Interaction of chlorhexidine digluconate with and adsorption of chlorhexidine on hydroxyapatite*

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It is well known that chlorhexidine digluconate provides an effective microbicidal activity during oral rinsing, and therefore, it was considered worthwhile to investigate its interaction with hydroxyapatite on a fundamental level. The kinetics of uptake (or reaction) of the compound from aqueous solutions by synthetic hydroxyapatite was studied at 23°C for four time periods by monitoring its concentration. There was no uptake at low concentrations for any time period. The uptake curves for higher concentrations shifted towards the lower concentrations as the period increased and became more and more vertically oriented to the concentration axis. The concentrations of calcium ions increased, phosphate ions decreased and hydrogen ions decreased a little for a given period as the concentration of the compound was increased. All of these experimental facts can be qualitatively explained on the basis of the solubility considerations of hydroxyapatite and of chlorhexidine phosphate, the reaction product that slowly precipitates out of the solution. The needle-shaped birefringent crystals of the phosphate salt are clearly visible in the apatite matrix under a microscope, and its refractive index and differential Fourier transform infrared spectra match almost exactly with those of a well-characterized, synthesized phosphate salt.

To explore the nature of interaction, the uptake of chlorhexidine base was studied from *p*-dioxane and it is irreversible. The uptake is total below a threshold equilibrium concentration and constant above it. © 1994 John Wiley & Sons, Inc.

INTRODUCTION

Chlorhexidine digluconate (ClHxDG) is an effective antimicrobial agent and prevents the growth of dental plaque, calculus, and gingivitis by reducing the mutans of *Streptococci* in patients' mouths.[1–5] The retentivity of ClHxDG in the oral cavity has been generally attributed to its adsorption on tooth mineral[6–8] and binding of positively charged chlorhexidine ion with phosphate groups in lipopolysaccharides and carboxyl groups in proteins.[9] The retention may, however, be caused by the precipitation of the insoluble salts, e.g., ClHx · H_3PO_4 and/or ClHx · 2HCl on tooth mineral and/or oral fluids surrounding it, and it is this aspect of the interaction of ClHxDG that was never properly studied by the previous investigators.

*Certain commercial materials and equipment are identified in this article to specify the experimental procedure. In no instance does such identification imply recommendation or endorsement by the National Institute of Standards and Technology or the ADA Health Foundation or that the material or equipment identified is necessarily the best available for the purpose.

Recently, Sodhi, Grad, and Smith[10] found, by x-ray photoelectron spectroscopy, the retention of ClHx moieties through electrostatic bonding with phosphate groups of hydroxyapatite, but they found no adsorption of gluconate ions. Unfortunately, these authors failed to notice or propose the precipitation of the phosphate salt as a separate phase.

In general, any ionic adsorption on an ionic surface will be conditioned by the charge-determining ions,[11–13] and specifically the hydrogen ion concentration of the solution,[14–18] if the solvent is water. Therefore, the constituent ions, calcium, phosphate, and hydroxyl or hydrogen, should play a very important role in any mechanistic evaluation of the uptake from the aqueous solution of chlorhexidine ion on hydroxyapatite. The impact of the interplay of hydrogen bonding between solute, solvent, and substrate on the uptake process should also be carefully explored.[19–26] In this report the interaction of ClHxDG with hydroxyapatite, the structural prototype for the principal inorganic crystalline constituent of tooth and bone, is presented to elucidate the role of the constituent ions on the process. The nature of this interaction was further explored by studying the up-

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take of chlorhexidine onto hydroxyapatite from a solvent in which chlorhexidine is soluble. Its uptake was, therefore, studied from *p*-dioxane on three different samples of hydroxyapatite. The reversibility of uptake was determined in each case. The isotherms were analyzed to reveal the orientation of maximally adsorbed molecules on the apatite surface.

