High-Resolution ^{1}H NMR Investigations of the Capacity of Dentifrices Containing a "Smart" Bioactive Glass to Influence the Metabolic Profile of and Deliver Calcium Ions to Human Saliva

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Received 29 July 2008; revised 19 January 2009; accepted 6 February 2009
Published online 22 April 2009 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.b.31377

Abstract: Dentifrices containing H_2O-reactive bioactive glasses alleviate hypersensitivity in teeth via the blockage of open dentinal tubules. Here, the ability of two such products to release Ca^{2+} ions into human saliva was investigated, together with their influence on the status of this biofluid's ^{1}H NMR-detectable biomolecules. Human salivary supernatants were equilibrated with increasing volumes of those derived from each dentifrice (5.00 min at 37°C). These biofluids were also equilibrated at 37°C with a preselected quantity of the intact products (samples were collected at increasing timepoints). Salivary Ca^{2+} concentrations were monitored by a ^{1}H NMR technique involving ethylenediamine tetra-acetate addition and/or atomic absorption spectrometry. Added Ca^{2+}- and dentifrice supernatant volume (DSV)-induced modifications to the salivary ^{1}H NMR profile were explored by spectral titration. Data acquired demonstrated added DSV-dependent increases in salivary Ca^{2+} concentrations and (Ca^{2+}-independent) modifications to the intensities of selected salivary ^{1}H NMR signals, particularly those of the malodorous amines methyl-, dimethyl-, and trimethylamines, which were diminished by up to 80% of their prior values. Time-dependent elevations in salivary Ca^{2+} level were observed on equilibration with the intact dentifrices. Added Ca^{2+} ions exerted a concentration-dependent influence on a range of resonances (including those of citrate, succinate, pyruvate, and lactate). These data provide valuable information regarding the mechanisms of action of the products tested. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 91B: 88–101, 2009

Keywords: bioactive glass; calcium sodium phosphosilicate; tooth hypersensitivity; Ca^2 ions; saliva

INTRODUCTION

Human tooth enamel remains highly susceptible to demineralization in view of its recurrent exposure to saliva, foods, and commonly consumed beverage drinks, the latter of which often having low-pH values and contain Ca^{2+} ion-chelating organic acids/anions, particularly citrate. These processes leach essential tooth minerals and give rise to an increased susceptibility to decay.[1,2] Indeed, incipient caries (i.e., very small enamel surface defects that are typically left untreated in the early stages) commonly arises from such demineralization phenomena and may also occur in patients with exposed regions of dentin, which derive from decay below the cementum–enamel junction. In view of these events, there has been a high level of research work conducted aimed at slowing this natural process, including the topical application of fluoride anion (F^-),[3] together with further agents.

This demineralization process gives rise to a time-dependent cavitation of the enamel coating with a contingent exposure of the underlying tooth structure. Such decay is commonly treated by drilling out the decayed region and the insertion of a semipermanent filling material. However, there is clearly a major requirement for a less-invasive pathway for the arrest and reversal of this process.

Although temporary prophylactic pit and fissure sealants are now frequently used for the prevention of decay at particularly susceptible loci,[4] such materials also require a fixing agent and dry application and do not result in the provision of an optimal level of sealing.[5] Similarly, liner and base materials are also used for the purpose of treating newly exposed tooth surfaces, including those arising from dental drilling. Indeed, such materials are designed and formulated in order to diminish dentine permeability at the

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tooth-material interface[6,7] and also offer protection against microleakage from the filling material and its insertion site, together with the sealing of dentinal tubules. Such materials include (1) liners or cavity varnishes[8] consisting of organic gums, which remain subsequent to the evaporation of organic solvents in which they are dissolved prior to treatment and (2) the stepwise application of a neutral oxalate solution (e.g. $K_2[C_2O_4]$) followed by an acidic one (e.g., one containing $KH[C_2O_4]$) to the layer.[9,10] However, many disadvantages of the former (gum) treatment have been revealed, such as acid vulnerability, junctional leakage, and lack of adherence,[11] and the latter (oxalate) process gives rise to only a poor level of tubular seal occlusion.

Novel advances in the area of bone replacement materials have led to the design and development of bioactive-, biocompatible-glasses as agents for the induction and/or facilitation of osteogenesis in physiological media.[12] Indeed, the bond-strength of the bone-glass junction has been shown to be high and resistant against physiological attack/insult,[13] and, to date, toxicological investigations have not revealed any deleterious effects of these agents in bone and soft tissue both *in vitro* and *in vivo*.[14]

Bioactive glasses comprise oxides of Ca^{2+} and Na^+, together with silicates and phosphates in a preselected proportion that ensures surface activity and, synchronously, the ability to chemically bond strongly to both teeth and bone. Indeed, the applications of many bioactive glasses as bone substitutes have been previously investigated in detail.[12,15,16]

Phosphopeptides (isolated from a tryptic digest of casein via aggregation with calcium phosphate and purified via ultrafiltration)[17] have the ability to stabilise nanoclusters of amorphous calcium phosphate,[18] whereas casein bioactive glasses consisting of sodium calcium phosphosilicate derivatives release Ca^{2+} and phosphate ions in aqueous environments, a process giving rise to localized elevations in pH and osmotic pressure.[19] Indeed, exposed dentin can provide a nucleation site for these ions, which locally generate hydroxycarbonate apatite species.[20]

The mechanism for the process observed on exposure of such bioactive glasses to aqueous biofluid systems primarily involves an exchange of glass-containing Na^+ ions with protons (H^+) therein, (e.g., those of human saliva) with a synchronous elevation in pH value. Ca^{2+} and phosphate ions are then transferred from this material to form a surface layer enriched with these species. Moreover, this process involves a further layer that becomes increasingly silicate-rich in view of the time-dependent removal of Na^+, Ca^{2+}, and phosphate ions from the glass.[21–23]

In studies involving the fabrication of replicated tooth forms and their implantation into the pre-extracted incisor sockets of adult baboons, Stanley et al.[24] found that a bioactive glass material significantly enhanced the attachment of the implant to surrounding bone, as noted by histological evaluation performed at a postimplant time-point of 6 months. Subsequent clinical investigations in humans have revealed that bioactive glass granules are valuable agents for the treatment of periodontal defects, together with further hard tissue-based problems in the oral environment.[25–29]

However, the materials tested in the above investigations did not demonstrate potential further advantages offered by an agent, which combines a facile method of application with tooth structure adherence, benefits which include the ready penetration of extremely small tooth structure defects and the longevity of both biophysical and biochemical interactions with tooth structure following treatment. In view of these critical, therapeutically important phenomena, a novel bioactive glass formulation available for delivery in selected dentifrices has been recently developed by Novamin Technology Incorporated (Alachua, FL). This agent (Novamin^R) consists of particles of <90 μm diameter (and also contains a therapeutically effective remineralizing content of those <10 μm) and was designed, formulated, and developed with regard to its potential and/or established therapeutic actions targeted at periodontal diseases, including its ability to seal pits and/or fissures, promote remineralization, line tooth structures, cap pulp, occlude dentinal tubules, treat post-surgical tooth structure, and provide an appropriate surface for the regeneration of tissue. This bioactive glass therefore serves as a "carrier" for Ca^{2+} and phosphate ions and is successful in maintaining them in an "amorphous" state in selected dentifrice products.

The therapeutic applications of such formulations are also focused on dentine hypersensitivity, and a recent investigation found that such products covered the surface of dentine and/or occluded open dentinal tubules[30] which, according to Brannstrom and Atrom's hydrodynamic hypothesis,[31] permit tubular fluid flow, a process giving rise to changes in pressure, which excite dental pulp nerve endings, and dentifrices containing bioglasses as active ingredients provided a greater surface coverage and tubular occlusion than those without these agents.[32] Indeed, the inclusion of bioactive glass particles in a selectively formulated vehicle has proven to be an effective formulation for the treatment of dentine hypersensitivity.[33]

This product has also been reported to exert bacteriostatic or bacteriocidal actions, a phenomenon probably related to the change in pH induced by the dissolution of the ions from the surface of the glass (as noted earlier) and lack of bacterial adherence to the glass surface.[34] Such actions are also putatively ascribable to their ability to elevate pH and osmotic pressure values at therapeutically relevant sites (the dentogingival margin). Indeed, Stoor et al.[35] also investigated the influence of a bioactive glass (S53P4) on the viability of *actinomycetumcomitans*, *Porphromonas gingivalis*, *Streptococcus naeslundi*, *Strepococcus mutans*, and *Streptococcus sanguis* and found that suspensions of this material exerted a broad spectrum of biocidal activity toward both supra- and subgingival plaque microorganisms. Hence, such agents may also offer carioststic benefits when present in oral health care products.

