen *et al*. (2001). The sugar-free chewing gums were provided by Warner Lambert Consumer Healthcare group of Pfizer Inc. (Piscataway, NJ, USA) as coded products. The gums tested and the form of the calcium additive are presented in the Table. We assayed the levels of water-soluble and acid-soluble calcium phosphate in each gum by extracting each gum cut into small pieces with 25 mL of distilled water or 25 mL of 1 M HCL, respectively, for 2 days at room temperature. The levels of calcium and inorganic phosphate in the extracts were determined by atomic absorption spectrophotometry and colorimetry, respectively, as described previously (Adamson and Reynolds, 1995). For the pellet gum study, subjects chewed for 20 min, 4 times *per* day for 14 days at the following times: 10:00 a.m., 11:30 a.m., 2:00 p.m., and 3:30 p.m. For the slab gum study, subjects chewed for 5 min, 7 times daily for 7 days at the following times: 7:00 a.m., 9:00 a.m., 11:00 a.m., 1:00 p.m., 3:00 p.m., 5:00 p.m., and 10:00 p.m. In both studies, subjects kept diaries of gum use and were instructed not to eat (except for the test chewing gums), drink, or perform oral hygiene procedures while wearing the appliances. When the appliances were not in subjects' mouths, they were stored in a sealed moist plastic bag at room temperature. After the completion of each treatment, the enamel half-slabs were removed from the appliances, paired with their control half-slabs, then embedded, sectioned, and microradiographed as described previously (Shen *et al*., 2001). Images of the lesions and the neighboring sound enamel were scanned and the percentage remineralization (%R) calculated as described by Shen *et al*. (2001). Data were statistically analyzed by a one-way ANOVA with *post hoc* Scheffé test (Norusis, 1993).

In another sugar-free gum study, subjects chewed 2 pieces of the Recaldent™ pellet gum containing 9.5 mg of CPP-ACP *per* piece for a 20-minute period 3 times daily at 10:00 a.m., 1:00 p.m., and 4:00 p.m. for 4 days while refraining from oral hygiene procedures. The purpose of this study was to determine how long CPP-ACP could be detected in plaque after short-term (5 days') use of a CPP-ACP-containing sugar-free gum. On the fifth day, the

Table. Remineralization of Enamel Subsurface Lesions *in situ* by Sugar-free Chewing Gum Containing Different Forms of Calcium

			Ca (mg/piece)		Pi (mg/piece)		
	Ca Additive	Weight (g/piece)	Water-soluble	Acid-soluble	Water-soluble	Acid-soluble	%R[a]
Pellet Gum Study[b]							
Lotte Xylitol	$CaCO_3$	1.5	0.5 ± 0.1[c]	9.9 ± 0.4[c]	ND[d]	ND	8.9 ± 1.4[e,f]
Lotte Xylitol + CP/FN[g]	$CaHPO_4/CaCO_3$	1.7	0.9 ± 0.1	9.2 ± 0.2	1.3 ± 0.1[c]	2.9 ± 0.1[c]	12.0 ± 1.6[e]
Recaldent	CPP-ACP	1.5	1.6 ± 0.1	1.9 ± 0.1	2.0 ± 0.1	2.5 ± 0.1	19.0 ± 2.5[e]
Slab Gum Study[h]							
Xylish Crystal Mint	$CaCO_3$	3.2	0.3 ± 0.1	20.7 ± 3.2	ND	ND	6.3 ± 1.2[f,i]
Lotte Xylitol + 2	$CaHPO_4/CaCO_3$	2.5	1.0 ± 0.1	33.1 ± 4.0	1.3 ± 0.1	3.5 ± 0.2	8.6 ± 1.0[i]
Recaldent	CPP-ACP	2.5	2.5 ± 0.1	2.6 ± 0.1	3.4 ± 0.1	3.6 ± 0.1	19.4 ± 1.6[i]

