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Kinetics of canine dental calculus crystallization: An *in vitro* study on the influence of inorganic components of canine saliva

Ballav M. Borah [a,1], Timothy J. Halter [a,1], Baoquan Xie [a], Zachary J. Henneman [a], Thomas R. Siudzinski [a], Stephen Harris [b], Matthew Elliott [c], George H. Nancollas [a,*]

[a] Department of Chemistry, University at Buffalo, The State University of New York, Buffalo, NY 14260, USA
[b] Waltham Centre for Pet Nutrition, Leicestershire LE14 4RT, UK
[c] Mars Care & Treats Europe, Oakwell Way, Birstall WF17 9LU, UK

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ABSTRACT

This work identifies carbonated hydroxyapatite (CAP) as the primary component of canine dental calculus, and corrects the long held belief that canine dental calculus is primarily $CaCO_3$ (calcite). CAP is known to be the principal crystalline component of human dental calculus, suggesting that there are previously unknown similarities in the calcification that occurs in these two unique oral environments. *In vitro* kinetic experiments mimicking the inorganic components of canine saliva have examined the mechanisms of dental calculus formation. The solutions were prepared so as to mimic the inorganic components of canine saliva; phosphate, carbonate, and magnesium ion concentrations were varied individually to investigate the roll of these ions in controlling the nature of the phases that is nucleated. To date, the inorganic components of the canine oral systems have not been investigated at concentrations that mimic those *in vivo*. The mineral composition of the synthetic calculi grown under these conditions closely resembled samples excised from canines. This finding adds new information about calculus formation in humans and canines, and their sensitivity to chemicals used to treat these conditions.

1. Introduction

Attempts have been made to study mineralization events that take place in canine saliva. The pH and inorganic ionic concentrations differ greatly, and are known to markedly influence reactions at tooth surfaces [1,2].

Dental calculus is composed primarily of mineral and organic components and is usually classified by location as supra- or sub-gingival calculus [3]. Supra-gingival and sub-gingival calculi differ from each other with respect to the principal crystal constituents and inorganic elemental components [4]. As soon as enamel is exposed to saliva, dental plaque is formed as the result of bacterial colonization, followed by subsequent mineralization of phases involving calcium, phosphate, carbonate, and other ions in the contacting saliva [5].

Canine calculi appear as granular, yellow–brown masses on the buckle surfaces of teeth and are largest on molar teeth of the upper jaw near salivary duct orifices. They are associated with gingivitis, periodontitis, and erosion of tooth surfaces. Four types of calcium phosphates, varying in relative abundance from sample to sample, are reported present in human dental calculi [6,7]. These calcium phosphates are: brushite (dicalcium phosphate dihydrate, DCPD, CaHPO4·2H2O), octacalcium phosphate ($Ca_8H_2(PO_4)_6{\cdot}5H_2O$), magnesium-containing whitlockite, β-TCP [$(Ca,Mg)_3(PO_4)_2$], and carbonate-containing hydroxyapatite [$(Ca,M)_{10}(CO_3,HPO_4,PO_4)_6(OH,X)_2$], where M represents other cations capable of substituting for the Ca^{2+}, e.g., Sr^{2+}, Pb^{2+}, K^+, Na^+, etc.; X = Cl or F. Calculus formation can be divided into two parts; (1), adhesion between calcified plaque and the tooth surface and, (2), the mineralization of this phase involving calcium, phosphate, carbonate, and other ions in the contacting saliva [8]. Calcification is also a kinetic process controlled by nucleation and growth of calcium-phosphate-rich phases on the tooth surface [9]. The rate of calculus formation can be elevated by salivary pH, salivary calcium concentration, bacterial protein and lipid concentration, and concentration of protein in the sub-mandibular salivary gland secretions as well as low inhibitory factors and higher total salivary lipid levels.

The most widely cited work on canine dental calculi identifies the main component the calculi as calcium carbonate, with minor amounts of calcium phosphate [1]. The carbonate concentration in

* Corresponding author. Fax: +1 716 645 6947.
E-mail addresses: ghn@buffalo.edu, ballavbo@buffalo.edu (G.H. Nancollas).
[1] These authors made equal contributions.

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canine saliva much is greater than that in humans and the lack of sufficient analysis of whole calculus samples led previous investigations likely led to conclusions. Preliminary speciation calculations showing relative supersaturation (σ) values for hydroxyapatite (σ_{HAP}) that are more than 10 times those for calcite ($\sigma_{calcite}$), suggested that canine calculus is predominantly composed of calcium phosphate minerals. Carbonate is known to markedly influence the mineralization of calcium phosphates; under pseudo canine conditions of relatively high carbonate and low phosphate concentrations, with high pH (7.7–8.5), the mineralization events were never studied quantitatively, nor the roles of specific inorganic components. An in-depth investigation of excised samples has revealed that the previously held views on canine dental calculus were incorrect and apatitic calcium phosphate minerals with minor inclusions of carbonate are principal components of canine dental calculus.

2. Materials and methods

2.1. Canine saliva collection

Canine saliva and calculus samples were collected from dogs housed at The WALTHAM Center (UK). Dogs were trained using a clicker for positive reinforcement to provide saliva samples by chewing or licking on large cotton wool pads. The pads were centrifuged at 500*g* for 5 min in a Salivette (Sarstedt), accumulated saliva was removed and the pads were spun at 1000*g* for 5 min to remove the remaining saliva fluid. The saliva samples were analyzed by Mars Care & Treats Europe, Oakwell Way, Birstall, WF17 9LU, UK.