MATERIALS AND METHODS

Apatite I

The synthetic hydroxyapatite was tribasic calcium phosphate (Fisher-certified, C-127) with a chemical formula given as approximately $Ca_{10}(OH)_2(PO_4)_6$. It was repeatedly washed with boiling water before use; the physical and chemical details of its characterization have been reported.[27] It has a surface area (BET, N_2) of 41 m^2/g, and Ca/P = 1.57 ± 0.03. The amount of physically adsorbed water on apatite was 1.58 wt %, which is equivalent to about 1.5 monolayers.

Apatite II

This material was prepared by Avnimelech[28] by titrating a calcium hydroxide slurry at boiling temperature with phosphoric acid. It has a surface area (BET, N_2) of 16.7 m^2/g and a Ca/P ratio equal to 1.67.

Apatite J

This high-purity material was obtained from Central Glass Company, Japan (courtesy Dr. K. Tanaka). It has a surface area (BET, N_2) of 3.5 m^2/g and Ca/P ratio equal to 1.67.

Chlorhexidine digluconate [ClHxDG]

Chlorhexidine digluconate [ClHxDG] was obtained as an aqueous solution (Sigma Chemical Co., St. Louis, MO). It contained 18.8 wt % ClHxDG, as shown by the peroxide titration suggested by Sigma Chemical Company. To the stirring solution of the compound (500 mg) were added glacial acetic acid (100 mL), mercuric acetate solution (6%, 10 mL), and two drops of crystal violet indicator, and the mixed solution was titrated with perchloric acid solution (0.1 N) until the end point was reached when the violet color changed to bluish green. Sigma supplied proton NMR spectra in D_2O confirming the proton distribution (Fig. 1).

Chlorhexidine phosphate [ClHx · H_3PO_4]

This compound was prepared by slowly adding an equimolar amount of aqueous potassium hydrogen phosphate (K_2HPO_4, Fisher-certified reagent) to a stirring aqueous solution of ClHxDG (5 mmol/L). The white precipitate was allowed to mature for a day, filtered, washed and dried at 35°C in a vacuum oven. The needle-shaped crystals were birefringent and had a refractive index of 1.476–1.480. The MP of the crystals was 72°C. Its elemental analyses agreed well with the calculated ones for $C_{22}H_{33}O_4N_{10}Cl_2P$ (found: C, 43.40; H, 5.54; N, 23.15; Cl, 11.60; P, 5.07; calculated: C, 43.79; H, 5.51; N, 23.21; Cl, 11.75; P, 5.13). The IR spectra had all the relevant functional group bands (Fig. 2). There was no precipitate when a dilute solution of KH_2PO_4 was added to ClHxDG. A sticky low-melting solid that had an indefinite composition precipitated when an aqueous solution of Na_3PO_4 was added to ClHxDG.

Uptake of chlorhexidine digluconate

Apatite I samples (1.000 g each) were shaken with standard aqueous solutions (10 mL each) of ClHxDG (2–50 mmol/L) for a given period (4 h, 1 day, 4 day, and 16 day) at room temperature (23.0 ± 0.5°C). The apatite slurry was filtered through a fine-pore fritted disc by applying a light suction for 5 sec. The concentration of ClHx in the filtrate was determined from its absorbance at 305 nm in a double beam spectrophotometer (Varian, DMS 80) by using a concentration vs. absorbance plot. Quartz spacers were used to reduce the path length of the cell. The uptake A (mol/g) of ClHxDG is derived by a relation $A = V\Delta C/W$, where V (liter) is the volume of solution in contact with W(g) of the apatite and ΔC (mol/liter) is the difference of the initial and final concentrations of solution. The uptake values obtained by repeating the experiments at least twice were reproducible within a range of 5–10%. The desorption of the solute was determined for a given set by repeatedly washing the apatite with distilled water (100 mL, total volume) after a predetermined period and determining carbon content of the apatite by combustion chromatographic analysis (standard deviation ± 2%).