[a] %R, Percentage Enamel Remineralization, $\%R = (1-\Delta Zr/\Delta Zd) \times 100$ (Shen *et al*., 2001).
[b] Pellet Gum Study—two pieces of pellet gum chewed for 20 min, 4 times *per* day for 14 days.
[c] Mean ± SD (n = 5).
[d] ND, not detected.
[e,i] Significantly different ($p < 0.01$, ANOVA/Scheffé) from all other values in column similarly marked.
[f] Mean ± SD (n = 30).
[g] CP/FN; CP indicates calcium hydrogen phosphate, and FN indicates Funoran, a sulfated polysaccharide extracted from *Gloiopeltis furcata* (red algae) (Saeki *et al*., 1996).
[h] Slab Gum Study—one piece of slab gum chewed for 5 min, 7 times *per* day for 7 days.

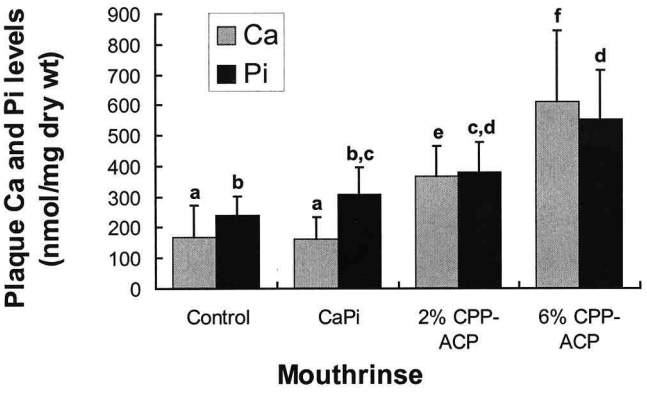

Figure 1. Calcium and inorganic phosphate levels in supragingival plaque after treatment with unstabilized calcium phosphate and CPP-ACP mouthrinses [data presented as mean ± SD (n = 30)]. **(a,e,f)** Ca values significantly different ($p < 0.05$) from other Ca values not similarly marked. **(b,c,d)** Pi values significantly different ($p < 0.05$) from other Pi values not similarly marked, as shown by a one-way classification ANOVA with a *post hoc* Scheffé test.

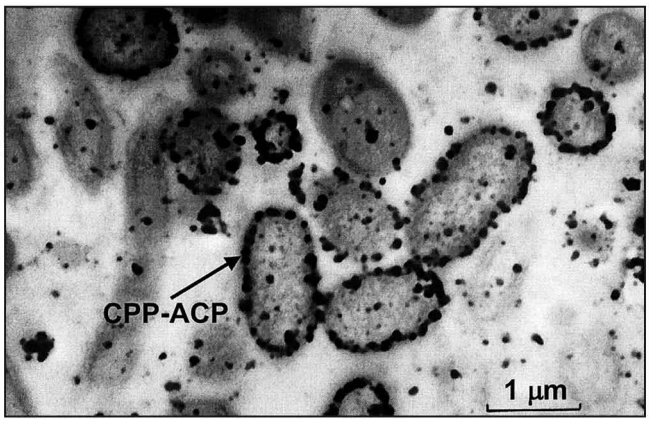

Figure 2. Representative electron micrograph of supragingival plaque showing CPP-ACP as electron-dense particles associated with the surface of bacteria and the intercellular matrix.

subjects chewed 2 pieces of gum at 10:00 a.m. and 1:00 p.m. Immediately after the 20-minute gum-chewing period at 1:00 p.m., supragingival plaque was collected from the upper-left quadrant into a pre-weighed microcentrifuge tube as described above. After a further 20 min, 60 min, and 180 min, supragingival plaque was collected from the lower-left, upper-right, and lower-right quadrants, respectively, into pre-weighed microcentrifuge tubes. The subjects did not eat or drink during this three-hour period. The microcentrifuge tubes were immediately re-weighed and then stored at -70°C. To obtain baseline measurements, we also collected control plaque from the subjects as described above after they had refrained from oral hygiene procedures for 5 days.