A further study[36] examined the anti-gingivitis and anti-plaque effects of a bioactive glass-containing dentifrice against those of a control one and found that the former product gave rise to significantly greater reductions in gingival bleeding and supragingival plaque throughout a 6-week study period.

Hence, these products offer valuable therapeutic advantages over previously developed formulations, specifically their facile application and rapid adherence to the tooth structure. Indeed, such advantages involve their ability to penetrate extremely small structural defects, together with the occurrence of prolonged, therapeutically relevant biochemical/biophysical interactions subsequent to their application.

Results concerning the applications of high-field proton (^{1}H) NMR spectroscopy to the multicomponent analysis of biomolecules (including inflammatory mediators) present in complex biological fluids have been previously presented (reviewed in Ref. 37). This technique offers many advantages over alternative time-consuming, labor-intensive analytical methods since it permits the rapid, non-invasive, and simultaneous study of a multitude of components present in biofluids such as human saliva and generally requires little or no knowledge of sample composition prior to analysis. Moreover, chemical shift values, coupling patterns, and coupling constants of resonances present in ^{1}H NMR spectra of such complex, multicomponent systems provide much valuable molecular information regarding both endogenous and exogenous chemical species detectable. The broad overlapping resonances, which arise from macromolecules present, are routinely suppressed by spin-echo pulse sequences, giving rise to spectra which contain many sharp, well-resolved signals attributable to a wide variety of low-molecular-mass (non-protein-bound) components and the mobile portions of macromolecules that are detectable at a sensitivity of $<5 \times 10^{-6}$ mol.dm^{-3} (at an operating frequency of 600 MHz) for samples not subjected to any form of pre-concentration technique.

High-resolution NMR techniques have also been previously used to (1) index salivary biomolecules and supply valuable metabolic data regarding intra- and inter-subject variability in the salivary concentrations of a range of components readily detectable by this technique, specifically organic acid anions and malodorous amines,[38] and (2) probe the complexation of Ca^{2+} ions by low-molecular-mass salivary biomolecules (results obtained revealed that the organic acid anion citrate acts as a powerful oxygen-donor complexant for this metal ion).[39]

In this investigation, high-resolution, ^{1}H NMR spectroscopy has, for the first time, been used to evaluate the time-dependent release of Ca^{2+} ions from two novel bioactive glass-containing dentifrices [products I (Denshield™ Toothpaste) and II (Oravive® Tooth Revitalising Toothpaste), Novamin Technology Incorporated] via the *in vitro* monitoring of salivary Ca^{2+} concentrations, determined by measurements of the intensity of the characteristic ethylenic resonances of its complex with ethylenediamine tetra-acetate (EDTA) present in the ^{1}H NMR profiles acquired on salivary supernatant samples treated with this chelator following equilibration with each product investigated (and/or an alternative atomic absorption spectrometric method not requiring pre-additon of this chelator). Modifications to the ^{1}H NMR spectra of human salivary supernatants induced by its equilibration (at 37°C) with increasing added (microliter) volumes of (1) supernatants derived from each dentifrice product examined and (2) a standard, stock Ca^{2+} ion solution were also evaluated. Furthermore, salivary supernatant specimens were equilibrated with pre-weighed quantities of the (solid) dentifrices at this temperature in order to monitor product- and time-dependent modifications to salivary Ca^{2+} ion concentrations (via the above techniques) and to determine their influence on salivary ^{1}H NMR profiles (samples were collected at increasing time-points). The biochemical, clinical, and therapeutic significance of the results acquired are discussed in detail.

MATERIALS AND METHODS

Materials

Products I and II (Denshield™ and Oravive® Tooth Revitalising Toothpastes respectively) were provided by Novamin Technology Incorporated, sodium dihydrogen citrate, EDTA, sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$] propionate (TSP), and deuterium oxide (^2H_2O) were obtained from the Sigma-Aldrich Chemical Co. (Milwaukee, WI), and calcium chloride hexahydrate, and 10% (w/v) lanthanum chloride ($LaCl_3$) solution were purchased from Fisher Scientific (Loughborough, UK).

Sample Collection and Preparation

Each dentifrice was prepared for ^{1}H NMR analysis via the addition of 2.00 ml of doubly distilled H_2O to 200 mg samples by thorough rotamixing, centrifugation (3,500 rpm for a 20-min. period) and removal of the clear supernatants. Subsequently, further dentifrice supernatants derived from the addition of 5.00 ml volumes of doubly distilled water to either 249 mg of dentifrice I or 247 mg of dentifrice II, followed by rotamixing and centrifugation as described earlier were prepared and used for experiments involving their addition to those derived from "whole" saliva specimens as outlined below.

According to the manufacturers specifications, dentifrice I contained (in order of decreasing content) glycerol, polyethyleneglycol, amorphous silica, calcium sodium phosphosilicate (Novamin®), sodium lauryl sulphate, titanium dioxide (TiO_2), carbomer, potassium acesulfame, and flavoring components (anethole, eucalyptol, L-menthol, and peppermint oil). Dentifrice II contained the same ingredients in the above order, but with a much larger number of flavouring agents (aldehyde C-9, anethole, anise oil,

anise aldehyde, L-carvone, dihydrocarvone, dihydrocarvyl acetate, eucalyptol, eucalyptus oil, eugenol, *cis*-3-hexenyl *iso*-valeric acid, lime oil, L-menthol, L-menthone, "orange juice carbonyls," "orange terpenese," peppermint oil, spearmint oil, terpinene-4-ol, α-terineol, and vanillin). Dentifrices I and II contained 7.5 and 5.0% (w/w) Novamin^R, respectively, with a mean particle size of 12 μm for this active agent in both products.

Unstimulated saliva samples were obtained from non-medically-compromised healthy human volunteers (n = 18) with an age range of 22–49 years (mean age ± standard deviation 32.8 ± 8.2 years). None of these volunteers had any active periodontal disease or active dental caries. To avoid any interferences arising from the introduction of exogenous and xenobiotic agents into the oral environment, all volunteers were requested to collect all saliva available (ca. 5–12 ml), i.e., "whole" saliva expectorated from the mouth, into a plastic universal container immediately after waking in the morning. Each volunteer was also asked to completely abstain from oral activities (i.e., eating, drinking, tooth-brushing and smoking) during the short period between awakening and sample collection (<5 min) on the morning of the study. Saliva samples were transported to the laboratory on ice and immediately centrifuged on their arrival to remove cells and debris. The supernatants were then stored at −70°C for a maximum duration of 20 h prior to NMR analysis.

Aliquots (6 × 0.50 ml) of each of a series of 5 human salivary supernatant specimens with sufficient total volumes (>8.0 ml) were treated with 0.00, 0.02, 0.04, 0.06, 0.08, and 0.10 ml volumes of the above dentifrice I supernatant, followed by (0.10 − x) ml of doubly distilled water [where x = volume of dentrifrice supernatant added (ml)], whilst a further 6 × 0.50 ml aliquots of each of the above group of such salivary supernatants (n = 5) were treated with equivalent volumes of dentifrice II supernatant [0.00–0.10 (x) ml] and correspondingly (0.10 − x) ml of doubly-distilled water.

In a further experiment, 12 × 0.50 ml aliquots of individual samples from an additional group of human salivary supernatants (n = 6) with limited citrate concentrations (i.e., little or no ^{1}H NMR-detectable citrate) were each treated with this biomolecule (final concentration 2.00 × 10^{-4} mol.dm^{-3}), and the first and second sub-groups of six of these were treated with 0.00, 0.02, 0.04, 0.06, 0.08, and 0.10 ml volumes of dentifrice I and II supernatants, respectively [and subsequently, corresponding (0.10 − x) ml volumes of doubly-distilled water as described earlier]. Each sample was thoroughly rotamixed prior to their preparation for ^{1}H NMR analysis (Sample Collection and Preparation section). About 10–100 μl additions of each of the two dentifrice supernatants tested were made to salivary supernatants using a pre-calibrated micropipette.

Each of the above pre-treated salivary supernatant samples were then equilibrated at a temperature of 37°C in a water bath for a period of 5.00 min.

Determination of Salivary pH

After collection, the pH values of aliquots of each saliva specimen were determined electrometrically using a Hanna HI 1270 combination pH electrode and meter. The mean (± standard error, SE) pH value of human saliva samples (n = 18) prior to treatment with dentifrice supernatants was 6.94 ± 0.09 (range, 6.58–7.34).

Equilibration of Human Salivary Supernatants with Untreated (Solid) Bioactive Glass Dentifrice Formulations

For further salivary supernatant samples with sufficient volumes available (n = 5), 48 mg of dentifrice I or II was added to individual 5.00 ml aliquots, the mixtures thoroughly rotamixed, and then equilibrated whilst shaking in a water bath at 37°C. 0.60 ml aliquots of these samples were collected prior and immediately subsequent to dentifrice addition and then at time-points of 30, 60, 90, 120, and 150 min. After collection, each aliquot was thoroughly centrifuged and the supernatants removed for ^{1}H NMR and/or atomic absorption analysis.