Determination of Ca and P in filtrates

KNO_3 (0.01 g) was added to a given volume (2 mL) of filtrate and was dried in a vacuum oven. This was

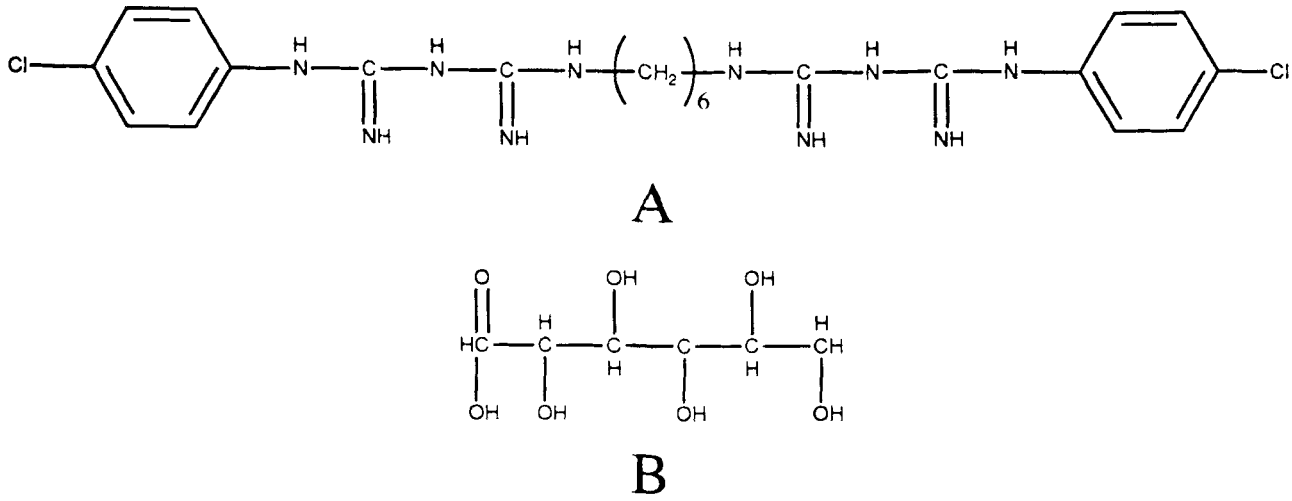

Figure 1. Schematic representation of: (A) chlorhexidine and (B) gluconic acid. A chlorhexidine molecule acquires two hydrogen ions from two gluconic acid molecules, becomes doubly charged, and remains associated with two gluconate ions.

digested with conc. HNO_3 and then with conc. $HClO_4$ (2 mL each), and the mixture was concentrated by evaporation (<1 mL). The colorless mixture, dissolved in water, was transferred into a volumetric flask and diluted to 25 mL. This solution was used for Ca or P analysis. The Ca was determined by the arsenazo III method[29] and P by the vanado-molybdate complex method.[30]

Solubility of ClHx · H_3PO_4

There was no discernible uptake of ClHxDG up to the concentration of 5 mmol/L for any of the four time periods. The concentration at this point (~5 mmol/L) may be taken as the solubility of ClHx · H_3PO_4 in this medium. The solubility of synthesized ClHx · H_3PO_4 in a similar medium (Table I, footnote c; also see Eq. [1]) is 1.05 mmol/L. The discrepancy in the two values may be attributed to supersaturation in the former case. The solubility of synthesized ClHx · H_3PO_4 in distilled water is 0.50 mmol/L (Table I, footnote b).

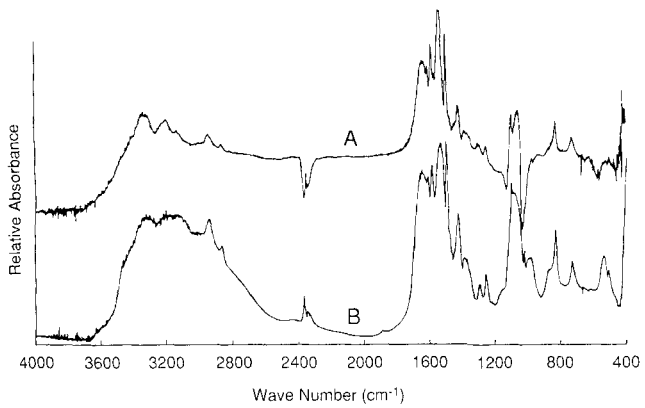

Figure 2. IR specta of chlorhexidine phosphate: (A) precipitated on Apatite I minus Apatite I (differential spectra) and (B) ClHx · H_3PO_4 (or $ClHx2H^+ \cdot HPO_4^{2-}$).