Plaque Calcium and Inorganic Phosphate Analyses

After the plaque samples were thawed, they were centrifuged for 5 min at 20,000 *g*, dried in a Jouan RC10.10 rotary evaporator (Societé Jouan, Saint Herblain, France) and then re-weighed so that dry weights could be determined. We then extracted the dry samples with 200 μL of 1 M HCl by mixing them in a vortex mixer for 1 min and then treating them in ice water in a Bransonic 12 ultrasonic bath (Consolidated Ultrasonic, Melbourne, Australia) for 8 hrs. After centrifugation (20,000 *g*, 5 min), calcium and inorganic phosphate concentrations in the supernatant were determined as described previously (Adamson and Reynolds, 1995). The calcium and inorganic phosphate plaque levels were statistically analyzed by a one-way classification analysis of variance (ANOVA) with a *post hoc* Scheffé test (Norusis, 1993).

Plaque CPP Analysis by Competitive ELISA

For CPP analysis, the plaque samples were dried and extracted with 100 μL of 1 M HCl as described above. After removal of the acid extract, the plaque residue was further extracted with 100 μL of 1 M NaOH. The alkaline extraction procedure was the same as that for the acid extraction. After centrifugation (40,000 *g*, 10 min), the supernatant was removed for analysis. Samples of each acid and alkali extract were adjusted to pH 7.4 with NaOH and HCl, respectively, followed by 1 M Tris-HCl (pH 7.4), and the neutralized samples were analyzed for CPP by rabbit, affinity-purified, anti-casein antibodies (1/15,000 dilution) in a competitive ELISA as described previously (Reynolds, 1987; Black and Reynolds, 1998; Perich *et al*., 1999). The CPP levels in plaque were statistically analyzed by a one-way classification ANOVA with a *post hoc* Scheffé test (Norusis, 1993).

Immunolocalization of CPP-ACP in Plaque

A sample of the supragingival plaque collected after each mouthrinse treatment was placed directly onto a celluloid strip (3 mm x 5 mm). The plaque samples were immediately immersed in liquid N_2, freeze-dried at -60°C under vacuum, and then infiltrated in L.R. White resin (London Resin Co. Ltd., Basingstoke, UK). The samples were then transferred to gelatin capsules filled with L.R. White resin and allowed to polymerize at 55°C for 18 hrs. The polymerized block was trimmed and 80-nm sections cut with a diamond knife onto a water bath by means of a microtome (Reichert-Jung Ultracut E model FC4, Heidelberg, Germany). The sections were transferred onto uncoated 200-mesh Nickel grids and dried, and non-specific binding sites were blocked by incubation in PBS (10 mM phosphate, pH 7.3, 120 nM NaCl) containing 2% v/v normal goat serum (NGS) and 0.05% v/v Tween 20 for 60 min. The sections were then washed twice in PBS for 30 min each and then incubated with PBS containing rabbit, affinity-purified, anti-casein antibody (1:10,000), 0.05% v/v Tween 20, and 0.5% w/v bovine serum albumin (BSA) for 20 hrs at 4°C. After being washed twice in PBS containing 0.05% v/v Tween 20 and 1% w/v BSA for 30 min each, the sections were then incubated with PBS containing horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:1000, 0.05% v/v Tween 20, and 1% w/v BSA for 3 hrs. The sections were again washed twice in PBS for 30 min each and incubated with 10 mL of 0.5 M Tris-HCl (pH 7.6) containing 5 mg of 3,3′-diaminobenzidine tetrahydrochloride (0.05% w/v) and 0.1 mL of 0.01% v/v hydrogen peroxide for 50 min. The sections were then washed in double-distilled water, allowed to dry at room temperature, and were then examined under an electron microscope (Phillips EM300) without being stained further. Sections incubated with PBS containing 0.05% v/v Tween 20 and 1.0% w/v BSA without the anti-casein antibodies constituted the negative controls.