NMR Measurements

Single-pulse ^{1}H NMR spectra were acquired on a Bruker Avance AM-600 spectrometer (Queen Mary University of London facility) operating at a frequency of 600.13 MHz, in quadrature detection mode and a probe temperature of 298 K. Each spectrum corresponded to 128 free induction decays (FIDs) for control and dentifrice supernatant-treated saliva samples, 5.6-μs pulses and a 1-s relaxation delay. The intense H_2O/HOD signal was effectively suppressed via presaturation with gated decoupling during the delay between pulses. Typically, 0.60 ml of each sample was placed in a 5-mm diameter NMR tube, and 0.10 ml of a 2.50 × 10^{-3} mol.dm^{-3} solution of TSP (internal chemical shift reference, δ = 0.00 ppm and quantitative ^{1}H NMR standard) in deuterium oxide (^2H_2O) was added, the latter to provide a field frequency lock.

For salivary supernatant specimens, the methyl group resonances of acetate (s, δ = 1.920 ppm), alanine (d, δ = 1.487 ppm), and/or lactate (d, δ = 1.330 ppm) served as secondary internal chemical shift references. The identities of biomolecules responsible for resonances present in the salivary ^{1}H NMR profiles acquired were routinely assigned by a consideration of characteristic chemical shift values, coupling patterns, and coupling constants. Where required, standard additions of authentic compounds were made to confirm assignments. Exponential line-broadening functions of 0.30 Hz were routinely used for the purpose of processing.

Acquisition of ^{1}H-^{1}H Correlation and Total Correlation Spectra

^{1}H-^{1}H TOCSY spectra were acquired on the Bruker Avance AX-600 spectrometer using the pulse sequence

RD-(90°-t_1-spin lock)-ACQ,[40] 90° on the Avance AX-600 spectrometer being equivalent to 8.4 μs for these experiments. The spin-lock used the MLEV-17 sequence,[41] with a typical mixing time of 70 ms (for experiments in which a restriction of carbon chain correlations was required, this mixing time was only 20 ms). Acquisition parameters were 256 t_1 increments, each of magnitude 2,048 data points, spectral width 12,019 Hz in each dimension, 64 transients in each case, four dummy scans, relaxation delay (RD) 3 s, and acquisition time 0.17 s. Sine-bell-squared window functions shifted by $\pi/2$ (i.e., a cosine squared function) were applied in each dimension, with zero-filling twice in f_1, prior to transformation of the matrix of size 2,048 × 1,024 data points. The standard phase-sensitive (Time Proportional Phase Incrementation) method[42] was used for optimal detection along the second dimension in these experiments.

Two-dimensional shift-correlated ^{1}H-^{1}H COSY of human salivary supernatants were also acquired on this NMR facility using the standard sequence of Aue et al.,[43] with 1,024 data points in the t_2 dimension, 512 increments of t_1, a 2.00-s relaxation delay, and 16 transients.

Determination of Salivary Ca^{2+} and Mg^{2+} Concentrations by a Complexometric Method Involving the ^{1}H NMR Detection of Their EDTA Complexes

Typically, 0.60 ml aliquots of human salivary supernatants were treated with 0.09 ml of a 6.80×10^{-1} mol.dm^{-3} solution of EDTA subsequent to their equilibration with the above dentifrice formulation supernatants at 37°C for 5.0 min. Salivary supernatants arising from experiments involving their equilibration with intact dentifrice products for increasing time-periods were treated with EDTA in the same manner. All samples were then equilibrated at an ambient temperature for a period of 30 min. prior to ^{1}H NMR analysis. The 600 MHz ^{1}H NMR spectra of these EDTA-treated samples were acquired in the manner described earlier, and the singlet ethylenic group proton resonances of free (uncomplexed) EDTA and its Ca^{2+} and Mg^{2+} complexes (δ = 3.28, 2.57, and 2.76 ppm, respectively) were electronically integrated, their intensities expressed relative to that of the TSP internal standard (final concentration 3.57×10^{-4} mol.dm^{-3}) and their concentrations determined, as previously described by Silwood et al.[39] Alternatively, salivary Ca^{2+} and Mg^{2+} levels were determined from the ratios of the intensities of their ethylenic group proton resonances to that of free EDTA, the latter's final added (total) concentration being 9.83×10^{-3} and 1.176×10^{-2} mol.dm^{-3} for salivary supernatants directly treated with those of the dentifrices tested, and those pre-equilibrated with specified weights of the intact formulations at 37°C for increasing periods of time, respectively.

Atomic Absorption Spectrometric Determination of Salivary Ca^{2+} Ion

About 0.60 or 1.00 ml aliquots of salivary supernatant samples collected at increasing time-points during their equilibration with pre-weighed quantities of the dentifrice products tested were added to 0.10 ml of a 10.0% (w/v) solution of $LaCl_3$ in order to alleviate interferences from oxyanions, and the mixture was then diluted to a final volume of 10.00 ml with doubly-distilled water. Ca^{2+} concentrations were determined on a Thermo Solaar S4 flame atomic absorption spectrometer operating at a wavelength of 422.7 nm, a bandpass of 0.5 nm, an air-acetylene flame with an acetylene flow-rate of 1.40 ml/min, and a sample uptake rate of 5.00 ml.min^{-1} with 4 s per reading and three resamples. A series of five $CaCl_2$ calibration standards containing 1.00, 2.00, 3.00, 4.00, and 5.00 mg.dm^{-3} Ca^{2+} and the above (final) $LaCl_3$ concentration [0.10% (w/v)] were prepared and also analyzed using this technique, and plots of signal absorbance versus Ca^{2+} concentration were linear (r = 0.99975, $p = 4.83 \times 10^{-6}$).

RESULTS

High-Field, High-Resolution Proton NMR Analysis of Human Saliva

^{1}H NMR spectra (600 MHz) acquired on human salivary supernatant specimens contained an abundance of prominent, sharp resonances ascribable to a wide range of low-molecular-mass components. Indeed, signals assignable to short-chain organic acid anions, amino acids, and carbohydrates were readily observable, as previously described.[38,44] In addition to considerations of chemical shift values, coupling patterns, and coupling constants, together with comparisons with established literature values (making allowances, where appropriate, for the analytical samples' pH values and the pH-dependence of selected resonances in spectra acquired therefrom), the identities of many of these resonances were confirmed or supported by (1) the acquisition of two-dimensional (2D) proton–proton (^{1}H-^{1}H) COSY and TOCSY NMR spectra, and (2) treatment of the salivary supernatants with standard additions of a range of authentic, pure components (involving the addition of microliter aliquots of ~5.0 mM aqueous solutions), which was particularly useful for ratifying components responsible for signals arising from uncoupled nuclei (i.e., singlets), or those with multiplets not readily detectable in either 1D or 2D spectra in view of their low concentrations.

The organic acid anions predominantly arise from microbial metabolism and hence these agents (either individually, or two or more in a multivariate sense) conceivably represent chemotaxonomic markers of microbial activity in the oral environment. Indeed, *n*-butyrate is produced in large quantities by the pathogenic micro-organism *P. gingivalis*.[45]