Uptake of chlorhexidine

The uptake of chlorhexidine (99 Wt %, Aldrich Chemical Co., Milwaukee, WI) was determined on Apatite I, Apatite II, and Apatite J from solutions in *p*-dioxane (Chemical number: 2144, Eastman Kodak Co., Rochester, NY) at 23°C by a method similar to the one outlined above for the uptake of ClHxDG. The concentration of a solution was determined from the absorbance at 330 nm using Beer's law plot. The uptake experiments were performed at least in duplicate and the values were reproducible within a range of 5–8%. The desorption of solute was determined from its uptake after repeated washing with excess dioxane (100 mL, total volume). The desorption was considered complete when final washings showed no absorbance. In order to determine whether chlorhexidine is desorbed by water, Apatite I and Apatite II samples with maximum uptaken amounts of chlorhexidine were repeatedly washed with 100 mL of distilled water for 15 min. The apatite samples were dried overnight at 45°C in a vacuum oven. The determination of carbon by combustion chromatographic analysis (Leco CS 244) showed that there was no discernible desorption of chlorhexidine by water. The used apatites did not show any indication of a distinctive foreign phase.

RESULTS

The uptake of ClHxDG from aqueous solutions (2–50 mmol/L) in the presence of Apatite I is shown in

TABLE I
Uptake of Chlorhexidine Digluconate on Synthetic Apatite I After 4 Days at 23°C and Concentrations of Ca, P, and H^+ in Filtrates

Init conc., (pH) mmol/L	[ClHx]* after 4 days, (pH) mmol/L	Uptake μmol/L	[Ca] mmol/L	[P] mmol/L	[H^+] μmol/L	[ClHx][P]
2 (5.73)	2.00 (5.15)	0.0	—	—	—	—
5 (5.71)	5.00 (5.16)	0.0†	1.23	8.44	6.92	42.2
10 (5.68)	7.09 (5.17)	29.1	2.40	7.06	6.76	50.1
15 (5.65)	8.75 (5.18)	62.5†	3.25	6.15	6.61	53.8
20 (5.64)	10.41 (5.19)	95.9	3.95	5.41	6.46	56.3
30 (5.61)	14.13 (5.21)	158.7†	5.30	4.58	6.17	64.7
40 (5.56)	18.80 (5.23)	212.0†	6.65	3.76	5.89	70.7
50 (5.51)	23.84 (5.24)	261.6†	7.95	3.12	5.75	74.4

Hydroxyapatite (1 g) was shaken with 10 mL solution of chlorhexidine digluconate for 4 days before the slurry was filtered. Total concentrations of chlorhexidine, calcium and phosphate ions in filtrates are represented, respectively, as [ClHx], [Ca], and [P].

*The solubility of synthesized ClHx · H_3PO_4 in distilled water is 0.50 mmol/L, [P] is 0.54 mmol/L and pH is 7.21. In a mixture containing 1 g Apatite I + 2.5 mg $Ca(OH)_2$ + 10 mL calcium gluconate (5 mmol/L) the solubility of synthesized ClHx · H_3PO_4 is 1.05 mmol/L, [P] is 4.21 mmol/L, and pH is 5.26 after 4 days of reaction.

†Uptake amounts after repeated washing with distilled water (100 mL) are: 0, 62, 158, 210, and 260 μmol/g (in descending order). They are determined by combustion chromatographic analysis.