RESULTS

Mouthrinse Study

The levels of Ca and Pi in the control (de-ionized water rinse) five-day-old supragingival plaque were 169 ± 103 and 242 ±

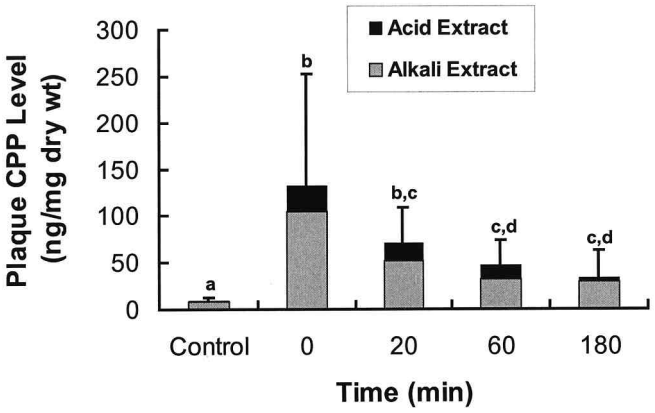

Figure 3. CPP levels in supragingival plaque detected by competitive ELISA [data presented as mean ± SD (n = 30)]. **(a-d)** Values significantly different ($p < 0.05$) from all other values not similarly marked, as shown by a one-way classification ANOVA with a *post hoc* Scheffé test.

60 nmol/mg dry weight, respectively (Fig. 1). The use of the unstabilized calcium phosphate mouthrinse did not significantly increase the plaque Ca and Pi levels (Fig. 1). The 2% and 6% w/v CPP-ACP mouthrinses significantly increased the plaque Ca and Pi levels in a dose-dependent fashion (Fig. 1). Electron-dense particles corresponding to the CPP-ACP were immunolocalized in the plaque samples to the surfaces of bacterial cells and to the intercellular plaque matrix (Fig. 2). These electron-dense particles were not observed in plaque untreated with the CPP-ACP mouthrinse or in plaque samples processed without the primary anti-casein antibody.

Sugar-free Chewing Gum Studies

(a) Enamel Remineralization

The total amount of calcium and inorganic phosphate *per* gum piece for all the gums studied was determined by analysis of acid extracts of the gums. This analysis revealed that the non-CPP-ACP-containing gums with added $CaHPO_4/CaCO_3$ or just $CaCO_3$ contained 5-13 times the total level of calcium *per* piece of gum than the Recaldent gums (Table). The Recaldent gums with the added CPP-ACP, although containing the lowest levels of total calcium, contained the highest levels of water-soluble calcium and inorganic phosphate (Table). The pellet and slab Recaldent gums containing the CPP-ACP also produced the highest level of enamel subsurface lesion remineralization in the two *in situ* studies (Table). In the slab gum study, the Recaldent gum produced more than twice the level of enamel remineralization than that of the other two gums that contained 8 and 13 times the total level of added calcium (Table). The level of enamel remineralization (%R) obtained with the 6 gums in the 2 studies was significantly correlated ($r = 0.96$, $p < 0.01$) with the level of water-soluble calcium phosphate *per* piece(s) of gum used *per* treatment.

(b) Incorporation into Plaque

The chewing of sugar-free gum containing CPP-ACP incorporated the CPP-ACP into plaque as detected by quantitative competitive ELISA of acid and alkali plaque extracts (Fig. 3). The plaque samples were extracted first with acid to dissolve the localized ACP nanoclusters, which should release CPP bound only to the ACP. The plaque residues were then extracted with alkali to extract CPP bound to bacterial cells and matrix molecules. The level of CPP in the alkali extract of the plaque samples, on average, represented 81% of the total CPP level extracted (Fig. 3). The plaque collected immediately after gum chewing contained 132 ± 121 ng of CPP *per* mg of plaque (dry weight). The CPP level in plaque decreased with time so that, 3 hrs after gum chewing, only 25% of the initial value could be detected; however, this level was still significantly higher (3.5-fold) than the baseline level (Fig. 3).