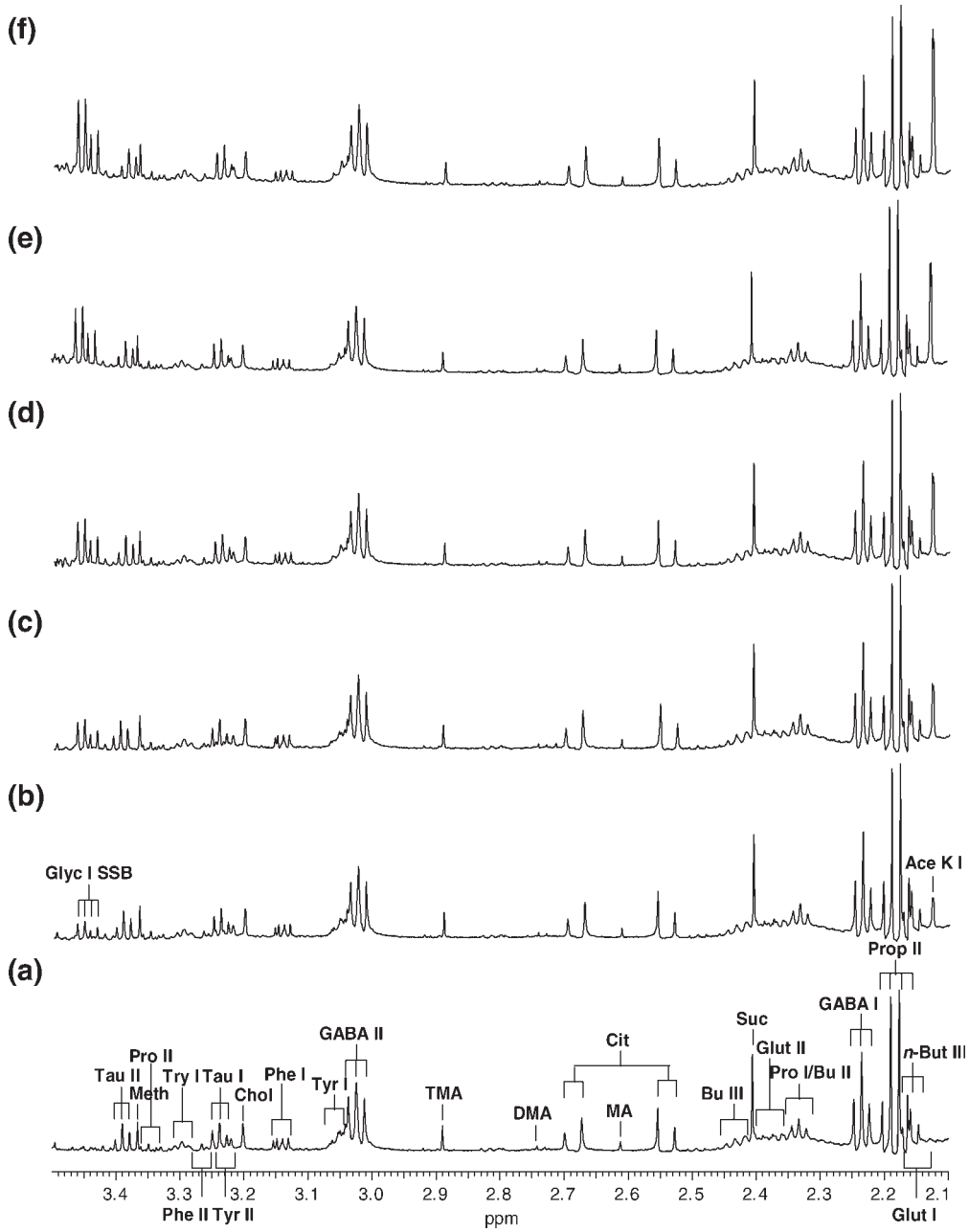

Figure 1. Expanded 2.10–3.50 ppm regions of 600.13 MHz single-pulse ^{1}H NMR spectra of an unstimulated salivary supernatant specimen treated with (a) 0 (untreated control), (b) 0.02, (c) 0.04, (d) 0.06, (e) 0.08, and (f) 0.10-ml aliquots of dentifrice I supernatant (and 0.10, 0.08, 0.06, 0.04, 0.02, and 0 ml of doubly distilled water, respectively), and (g) 0, (h) 0.02, (i) 0.04, (j) 0.06, (k) 0.08, and (l) 0.10 ml of dentifrice II supernatant (and 0.10, 0.08, 0.06, 0.04, 0.02, and 0 ml of doubly distilled water, respectively). Typical spectra are shown. Abbreviations: Ace K I, acesulfame K-CH_3; Bu II and III, β-hydroxybutyrate β and β' protons, respectively (*AB*X coupling system); *n*-But III, *n*-butyrate α-protons; Chol, choline-$N^+(CH_3)_3$; Cit, citrate$-CH_2-C(OH)(CO_2^-)-CH_2-$ AB protons; DMA, dimethylamine-CH_3′s; GABA I and II, γ-amino-*n*-butyrate α- and γ-CH_2 protons, respectively; Glut I and II, glutamate β-and γ-CH_2 protons, respectively; Glyc I SSB, glycerol-C$-$CH_2OH spinning sideband signal; MA, methylamine-CH_3; Meth, methanol-CH_3; Phe I and II, phenylalanine *AB*X β- and α-protons, respectively; Pro I and Pro II, proline β- and δ-CH_2 protons respectively; Prop II, propionate-CH_2 group protons; Suc, succinate-CH_2; Tau I and II, taurine $H_3N^+CH_2-$ and $-CH_2SO_3^-$ protons, respectively; TMA, trimethylamine-CH_3′s; Try I, tryptophan β-CH_2 protons; Tyr I and II, tyrosine *AB*X β protons.

Treatment of the salivary supernatants with those derived from each oral health care product revealed that agents present in the latter formulations were also readily detectable in the ^{1}H NMR spectra acquired. Indeed, the results acquired demonstrated that each ^{1}H NMR-responsive component present in either dentifrice (glycerol, stea-

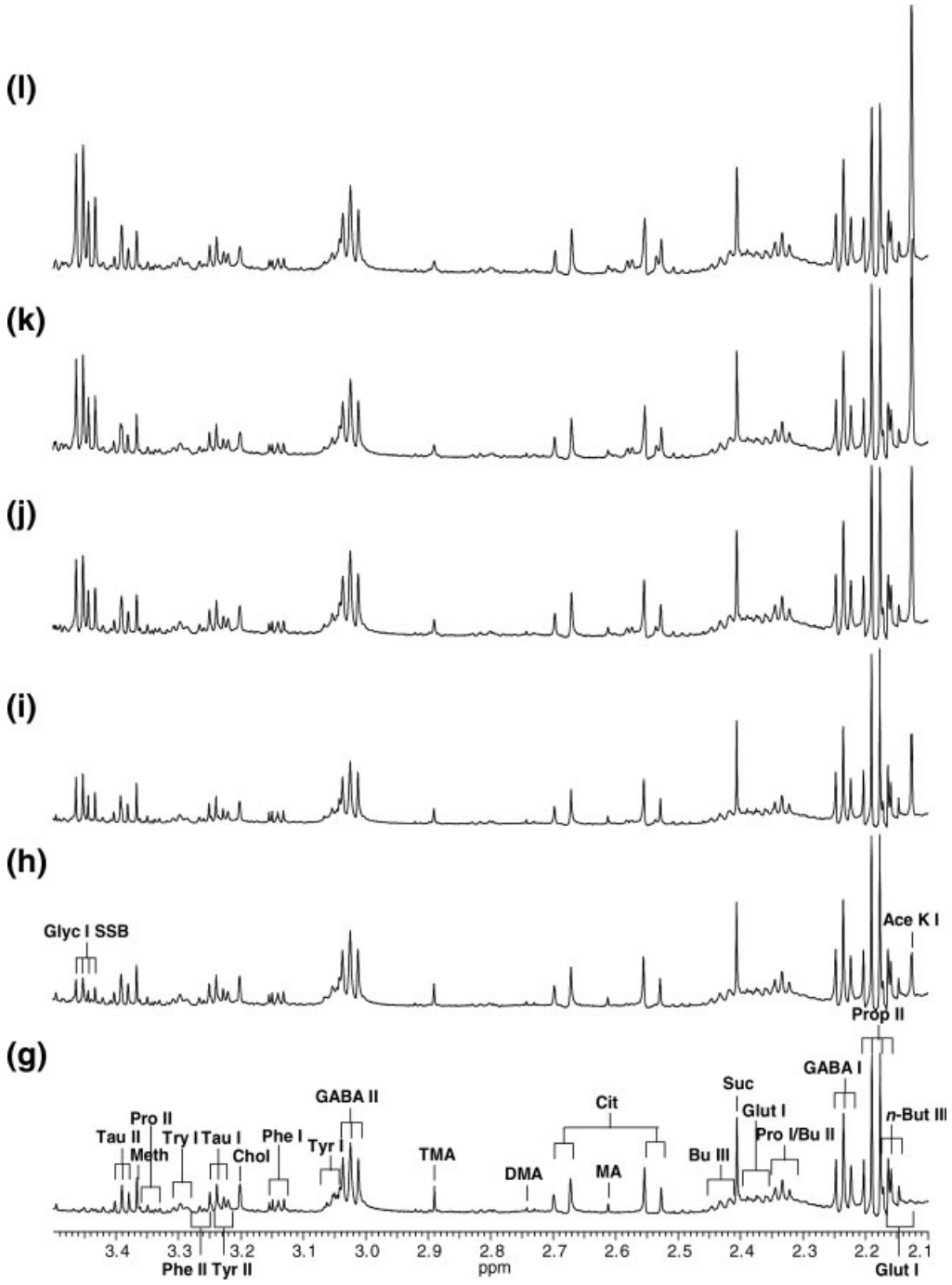

Figure 1. (*Continued*)

rate, sodium lauryl sulphate, and acesulfame) was readily observable in the ^{1}H spectra acquired with little or no interference from resonances arising from salivary components (data not shown). As expected, the ratios of the electronically integrated intensities of each dentifrice component's ^{1}H NMR resonance to that of an added internal standard increased linearly with added dentifrice supernatant volume (DSV).