Figure 3 for four time periods at 23°C. There is no uptake below 5 mmol/L for all four time periods. At higher concentrations the uptake slowly rises for smaller time periods and becomes almost vertical to the concentration axis for longer periods. The uptake amounts are irreversible since they cannot be removed by repeated washing of the apatite with distilled water (Table I, footnote d). When washed and dried apatite powders, which had interacted with concentrated solutions of ClHxDG, were examined under a microscope, distinct birefringent needle-shaped crystals were observed (Fig. 4). These crystals had the same refractive index (1.476–1.480) as that of the synthesized phosphate salt. The differential Fourier transform infrared (FT-IR) spectra of the reacted apatite powder is nearly identical to the FT-IR spectra of ClHx · H_3PO_4 (Fig. 2). Both spectra were obtained on a Nicholet 7199 spectrometer. The vibrational spectra of the main functional groups can be identified: C–H stretching of methylene groups (2934, 2859 cm^{-1}), C–H stretching of chlorophenyl group (3200–3100 cm^{-1}), C–H out of the plane bending with respect to the benzene ring (826 cm^{-1}), N–H symmetrical and antisymmetrical stretching of $-NH_2$-salt (3315–3280 cm^{-1}), C–N stretching of $-NR_2$ group (723 cm^{-1}), C=N stretching of N_2–C=N– group (1640 cm^{-1}), $-CH_2-$ bending of methylene group (1490 cm^{-1}), and P–O stretchings of PO_4 and HPO_4 group (ν_1 at 972, ν_3 at 1091, and ν_4 at 532 cm^{-1}).

Table I presents the uptake, from solutions of different concentrations, of ClHxDG on Apatite I after 4 days. Total concentrations of calcium, phosphate and hydrogen ions are also presented in Table I. The amount of calcium ions released to the solution increases with concentration of ClHxDG, and the phosphate ions decreases with concentration (Fig. 5) of ClHxDG.

The isotherms of chlorhexidine from *p*-dioxane solutions on Apatite I, Apatite II, and Apatite J at 23°C are shown in Figure 6 and most probably represent irreversible adsorption as is subsequently discussed. The abscissa represents the equilibrium concentration of chlorhexidine in contrast to that of a time-dependent (or kinetic) concentration for ClHxDG. The uptake is total and exhaustive below a threshold concentration and constant above it. The uptaken solute cannot be removed by repeated washing with 100

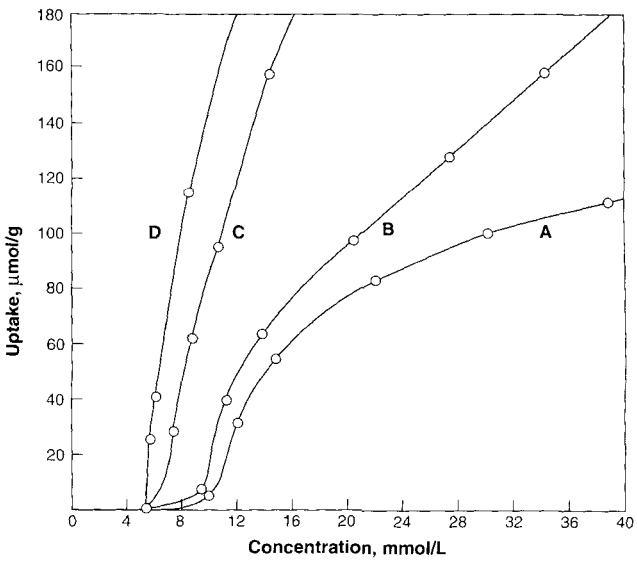

Figure 3. Reaction of chlorhexidine digluconate in aqueous solution with Apatite I (uptake vs. concentration) at 23°C after: (A) 4 h, (B) 1 day, (C) 4 days, and (D) 16 days.

