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Research Paper

Exploration of Intestinal Calcium Precipitation as a Barrier to Absorption at High Calcium Doses

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Purpose. To investigate the hypothesis that intestinal bicarbonate secretions precipitate calcium as the carbonate salt, thereby resulting in poor absorption (20–40%) from calcium supplements.
Methods. The *in vitro* effect of calcium dose and bicarbonate secretion rate on soluble calcium was determined by neutralizing elemental Ca^{2+}(250, 475, and 630 mg) in 0.1 N HCl to pH 7 with a bicarbonate secretion rate of 0.12 or 1.2 mEq/min. P_{CO2} and pH of the solutions were monitored. Soluble calcium was analyzed using atomic absorption spectrometry. Additionally, the transport of calcium across Caco-2 cell monolayers was determined.
Results. Calcium from a 250 mg dose remained soluble during bicarbonate secretion, regardless of rate. Once the dose increased, the calcium remaining in solution decreased during neutralization with bicarbonate. The $Ca^{2+}/CaHCO_3^+$ ratio had no effect on calcium permeation across Caco-2 cell monolayers.
Conclusions. The physicochemical mechanism of intestinal calcium precipitation supports published clinical data by suggesting that once the solubility product of calcium carbonate is reached, increasing the calcium dose results in significant precipitation at intestinal pH values.

KEY WORDS: calcium absorption; calcium supplements; intestinal CO_2; intestinal HCO_3^- precipitation; Caco-2 transport.

INTRODUCTION

The administration of calcium supplements is used as a preventive measure to improve bone mineral density (1,2) for the prevention and treatment of osteoporosis. The average calcium intake (from food sources alone) for adult women is approximately 600–700 mg/day. If women are to meet their calcium requirement (1,000–1,200 mg/day), then approximately 50% of the required calcium must originate from supplements (3). Unfortunately, there is low and highly variable absorption of calcium from the gastrointestinal tract (GI; 4–40%) (4–6), which is largely attributed to biological variation. Potentially rate-limiting steps in calcium bioavailability are the calcium salt dissolution in the stomach, followed by subsequent precipitation and complexation/chelation in the gastrointestinal lumen.

Maintaining dissolved nutrients and drugs in the GI tract, specifically the small intestine, is a necessary step to oral absorption (7) and is greatly influenced by solubility (8). Calcium absorption studies on rats have shown that calcium transporters, specifically in the duodenum, are saturated at approximately 100 mM of calcium, suggesting that they are responsible for only about 1/3 of the approximately 120 mg of calcium absorbed from a standard 600 mg supplement (9,10). The remaining 2/3 of the soluble calcium is absorbed by passive diffusion in the ileum and jejunum. Whether calcium is transported actively or passively, it must be in solution to be absorbed (11). Therefore, the remaining 480 mg is not absorbed. The rate of passive absorption across the lumen typically increases linearly with concentration; however, increases in calcium salt solubility do not result in comparable increases in absorption (12,13). Therefore, other factors besides aqueous solubility measured in water must influence calcium absorption.

When the acidic contents of the stomach move into the small intestine, various mechanisms offer protection of the intestinal mucosa against the low pH. Two such mechanisms are the alteration of mucosal blood flow and release of neurotransmitters that control secretion of sodium bicarbonate ($NaHCO_3$) (14,15), which increases pH. The reaction between the intestinal bicarbonate and HCl from the gastric contents which is emptied into the duodenum produces carbonic acid (H_2CO_3) and is subject to the following equilibrium:

$$CO_{2(g)} \Leftrightarrow CO_{2(aq)} + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow HCO_3^- + H^+ \Leftrightarrow CO_3^{2-} + 2H^+$$

Depending upon pH, carbonic acid, a very unstable species, can either ionize to bicarbonate (HCO_3^-) and

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carbonate (CO_3^{2-}) or dehydrate to carbon dioxide dissolved in the aqueous intestinal contents.

The carbon dioxide dissolved in the intestinal lumen eventually diffuses to the bloodstream and is expired primarily from the lungs. However, during HCO_3^- secretion, the dissolved CO_2 concentration can accumulate to a significant concentration. Intestinal values of the partial pressure of carbon dioxide, P_{CO2}, (95–680 mmHg) (16,17) are significantly higher than atmospheric P_{CO2} or gastric P_{CO2} levels (18,19), 0.3 mmHg and 38 mmHg, respectively (Table I). The intestinal P_{CO2} values from dogs show that P_{CO2} nearly doubles in the fed state, due to the increased concentration of HCO_3^- secreted during digestion. While the human values shown in Table I are also in the fed state, and lower than dog values, it should be noted that the liquid meal administered to the subjects in that study had a pH of 6.6, with protein acting as a buffer to help maintain the relatively neutral pH even in the stomach. If the pH was reduced, higher HCO_3^- secretion would be expected, leading to a higher P_{CO2}. Furthermore, the effect of food on HCO_3^- secretion might be greater than that in dogs, since pentagastrin was used in dogs to stimulate greater acid production to mimic the fed state in humans (20). In any case, the accumulation of intestinal CO_2 indicates that the GI tract is not an open system, where CO_2 evolves quickly from solution. Instead there is a high concentration of dissolved CO_2 which is accompanied by high concentrations of carbonate species according to the equilibrium shown above.