2.2. Driving force and solution speciation

Solution speciation calculations were made to determine the relative supersaturations, σ, for mineral phases under canine salivary conditions (Table S3). These calculations were made using the extended Debye–Hückel equation proposed by Davies, with mass balance expressions for total calcium and phosphate incorporating appropriate ion pair equilibrium constants by successive approximation for the ionic strengths, I (Eq. (1)).

$$\sigma = S - 1 = [IAP/K_{SP}]^{1/\nu} - 1 \quad (1)$$

where IAP is the ionic activity product of free lattice ions in solution, K_{SP} the activity solubility product (HAP 2.88×10^{-112} mol^{18} L^{-18}) [10] and υ the number of ions in a formula unit (HAP $\nu = 18$). Conditions used for the calculations are shown in Table 1. The aim of these calculations was to use the analytically determined total concentrations of the dissolved inorganic calculus components and estimate the extent of ion-pairing, as well was the mineral phases which form under canine oral conditions to form dental calculus.

2.3. FE-SEM and TEM

Crystallites were collected from solution by filtration using polycarbonate membrane filters (200 nm pore size). The samples were then dried, sputter coated with graphite under vacuum, and examined by field-emission scanning electron microscopy (FE-SEM, Hitachi SU-70) at 20 kV. Transmission electron microscopy (TEM) investigations of nucleated particles removed at various time intervals in Ultrathin Carbon type A (400 mesh copper grids) were made using a JEOL-2010 TEM at an accelerating voltage of 200 kV.

2.3.1. Experimental method

Reactions were made in Teflon® covered, 150 mL double-walled Pyrex® jacketed cells, thermostated at 38.0 ± 0.1 °C by a circulating water bath. Supersaturated solutions were prepared by first adding triple distilled water (TDW) to the cell, followed by slowly mixing the appropriate amounts of $CaCl_2$, $MgCl_2$, KH_2PO_4, and $NaHCO_3$with NaCl to established the ionic strength, I. The pH was adjusted to the desired value (pH = 8.50) using 0.05 M KOH solutions. Titrant addition was potentiometrically controlled by a glass electrode (Orion 91-01) and reference electrode (Orion 900100). During growth, the electrode potential was continuously compared with the pre-set voltage bias and a difference in signal ($\Delta \geqslant +0.10$ mV) activated the motor-driven titrant burettes, which maintained a constant thermodynamic driving force by the addition of titrant solutions. The final reaction solution concentrations approximated those of canine saliva.

Hydrogen ion activity was monitored by a pH electrode coupled to a Ag/AgCl single junction reference electrode and a pH meter. Data were analyzed using the Nernst equation and calibration of the electrodes was performed using the following buffers: (1) pH 9.088 (9.96 mM $Na_2B_4O_7$) and (2) pH 7.385 (8.70 mM KH_2PO_4 and 30.43 mM Na_2HPO_4, at 37 °C). After calibration, the electrodes were washed extensively with triple distilled water (TDW) and allowed to stabilize in the reaction cell. When a stable *emf* value had been reached, the automatic titrator potentiostat was set to this value (i.e. the "voltage bias"). As nucleation and growth took place, the removal of ions from the bulk solution caused a deviation in the cell solution concentration and *emf* from the set value. If greater than ±0.050 mV, the addition of titrant solutions from mechanically coupled burets was triggered to restore the *emf* to the "voltage bias". A computer program recorded the titrant addition volume as a function of time (Fig. S4). Prior to the start and at the end of each experiment, a weighed aliquot of solution was removed for calcium and phosphate analysis. At the end of the experiment, cell solutions were vacuum filtered and the remaining precipitates were saved for further solid phase analysis. Phosphate and calcium analyses were made using UV–Vis (as the vanadomolybdate complex, Perkin Elmer Lambda 25) and atomic absorption spectroscopy (AAS) (Perkin Elmer 3100), respectively.

2.3.2. Titrant solutions

Addition of the titrant solution containing KOH and NaCl was triggered to maintain constant pH and ionic strength. The reaction solution concentration (W_i), of the free ion, i, following titrant addition was given by:

$$W_i = \frac{W_i V_T + T_i dV_t - xdn}{V_T + n_b dV_t} \quad (2)$$

Table 1
Solution concentration for synthetic canine dental calculus growth experiments (I = 0.10 M, pH = 8.50 at 38.0 °C).

Component	Concentration (mmol L^{-1})	Mole ratio	Calculated relative supersaturation	
			Phase (xx)	$\sigma_{(xx)}$
Calcium (as $CaCl_2$)	2.57	[Ca]/[HCO_3] = 0.25	Calcite ($CaCO_3$)	3.27
Phosphate (as KH_2PO_4)	0.56	[Ca]/[PO_4] = 4.59	Hydroxyapatite (HAP)	47.70
Magnesium (as $MgCl_2$)	2.29	[Ca]/[Mg] = 1.12	Tricalcium phosphate (TCP)	9.06
Bicarbonate (as $NaHCO_3$)	10.10		$MgCO_3$	0.66
Sodium chloride	84.23			