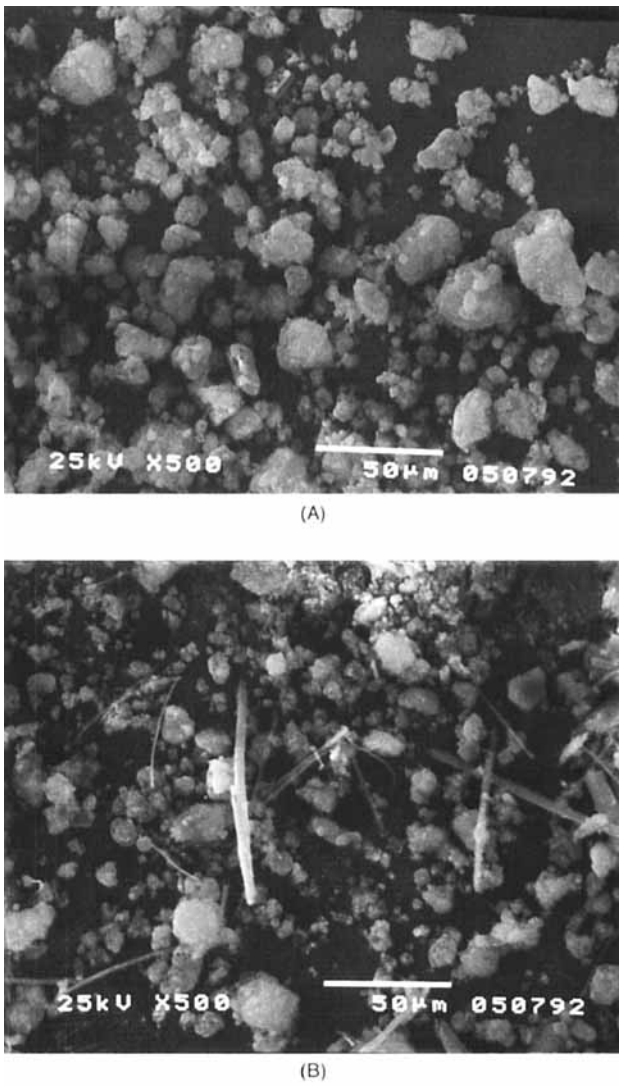

Figure 4. Electron photomicrographs of: (A) hydroxyapatite and (B) hydroxyapatite reacted with chlorhexidine digluconate; the needles are the phosphate salt of chlorhexidine, ClHx · H_3PO_4.

mL *p*-dioxane. The maximum amounts uptaken on Apatite I, Apatite II, and Apatite J are 63.6, 26.8, and 5.9 µmol/g, respectively, and their ratio (1:0.421: 0.093) compares favorably with the ratio of their surface areas (1:0.407:0.085) when the experimental variation (5–8%) is taken into account.

DISCUSSION

That the reaction product of ClHxDG and Apatite I is most probably ClHx · H_3PO_4 is demonstrated by the identity of its refractive index with the synthesized product and by its differential IR spectra (Fig. 2). The reaction may, therefore, presumably be represented as:

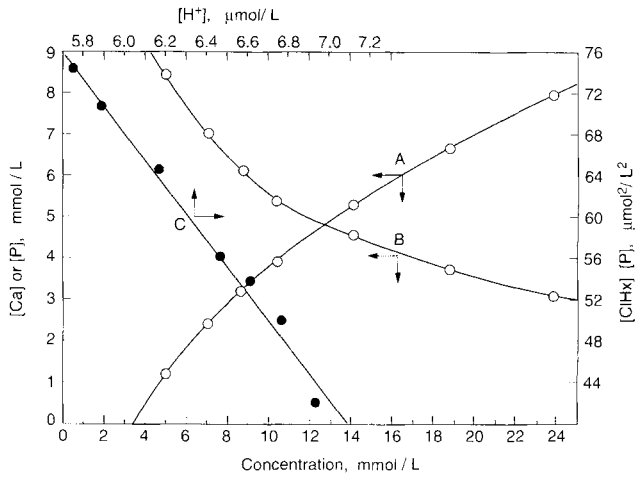

Figure 5. Concentration of: (A) total calcium [Ca] and (B) total phosphate [P] ions upon interaction of chlorhexidine digluconate with Apatite I at 23°C. Dependence of solubility of chlorhexidine phosphate with [H^+]: curve (C). The solubility is expressed as the product of total concentrations of chlorhexidine and phosphate ions.