Clearly added dentifrice supernatant-induced modifications to the ^{1}H NMR profiles of human saliva were also observed (Figure 1). Indeed, particularly notable were decreases in the intensities of resonances assigned to methylamine (*s*, δ = 2.61 ppm), dimethylamine (*s*, δ = 2.74 ppm), and trimethylamine (*s*, δ = 2.89 ppm), which were clearly dependent on added DSV for both products investigated here. Plots of the salivary concentrations of methylamine and trimethylamine (determined from the TSP-normalized ^{1}H NMR resonance intensities of each of these malodorous amines) versus added supernatant volume are shown for each product in Figure 2: both mean $\pm$ standard

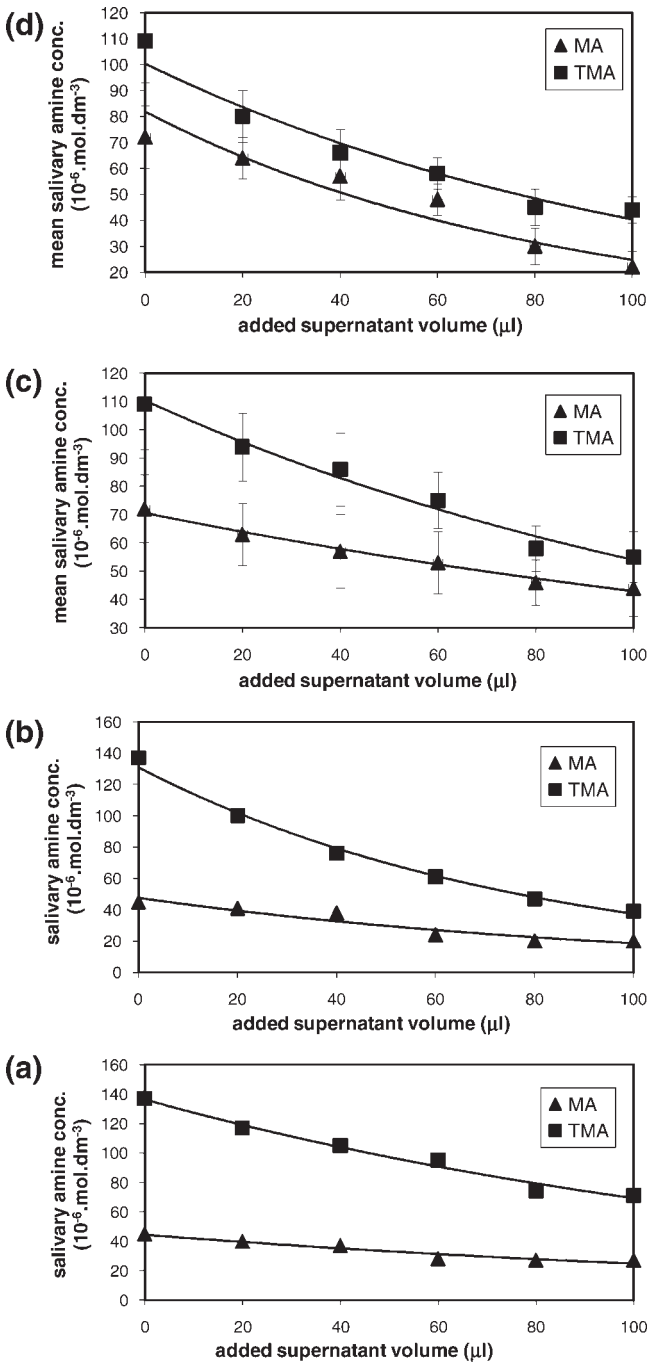

Figure 2. Plots of ^{1}H NMR-determined salivary methylamine and trimethylamine concentrations versus added dentifrice supernatant volume for products I and II. Results for a typical saliva specimen are shown in (a) and (b) for products I and II, respectively, whereas mean ± SE values for all five samples used in these experiments are exhibited in (c) and (d) (corresponding to products I and II, respectively).

error (s.e.) data for the five salivary supernatant specimens used for these experiments, and results for a typical sample, are shown. These decreases in amine concentrations were also observed in the salivary supernatant specimens equilibrated for increasing periods of time with both of the intact (solid) dentifrices, the magnitude of the reductions observed increasing with increasing equilibration time, especially at the lower time-points (data not shown).

As expected, equilibration of human salivary supernatants with those derived from each of the bioactive glass-containing dentifrices examined here (as described in the Materials and Methods section) also gave rise to detectable modifications to the chemical shift values and AB system-coupling patterns of salivary citrate resonances in specimens in which this biomolecule was ^{1}H NMR-detectable. Indeed, for selected samples, addition of supernatants obtained from either dentifrice product resulted in a downfield shift of citrate's A proton signal, and an upfield shift for that of its B proton. However, for all samples with ^{1}H NMR-detectable citrate examined, there were small but nevertheless significant broadenings of these resonances (data not shown). These data are consistent with an increase in its level of saturation by Ca^{2+} ions, which are released from the bioactive glass formulation present therein.[39] Similarly, the A and B citrate ^{1}H resonances of selected salivary supernatant samples that were pretreated (i.e., "spiked") with this powerful Ca^{2+} complexant (added concentration 2.00×10^{-4} mol.dm^{-3}) also displayed this behavior following addition of increasing volumes of either dentifrice supernatant (in view of the much higher [citrate]:[Ca^{2+}] concentration ratios, the magnitude of such shifts were much smaller than those observed with untreated salivary supernatant specimens and were only observable in ~30% of samples investigated).

Treatment of control (untreated) salivary supernatant samples with a final concentration of 9.83×10^{-3} mol.dm^{-3} EDTA, i.e., a concentration greater than that of salivary Ca^{2+} (mean level 1.55×10^{-3} mol.dm^{-3} [39]) gave rise to the generation of intense ethylenic ^{1}H NMR singlet resonances located at 2.57 and 2.71 ppm attributable to its Ca^{2+} and Mg^{2+} complexes, respectively; the corresponding signal of free (uncomplexed) EDTA had a chemical shift value of 3.27 ppm (Figure 3). Moreover, spectra acquired on salivary supernatants pre-treated with increasing microliter aliquots of the supernatant derived from products I and II gave rise to added volume (i.e., released Ca^{2+} ion concentration)-dependent elevations in the intensity of the ethylenic group ^{1}H NMR resonance of the $Ca^{2\pm}$ EDTA complex, an observation accompanied by corresponding decreases in that of free EDTA. Hence, these experimental data provide clear evidence for dentifrice-mediated increases in salivary Ca^{2+} ion concentrations (Figure 3 reveals clear increases in the $Ca^{2\pm}$ EDTA: free EDTA resonance intensity ratio for a typical sample pre-treated with a specified volume of each dentifrice supernatant). Plots of salivary Ca^{2+} concentration versus volume of added dentifrice (μL) were clearly linear, typical correlation coefficient (r) values being 0.9845 ($p = 3.57 \times 10^{-4}$) for product I and 0.9943 ($p = 4.82 \times 10^{-5}$) for product II (Figure 4). The salivary Mg^{2+} level remained independent of added supernatant volume for both products tested, an observation clearly indicating that no available Mg^{2+} ions are present in these media.