DISCUSSION

The results of the mouthrinse study showed that CPP-ACP was incorporated into supragingival dental plaque by binding onto the surfaces of bacterial cells, as well as to components of the intercellular plaque matrix, and significantly increased the plaque levels of calcium (Ca) and inorganic phosphate (Pi). The baseline levels of calcium and inorganic phosphate determined in this study are similar to those reported in other studies (Dawes and Jenkins, 1962; Ashley, 1975a,b). The 2% CPP-ACP mouthrinse increased plaque Ca and Pi levels by 118% and 57%, respectively, which are similar to the increases in plaque Ca and Pi levels obtained with the use of plaque-mineralizing solutions containing Ca, Pi, and urea (Pearce and Moore, 1985). A concern with plaque-mineralizing solutions is the promotion of calculus. However, CPP inhibit the transformation of amorphous calcium phosphate into crystalline phases (Holt and van Kemenade, 1989) such that they should not directly promote calculus formation but instead provide a plaque reservoir of soluble calcium phosphate ions capable of diffusing into subsurface enamel and promoting remineralization. This is consistent with the results of the chewing gum *in situ* remineralization studies which showed that the CPP-ACP-containing gums, although not containing the highest level of total calcium, did contain the highest level of water-soluble calcium phosphate and produced the highest level of enamel subsurface lesion remineralization, independent of chewing frequency or duration.

The increase in plaque Ca and Pi levels over the baseline plaque levels by use of the CPP-ACP rinses is consistent with the results of Rose (2000a,b), who has shown that CPP-ACP binds to a model streptococcal plaque *in vitro*, with twice the affinity of free calcium ions, providing a large calcium reservoir. It is interesting to note that the unstabilized calcium phosphate mouthrinse did not significantly increase plaque Ca and Pi levels. Since the predominant form of calcium in the unstabilized calcium phosphate solution would not have been free calcium ions but unstabilized ACP, then this result is also consistent with the work of Rose *et al.* (1997, 2000a,b), who showed that the major Gram-positive bacteria of supragingival plaque bind calcium as free ions interacting with surface phosphoryl and carboxylate groups. These results therefore suggest that the CPP are responsible for not only the stabilization and water solubility of ACP but also the incorporation of ACP into plaque by binding to bacterial cell surfaces and onto adsorbed macromolecules on the tooth surface. The immunolocalization study revealed that CPP were bound to the surfaces of bacterial cells as well as to the intercellular matrix. However, some bacterial species were