$$Ca_{10}(OH)_2(PO_4)_6 + 6ClHx2H^{2+} + 12G^- + 6H_2O \rightarrow 6ClHx \cdot H_3PO_4 \downarrow + 4Ca(OH)_2 \downarrow + 6Ca^{2+} + 12G^- \quad (1)$$

where $ClHx2H^{2+}$ and G^- are protonated chlorhexidine and gluconate ions. This reaction also explains the work of Sodhi et al.[10]: that the chlorhexidine moieties are retained through electrostatic bonding with phosphate groups of hydroxyapatite and that the gluconate ions are not adsorbed.

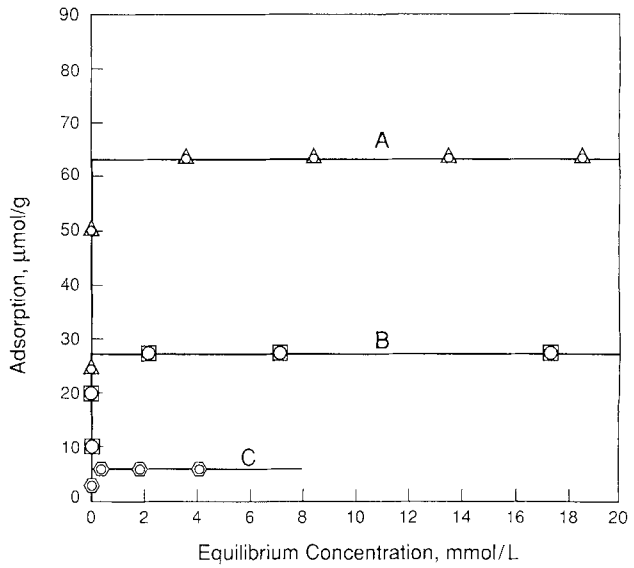

Figure 6. Adsorption isotherms of chlorhexidine from p-dioxane solution at 23°C: (A) Apatite I, (B) Apatite II, and (C) Apatite J. Adsorption points ○, desorption points △, or □, or ○. The surface areas (BET, N_2) of the apatites are (in order) 41, 16.7, and 3.5 m^2/g.

The characteristics of the uptake curves representing the reaction of ClHxDG with Apatite I (Fig. 3) could be qualitatively explained on the basis of the solubility considerations of the product, $ClHx \cdot H_3PO_4$, which is a sparingly soluble salt. In the lower concentration region the salt is soluble; the solution is probably supersaturated (Table I, footnote c), and there is no uptake. As the amount of ClHxDG is increased, the uptake becomes dependent on the rate of crystallization of the phosphate salt, and, therefore, as time increases, the uptake becomes more and more vertical to the concentration axis.

These characteristics of the uptake curves cannot be explained on the basis of chemisorption or irreversible adsorption of ClHxDG on hydroxyapatite. Caution should be exercised to interlink any adsorptive property to uptake curves since the former is an equilibrium and the latter a kinetic process. Nevertheless, chemisorption or irreversible adsorption of ClHxDG, were it so, cannot explain: (i) absence of any adsorption below a threshold concentration (Fig. 3), (ii) equilibrium concentration that is not reached even after 16 days, (iii) saturation value for adsorption that is not reached at all, and (iv) the adsorption of $ClHx2H^{2+}$ at pH $\cong$ 5 because the apatite will itself be positively charged below its point of zero charge (pH $\cong$ 7).[12–16] The reason that the chlorhexidine molecule gets adsorbed from *p*-dioxane is that the molecules possess no charge and that their adsorption is irreversible because the adsorbate molecules are multiply hydrogen-bonded to the hydrated substrate as is subsequently discussed. The term uptake is used in a general sense, and it represents the amount that is removed from solution by a reactive process, in the case of ClHxDG from water, and by an adsorptive process, in the case of ClHx from *p*-dioxane.