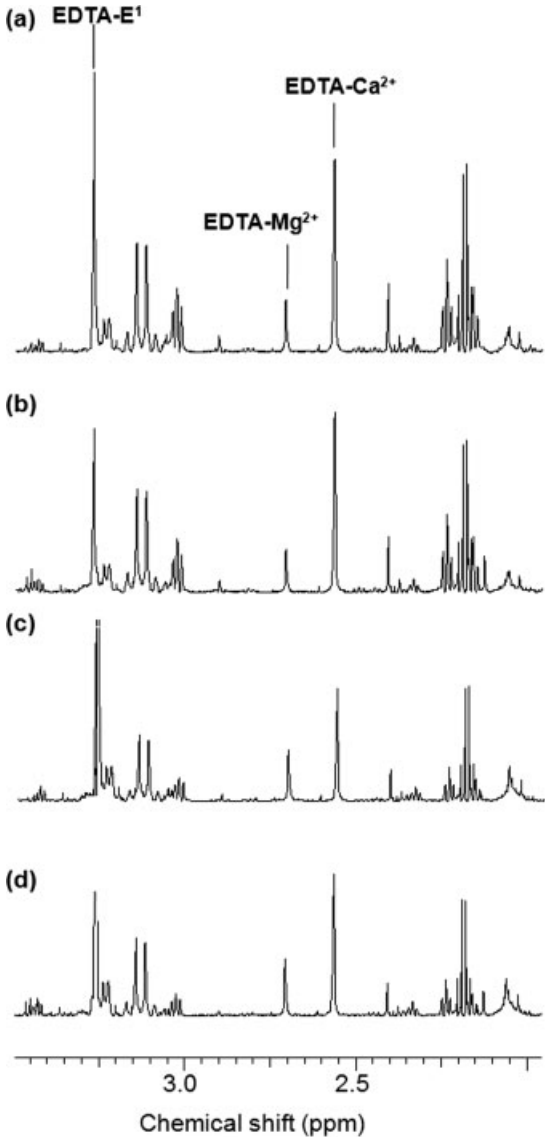

Figure 3. Estimation of salivary Ca^{2+} and Mg^{2+} ion concentrations by the ^1H NMR detection of their complexes with EDTA. Expanded 1.96–3.50 ppm regions of the 600.13 MHz ^1H NMR spectra of a typical salivary supernatant sample (a) before and (b) after equilibration with 0.10 ml of dentifrice I supernatant (and, correspondingly, 0 ml of doubly-distilled water). Subsequent to equilibration, samples were treated with EDTA [final added (total) concentration 9.83 mmol.dm^{-3}]. (c) and (d), as (a) and (b), respectively, but prior and subsequent to equilibration of the sample with 0.10 ml of dentifrice II supernatant. Typical spectra are shown. Abbreviations: as Figure 1, with EDTA-Ca^{2+} and EDTA-Mg^{2+} representing singlet signals ascribable to the ethylenic group protons (>N−CH_2CH_2−N<) of the Ca^{2+} and Mg^{2+} complexes of EDTA, respectively, and EDTA-E^1 that of free (uncomplexed) EDTA.

For selected salivary supernatant specimens containing citrate, or those pre-treated with this ^1H NMR-detectable Ca^{2+} complexant (added concentration 2.00×10^{-4} mol.dm^3), modifications in the chemical shift values of both the A and B protons of citrate's two —CH_2 groups were noted on addition of EDTA, the former to lower and the latter to higher frequency. This observation was accompanied by the appearance of the relatively intense ethylenic ^1H singlet resonance (δ = 2.57 ppm) attributable to the $Ca^{2\pm}$ EDTA complex, an observation corresponding to the complete transfer of Ca^{2+} from citrate to the hexadentate chelator EDTA. Following such treatment, the chemical shift value of the citrate A and B ^1H nuclei signals were very similar to that of free, uncomplexed citrate.

Moreover, using the above ^1H NMR/EDTA method for the determination of salivary Ca^{2+} levels (with a similar added concentration of EDTA), clear time-dependent elevations were noted in this parameter following equilibration of each of these intact dentifrice formulations with the 5 of the 18 human salivary supernatant samples of sufficient volume. Indeed, plots of salivary Ca^{2+} and Mg^{2+} concentrations versus equilibration time (Figure 5) revealed that for product I there was an immediate rise of Ca^{2+} level [from 0.57 to 0.90×10^{-3} mol.dm^{-3} for the example shown in Figure 5(a)] on addition of the dentifrice, a value which then further increased to a maximum of 0.99×10^{-3} mol.dm^{-3} in the 60–90 min. time period and then slightly decreased to 0.96 and 0.92×10^{-3} mol.dm^{-3} at 120 and 150 min., respectively. As noted for product I, addition of intact product II to human salivary supernatants also gave rise to an immediate elevation of supernatant Ca^{2+} concentration [to 0.98×10^{-3} mol.dm^{-3} in the ^1H NMR/EDTA-determined example shown in Figure 5(b)], which then further

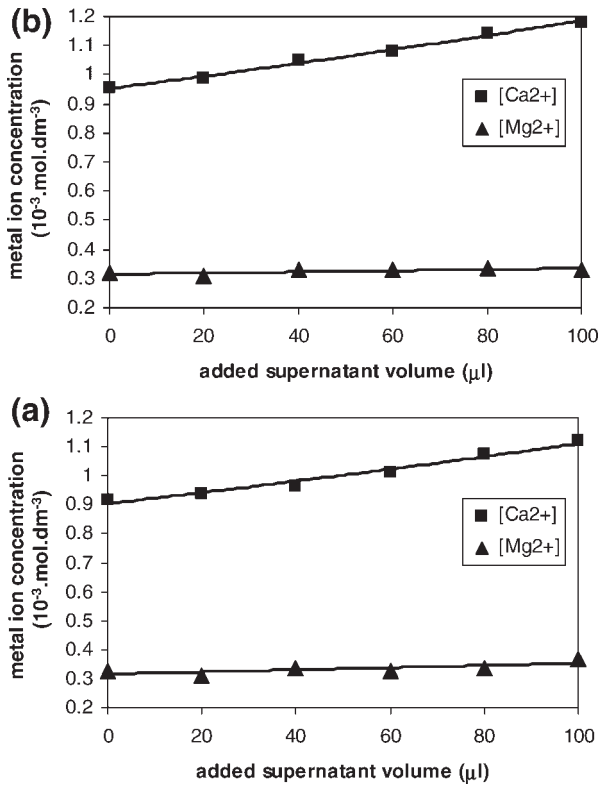

Figure 4. (a) and (b): Plots of salivary Ca^{2+} and Mg^{2+} concentrations versus volume of added dentifrice (μl) for products I and II, respectively. Ca^{2+} and Mg^{2+} levels were determined by the ^1H NMR/EDTA method (Section 2). Typical results are shown.

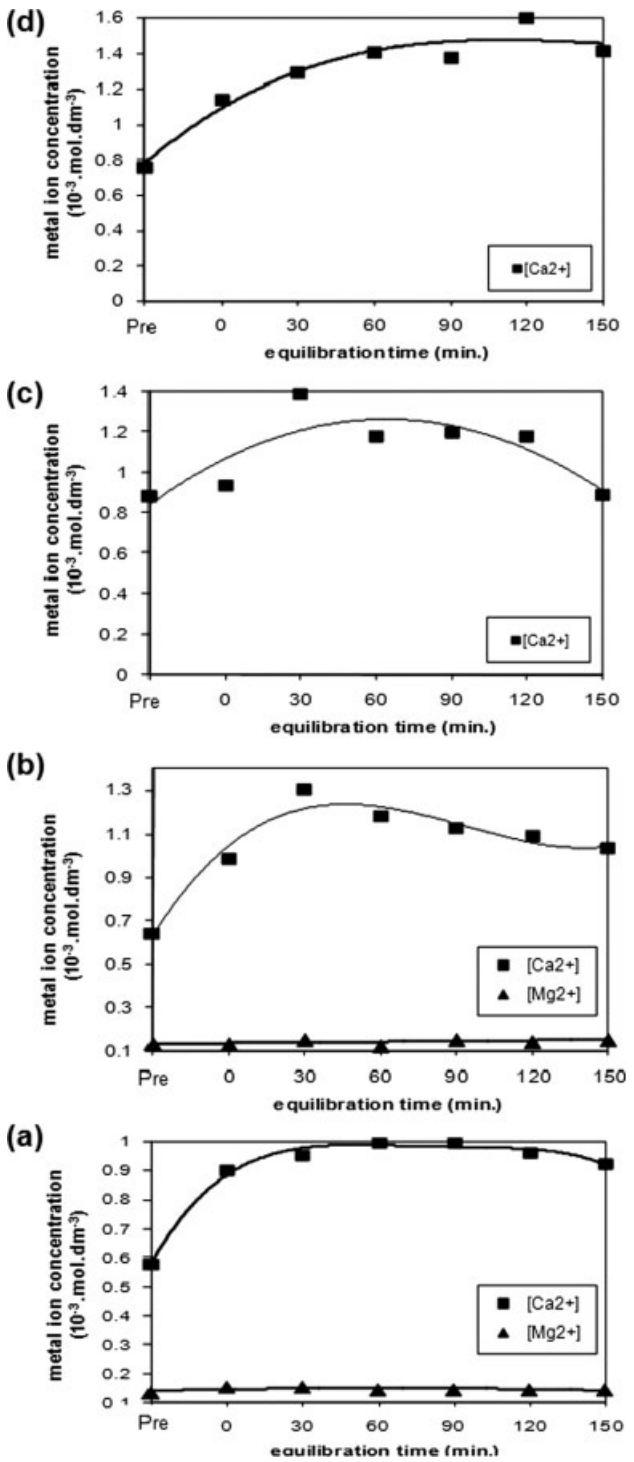

Figure 5. Plots of salivary Ca^{2+} and Mg^{2+} concentrations versus equilibration time for salivary supernatant specimens incubated with untreated (solid) dentifrices I [(a) and (c)] and II [(b) and (d)] at 37°C. For (a) and (b), Ca^{2+} and Mg^{2+} levels were determined by the ^{1}H NMR/EDTA method, whereas for (c) and (d), Ca^{2+} concentrations were estimated by atomic absorption spectrometry (Section 2). Typical results are shown.

increased to a value of $\sim 1.30 \times 10^{-3}$ mol.dm^{-3} after 30 min.; however, slow decreases in salivary Ca^{2+} were also noted during the 30–150 min time period.