intensely stained at the cell surface (Fig. 2), suggesting a high affinity of the CPP for molecules on the surfaces of some bacterial species. The bacterial cell contains both hydrophilic and hydrophobic molecules on its surface (Rose *et al*., 1997). The CPP molecules also contain hydrophilic and hydrophobic regions, and it is possible that binding to the bacterial cell surface is mediated by Ca^{2+} cross-linking of the negative charges on the peptide and the cell surface molecules (*e.g.*, phosphoryl and carboxylate groups) as well as by hydrophobic- and hydrogen-bond-mediated interactions. For this reason, to extract CPP from plaque, we first extracted the plaque samples with acid to dissolve the ACP nanoclusters and to break any Ca^{2+} cross-links with plaque macromolecules. The plaque residues were then extracted with alkali in an approach to solubilize the CPP bound through hydrophobic- and hydrogen-bond-mediated interactions. The percentage of total CPP detected was considerably higher in the alkali extract (81%) than in the acid extract (19%), suggesting that the major bonds localizing the CPP at the bacterial cell surface were directly between the peptides and the bacterial surface molecules and were not predominantly mediated by acid-labile Ca^{2+} or ACP cross-linking. This could explain the efficient incorporation of the CPP-ACP nanocomplexes into plaque, where the CPP carrying the stabilized nanoclusters of hydrated ACP bind onto bacterial cell and intercellular matrix macromolecules localizing the bioavailable calcium and phosphate ions at the tooth surface. It is very likely that the level of CPP in plaque was underestimated, since the acid and alkali extraction procedures would be unlikely to release all the CPP from the bacterial cell surface, particularly if strong hydrophobic interactions were involved. Also, the anti-casein antibodies (Perich *et al*., 1999) used in the assay would not have detected CPP with minor structural modifications that would be expected in plaque through the presence of peptidase and phosphatase activity (Reynolds, 1987; Reynolds and Riley, 1989). Epitope mapping of the CPP with these anti-casein antibodies has shown that the phosphorylated residues as well as others are critical for recognition by the antibodies (Perich *et al*., 1999). Antibody recognition of the CPP is therefore extremely sensitive to the integrity of the peptides, such that the truncation, deamidation, and dephosphorylation that will occur in dental plaque through the activity of peptidases, amidases, and phosphatases, respectively (Reynolds and Riley, 1989), would reduce detection. The marked decrease in the ability of the anti-casein antibody to detect CPP in plaque after only 20 min (Fig. 3) is consistent with structural modifications of the CPP by bacterial enzymes in plaque and, with this methodology, underestimates the true level of CPP incorporated.

The low level of CPP in the baseline plaque sample could indicate cross-reactivity, non-specific binding by the antibody, or the incorporation of casein from dietary sources, since all participants consumed dairy products as part of their normal diets. However, the baseline levels were very low relative to the 39-fold increase that was obtained immediately after subjects chewed gum containing CPP-ACP (Fig. 3). The large variance in the plaque levels of CPP precluded any meaningful analysis of the half-life for CPP in plaque for each subject. This large variance is likely to be attributable to the CPP-ACP not being uniformly incorporated into the plaque, and to the variable nature of plaque resulting in variable binding and breakdown of the CPP. However, notwithstanding the large differences in the plaque CPP levels among subjects and among quadrants, the plaque sampled 3 hrs after gum chewing for all subjects still contained significantly ($p < 0.01$) higher amounts (4.6-fold higher) of CPP than the control baseline plaque.

The results of these studies have shown that CPP-ACP become incorporated into dental plaque and significantly increase the levels of plaque Ca and Pi ions. This result is therefore consistent with the proposed anticariogenic mechanism of the CPP, which is the localization of ACP at the tooth surface. Several authors have observed an inverse association of plaque Ca and Pi levels and caries experience, with higher plaque Ca and Pi levels being associated with lower caries experience (Dawes and Jenkins, 1962; Ashley, 1975a,b; Schamschula *et al*., 1977; Shaw *et al*., 1983). Higher plaque levels of Ca and Pi ions may result in a higher degree of saturation with respect to enamel mineral in plaque fluid, thereby lowering the risk of enamel demineralization and facilitating remineralization (Moreno and Margolis, 1988).

These studies highlight the importance of the CPP in delivering ACP to the tooth surface. However, the *in situ* remeralization results also demonstrate the importance of the CPP in stabilizing ACP and producing a highly water-soluble calcium phosphate phase. The Recaldent gums were superior to the other sugar-free gums in remineralizing enamel subsurface lesions *in situ*, even though the other gums contained from 5 to 13 times the level of total calcium. These remineralization results confirm and extend the results of Shen *et al*. (2001), who demonstrated the efficacy of CPP-ACP in sugar-free gum in remineralizing enamel subsurface lesions in an *in situ* model. The results of the current study indicate that this efficacy is superior to that achieved with other forms of calcium and highlight the important role of the CPP as an ACP-carrier localizing the highly soluble calcium phosphate phase at the tooth surface. This localization maintains high concentration gradients of calcium and phosphate ions in the subsurface enamel, thereby facilitating remineralization (Reynolds, 1999).

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