As required by solubility considerations, it is observed that the concentration of total phosphate ions in solution decreases with the increase in ClHx ions in solution (Table I, Fig. 5). It is difficult to show quantitatively the constancy of the solubility product of the phosphate salt on the basis of the present measurements since the concentrations of the relevant species $ClHx2H^{2+}$ and HPO_4^{2-} are not known. The solubility product, as represented in Table I, column 7, reflects the total concentrations of all ClHx species and phosphate ions in solution. Therefore, the solubility product is not constant but varies directly with the concentration of ClHx and indirectly with the concentration of H^+ in solution (Fig. 5).

The constancy of the solubility product of hydroxyapatite, like that of $ClHx \cdot H_3PO_4$, cannot be determined since the concentrations of the relevant species Ca^{2+} and PO_4^{3-} are not known in the reaction mixture. The concentration of calcium ions in the filtrates (Table I, column 4), however, bear an inverse relationship to the concentration of phosphate ions (the change in pH is minimal), in accord with the solubility requirements of hydroxyapatite. It is discerned, therefore, that all the relevant features of the uptake curves and ionic composition of the solutions can, at least, be qualitatively explained on the basis of the solubility considerations of the product and the substrate. Previous investigators falsely characterized the uptake as adsorption,[6–8] probably because they did not: (i) study the time-dependence of the interaction, (ii) monitor the calcium and phosphate ions released to the solution, (iii) correlate the adsorbed amount with the surface area of the apatite, and (iv) examine the interacted apatite for the presence of other phases.

To explore further the nature of interaction of chlorhexidine with hydroxyapatite, the uptake of chlorhexidine on apatite was studied from *p*-dioxane, a solvent in which it is soluble. Uptake on three different apatites (Fig. 6) showed that it is irreversible, and the maximum amounts, obtained from the horizontal plateau of the isotherms, are proportional to the surface areas of the apatites. The maximum or saturation amounts (M) could be used to calculate the effective cross-sectional area, σ (σ = S/NM, where S is surface area of substrate and N is Avogadro's number) of the chlorhexidine molecule (varies from 1.00–1.07 nm^2). The molecular model that matches this area has an appropriate configuration where it is most likely hydrogen-bonded[24] to the surface with its amino and imido moieties, and its hydrophobic chlorophenyl groups stand vertically to the surface. The chemical reaction with the apatite, were it involved in the uptake of ClHx, would have required formation of an impervious monomolecular layer of the phosphate salt on the substrate, which had to satisfy certain configurational and charge requirements. This seems to be highly unlikely, and the uptake may, therefore, be considered as irreversible adsorption or chemisorption.

The irreversibly adsorbed chlorhexidine molecules are not desorbed by limited washing with water because the presence of chlorophenyl and methylene moieties render the molecules hydrophobic, and in the adsorbed state these moieties are likely to be exposed to the solvent. To reiterate, these adsorptive characteristics show that the chlorhexidine molecules may be irreversibly adsorbed or chemisorbed and do not precipitate out as a separate phase, whereas the chlorhexidine ions are not adsorbed, but react with the apatite to form $ClHx \cdot H_3PO_4$.

In conclusion, it may be asserted that the interaction of the ClHxDG with apatite is not adsorptive but reactive. The mechanism of retention of chlorhexidine is, therefore, dependent on the precipitation of the sparingly soluble phosphate salt in the oral cavity, a condition that may involve phosphate ions belonging to tooth mineral and phosphate groups of

lipopolysaccharides in plaque.[9] Clearly, any treatment with phosphate-containing mouthwash before rinsing with ClHxDG should enhance the retentivity of ClHxDG as a phosphate salt and, thus, its efficacy as an antimicrobial agent. In fact, a treatment with soluble nitrate or chloride salts could also be used to improve the retentivity or control the release of chlorhexidine in the mouth since chlorhexidine salts with these ions are also sparingly soluble.

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