TABLE I. Comparison of Atomic Absorption (AAS)- and ^{1}H NMR-Determined Percentage Increases in Ca^{2+} Ion Concentrations at Increasing Time-Points for a Typical Salivary Supernatant Specimen Equilibrated with Intact (Solid) Dentifrices I and II at 37°C

	Product I		Product II	
Equilibration Time (min.)	AAS (%)	^{1}H NMR (%)	AAS (%)	^{1}H NMR (%)
0	0	0	0	0
30	5.3	7.1	51.2	48.6
60	58.1	57.2	71.5	74.1
90	34.6	35.0	85.4	89.2
120	36.0	39.1	66.1	70.1
150	34.3	32.9	111.9	108.4
180	0.5	2.1	86.8	89.7

For salivary supernatant samples subjected to Ca^{2+} ion determination by both the ^{1}H NMR/added EDTA and atomic absorption spectrometric techniques, there was an excellent agreement between the two methods used. Table I shows time-dependent percentage increases in salivary Ca^{2+} concentration determined by both atomic absorption spectrometry and our ^{1}H NMR technique for a typical salivary supernatant sample equilibrated with intact products I and II in the above series of experiments. As noted for the above spectral titration experiments, the salivary Mg^{2+} concentration was completely unaffected by equilibration at 37°C with both of the solid products, observations confirming that, unlike Ca^{2+}, the dentifrices do not supply this metal ion to human saliva.

In order to ascertain whether or not the added dentifrice supernatant-dependent ^{1}H NMR spectral modifications noted in experiments involving the addition of increasing volumes of each of the dentifrice supernatants to those arising from human saliva (Figures 1 and 2) were attributable to Ca^{2+} ions released from the products, we also performed an investigation involving an ^{1}H NMR titration of human salivary supernatants with increasing added concentrations of this metal ion (added level 0.00–14.30 mmol.dm^{-3}).

In addition to the Ca^{2+} ion-mediated modifications to the chemical shift values and coupling patterns of the salivary citrate resonances observed, additional changes to the ^{1}H NMR profiles of this biofluid were also noted for samples pretreated with increasing added concentrations of this metal ion (Figure 6). These modifications included (1) substantial reductions in intensity or complete removal of the succinate ($—CH_2—CH_2—$) resonance (*s*, δ = 2.41 ppm), an observation consistent with its involvement in Ca^{2+} complexation, (2) significant decreases in that of the pyruvate-CH_3 group (*s*, δ = 2.388 ppm) and glycine's α-CH_2 protons (*s*, δ = 3.59 ppm), with smaller reductions in signals ascribable to the methyl groups of 3-D-hydroxybutyrate (*d*, δ = 1.24 ppm) and alanine (*d*, δ = 1.487 ppm), data indicating their role in the chelation of added Ca^{2+}, and (3) a marked broadening (and consequent reduction in height) of the lactate-CH_3 group resonance (*d*, δ = 1.330

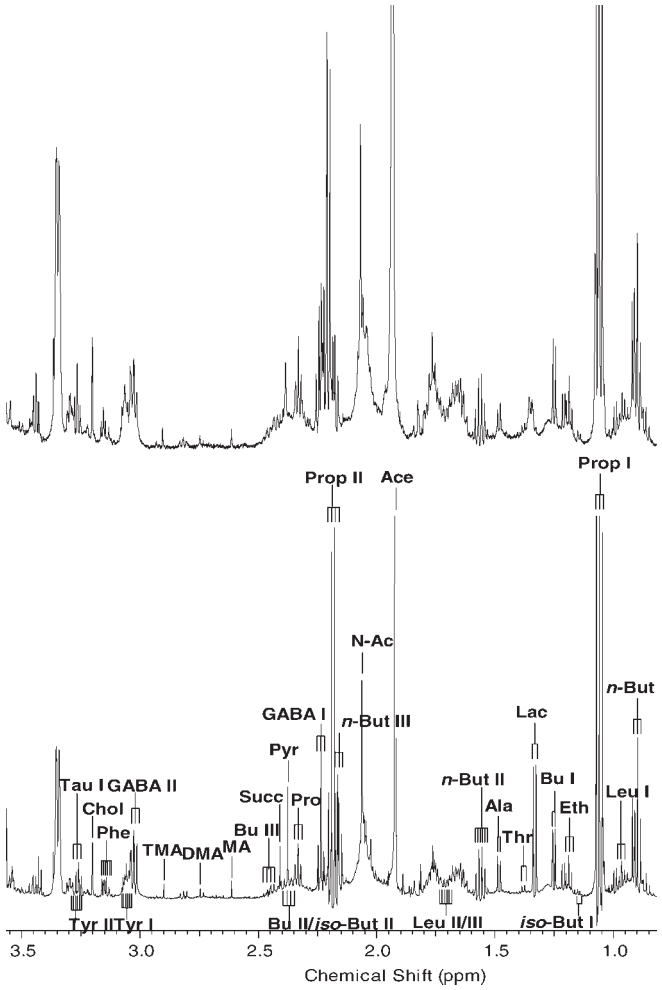

Figure 6. Expanded 0.72−3.30 ppm regions of 600.13 MHz single-pulse ^{1}H NMR spectra of an unstimulated salivary supernatant sample (a) before and (b) after treatment with Ca^{2+}ion (final added level 14.30 mmol.dm^{-3}). Typical spectra are shown. Abbreviations: as Figure 1, with Ace, acetate-C*H*$_3$; Ala, alanine-C*H*$_3$; Bu I, 3-D-hydroxybutyrate-γ-C*H*$_3$ protons; *iso*-But I and II, *iso*-butyrate-β-C*H*$_3$ and-α-C*H* protons, respectively; *n*-But I, II, and III, *n*-butyrate-γ-C*H*$_3$, -β-C*H*$_2$, and γ-CH$_2$ protons, respectively; Eth, ethanol-C*H*$_3$; Lac, lactate-C*H*$_3$; Leu I, II, and III, leucine δ, γ, and β protons, respectively; *N*-Ac, acetamido-C*H*$_3$ groups of free or low-molecular-mass oligosaccharide-containing *N*-acetylsugars (sharp signals)—the underlying broader resonances are ascribable to hyaluronate *N*-acetylglucosamine residues and *N*-acetylneuraminate/*N*-acetylglucosamine sugars present in the molecularly mobile carbohydrate side chains of glycoproteins; Prop I, propionate-C*H*$_3$ protons; Pyr, pyruvate-CH$_3$; Thr, threonine-CH$_3$.

ppm), together with an upfield shift (from 4.13 to 4.005 ppm at an added Ca^{2+} concentrations of >8.60 m*M*) and associated broadening of its —C*H* proton quartet resonance. Further modifications to the ^{1}H NMR profiles of human salivary supernatants arising from the addition of increasing levels of Ca^{2+} included a significant, reproducible broadening of the sharp singlet signal attributable to the acetamido group methyl protons (—NHCOC*H*$_3$) of low-molecular-mass *N*-acetylsugar species [e.g., *N*-acetylglucosamine and/or *N*-acetylneuraminate (sialate) present as monosaccharides or small oligosaccharide species] (*s*, δ = 2.04 ppm)[38] and elevations in choline's —$N^+(CH_3)_3$ head-group singlet resonance (δ = 3.20 ppm), a possible consequence of its displacement from negatively charged protein binding-sites (putatively by positively charged Ca^{2+}-biomolecule complexes).

Notwithstanding, since the salivary methyl-, dimethyl-, and trimethylamine ^{1}H NMR signals were unaffected by added Ca^{2+} ion up to a level of 14.30 mmol.dm^{-3}, it appears that both the solid dentifrice materials and water-soluble components derived therefrom in corresponding aqueous supernatants exert a unique means of removing such malodorous metabolites from human saliva (the former by adsorption, i.e., physisorption), processes which indicate their ability to combat and alleviate oral malodour.

DISCUSSION

^{1}H NMR data acquired revealed significant elevations in salivary Ca^{2+} levels following treatment of supernatants derived from this biofluid with calcium sodium phosphosilicate-containing dentifrices, an observation consistent with their mechanism of action. Indeed, release of Ca^{2+} ions from the products tested here may effectively serve to diminish the quantity of open dentinal tubules in patients, a parameter reflecting tooth hypersensitivity.

Although, as expected, there was a clear linear dependence of increase in salivary Ca^{2+} concentration on volume of added dentifrice supernatant for both products investigated, the time-dependence of this metal ion's release from the solid dentifrices into this biofluid matrix was more complex. Indeed, for these experiments, the salivary Ca^{2+} ion level increased to values approximately double their initial, untreated (control) ones within a 90-min equilibration period at 37°C and then decreased to a small extent from 90 to 150 min for both products I and II. These subsequent decreases in Ca^{2+} ion concentration are not simply explicable; however, they suggest a time-dependent shift in the complex equilibria for this metal ion between solid sodium calcium phosphosilicate and a potentially very wide range of solution-phase biomolecules with which it forms complexes. Moreover, the stoichiometry and thermodynamic stability constants of such complexes and the Ca^{2+} ion saturation status of the complexants involved also represent further physiochemical considerations, as do the pH and ionic strength values of the salivary supernatants examined here.

As expected, salivary Mg^{2+} ion concentrations remained unaffected by these dentifrices in both the supernatant titration and time-dependent equilibration experiments.

Data acquired also revealed that the ^{1}H NMR signals of malodorous salivary amines (methyl-, dimethyl-, and trimethylamines) were substantially diminished in intensity after equilibration with increasing volumes of the dentifrice supernatants or the specified added weights of the intact, untreated

solid products, an observation clearly indicating that such species become "^{1}H NMR-invisible" or are removable from this biofluid medium by one or more available, partially or wholly water-soluble components in the products tested. Indeed, in the titration experiments performed with the product supernatants, the decrease observed in amine resonance intensities was clearly dependent on added volume (Figure 2). The process involved clearly does not involve the complexation of released Ca^{2+} ions by these salivary biomolecules since an ^{1}H NMR spectral titration with increasing added levels of this metal ion was found not to exert an influence the intensities of their resonances (Figure 6). Indeed, the high pK_a values of these species (10.66, 10.73, and 11.01 for methyl-, dimethyl-, and trimethylamine, respectively[46]), would, of course, extremely limit their abilities to complex Ca^{2+} ions at physiological pH values.

In principle, polyethylene glycol present at high levels in both dentifrice products may hydrogen (H)-bond to these malodorous amines, a process, which, in view of the former's polymeric nature, may substantially broaden their resonances and render them "^{1}H NMR-invisible." Moreover, carbomer (a cross-linked polymer containing linear polyacrylate chains) may also exert this effect on the ^{1}H NMR resonances of these amines (which are fully protonated under our experimental conditions, i.e., a mean salivary pH value of 6.92 in this study) via their electrostatic binding to negatively charged acrylate carboxylate groups of this agent. A further, although less-plausible explanation involves the formation of an ion-pair complex with the high levels of dentifrice supernatant-containing lauryl sulphate, with a fast exchange between the "free" and "bound" forms on the NMR timescale. Although the reaction of salivary methylamine with aldehydes present in the flavoring ingredients of product II to form one or more Schiff-base adducts may also partially account for the decrease in intensity of its ^{1}H NMR signal, such reactive aldehydes were absent from product I (and furthermore, they are only very minor components present in product II).

With regard to salivary supernatant specimens equilibrated with pre-weighed quantities of the intact (solid) dentifrice formulations, it is also plausible that solid-phase polyethylene glycol and carbomer (i.e., those fractions which remain water-insoluble during the 150-min equilibration period) bind these positively charged amines via H-bonding or electrostatic interactions, respectively, at salivary pH values. Moreover, negatively charged, solid-phase silicate and phosphosilicate may also attract these amine species and effectively remove them from the solution phase via physisorption processes.

TMA present in the oral environment is potentially of much importance regarding the aetiology of recalcitrant oral malodour.[47] Indeed, a low but nevertheless significant proportion of subjects with this condition have an extra-oral aetiology and, in view of its high level of volatility, TMA can be delivered to exhaled air (and therefore saliva in which it is also readily soluble) from the circulatory system via the lungs. Hence, a further novel therapeutic activity of the two products tested here appears to be their capacity to alleviate oral malodour.

Intriguingly, for these salivary supernatant specimens equilibrated with the solid products I and II, the reductions observed in the intensities of the ^{1}H NMR resonances of these salivary amines displayed a dependence on equilibration period for those samples treated with either dentifrice. Although this time-dependence approximately correlated with released Ca^{2+} ion concentrations at the lower time-points, as noted earlier, this metal ion did not exert an influence on these singlet signals when independently added to salivary supernatants up to a concentration of 14.3 mmol.dm^{-3}.

In addition to the previously documented influence of added Ca^{2+} ions on the ^{1}H NMR resonances of salivary citrate,[39] spectral titration of human salivary supernatants with increasing concentrations of this metal ion revealed further modifications to the ^{1}H NMR profiles of this biofluid, specifically reductions in the intensities, increases in the line-widths and/or changes in the chemical shift values of a series of common salivary biomolecule resonances, including those of succinate, pyruvate, lactate, 3-D-hydroxybutyrate, and low-molecular-mass *N*-acetylsugar species [the release of positively charged choline from macromolecular (e.g., protein) binding sites was also notable]. These modifications to the salivary ^{1}H NMR resonances are key features, which provide valuable evidence for the involvement of their corresponding biomolecules in the complexation/chelation of this added metal ion, as noted in our previous investigations.[39,48–52] Moreover, the "speciation" of Ca^{2+} in this biofluid represents a phenomenon of much relevance to demineralization/remineralization processes and the pathogenesis of oral diseases in general. Indeed, the extent of salivary biomolecule saturation with Ca^{2+} is of critical importance to tooth enamel erosion (superficial losses of dental hard tissue via a chemical process that is independent of microbial activity), the basic mechanism of which involves a decrease in the degree of saturation of the fluid surrounding the enamel with respect to calcium phosphate species.[53] Therefore, selected biomolecular Ca^{2+} complexants can effectively diminish the concentration of "free" (i.e., uncomplexed) Ca^{2+} in saliva. Our further observations regarding salivary Ca^{2+} ion speciation and their physicochemical and clinical significance will be reported in detail elsewhere.

That added Ca^{2+} up to a level of 14.3 mmol.dm^{-3} did not affect the methyl-, dimethyl-, and trimethylamine signals confirms that the marked reduction in their intensities observed in salivary spectra with increasing added volume of dentifrice supernatant, or time when this biofluid was equilibrated at 37°C with the solid, untreated products, confirmed that components other than released or releasable Ca^{2+} were responsible for this observation (the high pK_a values of these amines represents an important factor, which serves as a barrier to their complexation of this

metal ion at physiological pH values, as does the availability of a wider range of oxygen donor atom-containing complexants present at much higher salivary concentrations).

Bioactive glasses such as those present in the two products investigated here readily liberate-free Ca^{2+} and phosphate ions in a time-dependent manner, a process facilitating a reduction in the level of open dentinal tubules in patients with tooth hypersensitivity and further oral diseases involving these phenomena in their aetiology. Moreover, data obtained here describe, for the first time, the facile application of high-resolution ^1H NMR analysis to the determination of bioactive-glass induced elevations in salivary Ca^{2+} concentrations subsequent to treatment with dentifrices containing this novel, "smart" biomaterial. In addition to confirming elevations in the concentration of available salivary Ca^{2+} following treatment with these novel products, these data also revealed that, in at least some of the samples examined, the degree of citrate saturation with this metal ion is enhanced subsequent to such treatment. As expected, this value was reduced to 0% on the addition of excess levels of the Ca^{2+} ion-chelator EDTA; such a transfer of Ca^{2+} is, of course, thermodynamically favorable under our experimental conditions. In principle, EDTA can also effectively remove Ca^{2+} from alternative salivary complexants such as phosphate, bicarbonate, lactate, succinate, and pyruvate.

Elevations in salivary Ca^{2+} levels (and also those of phosphate) at exposed dentinal sites may serve to offer a valuable means for the arrest and, when in its primary stages, reversal of tooth demineralization and hence dental caries.[54]

CONCLUSIONS

High-resolution ^1H NMR analysis serves as a useful analytical tool for evaluating the mechanisms of action of dentifrices containing H_2O-reactive phosphosilicates, specifically their concentration- and time-dependent release of Ca^{2+} ions into human saliva, together with its therapeutic fate in this biofluid (notably its complexation by salivary biomolecules): results acquired in this study indicate that for non-protein-bound Ca^{2+}, salivary citrate, succinate, lactate, and pyruvate can be used as probes for salivary Ca^{2+} availability. These observations provide valuable mechanistic information regarding the therapeutically effective delivery of this metal ion by the products tested to active sites involved in the repair of caries-susceptible dentinal tubules. Data obtained also provide evidence for the consumption of deleterious, malodorous salivary agents (methyl-, dimethyl-, and trimethylamines) by further agents present in these dentifrice products, an observation indicating that they offer an additional mode of action (i.e., they appear to have the ability to combat halitosis).

The authors are very grateful to the Department of Biological and Chemical Sciences, Queen Mary, University of London, for the provision of NMR facilities.

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