The presence of carbonate species may affect the bioavailability of calcium ions. The HCO_3^- secreted into the duodenum can act as an overwhelming source for CO_3^{2-}, depending upon local pH. Once the concentrations of calcium and carbonate exceed the solubility product (K_{sp}), calcium carbonate ($CaCO_3$) will precipitate out of solution. The solubility product for calcium carbonate is defined as:

$$K_{sp} = \left[Ca^{2+}\right]_{eq}\left[CO_3^{2-}\right]_{eq} \quad (1)$$

where $[Ca^{2+}]_{eq}$ and $[CO_3^{2-}]_{eq}$ are the concentrations of the species at the limit of solubility. The intestinal precipitation of $CaCO_3$ has been reported in many marine species that secrete HCO_3^- as an osmoregulatory mechanism (21).

In humans, data from a clinical study that compared calcium absorption after administration of calcium carbonate tablets and calcium citrate solutions to normal subjects and achlorhydric patients (i.e., those with little to no gastric hydrochloric acid secretion) (22) also suggest calcium precipitation. As expected, the fraction of calcium absorbed from the calcium carbonate tablets was significantly higher in normal subjects due to the low stomach pH needed to dissolve the tablets. Surprisingly, however, after ingesting a calcium citrate solution, the absorption of calcium increased from approximately 25% in healthy subjects to 40% in achlorhydric patients. In patients with achlorhydria, the stomach pH ranges from 5 to 7. The chyme that empties into the duodenum does not have a pH low enough to induce appreciable secretin-mediated intestinal bicarbonate, and therefore, less carbonate is expected to be available to precipitate the calcium. Under these conditions, the calcium should be readily absorbed.

The hypothesis explored in the current work is that under various conditions in the intestine, calcium will precipitate as calcium carbonate. An *in vitro* system which simulates the relevant aspects of gastric and intestinal environments was devised to test this hypothesis. According to Eq. 1, precipitation of $CaCO_3$ depends on the concentration of Ca^{2+}, which depends on the dose and volume of water with which it is taken, as well as CO_3^{2-} in the intestine. Due to the carbonate equilibria described above, the concentration of CO_3^{2-} changes with HCO_3^- secretion rate. When acidic Ca^{2+} solutions from the stomach are neutralized with intestinal HCO_3^-, precipitation of $CaCO_3$ is just one of the possible fates of Ca^{2+}. The concentration of calcium bicarbonate ($CaHCO_3^+$) is expected to be quite high at certain conditions of pH and P_{CO2}. However, the absorption of associated (e.g. $CaHCO_3^+$) *versus* free calcium (e.g. Ca^{2+}) is not known.

Permeation of compounds across human colorectal adenocarcinoma (Caco-2) cell monolayers has been correlated with their *in vivo* rate of absorption (23). The relative effects of active and passive transport of calcium in Caco-2 cells were previously studied (24), with active transporters nearly saturated at a concentration of approximately 5 mM. The transport of calcium through Caco-2 monolayers under different pH and P_{CO2} conditions is presented to understand the effect of the carbonate species on intestinal calcium absorption.

The data presented are an initial step to investigate *in vitro* methodology and equilibrium relationships that may give insight into *in vivo* calcium absorption. If the precipitation of calcium as the carbonate salt can be understood and quantified under the changing pH and P_{CO2} conditions of the small intestine, it could lead to the development of more efficiently absorbed calcium formulations. The intestinal concentration of CO_3^{2-} may also influence the solubility, and therefore, absorbability of other minerals, such as iron.

Table I. Intestinal P_{CO2} Values for Human and Dog

Species	Segment	Average P_{CO2} in mmHg (range)	Method	Subjects
Human	Duodenum	280 (225–340)	Aspirations (17)	Healthy fed: liquid meal 10 g protein, 17 g lipid, 50 g carbohydrate in 300 ml water (pH 6.6)
Dog	Duodenum	Fasted: 263 (160–450) Fed: 413 (230–680)	Electrode (*in situ*) (16)	Healthy fed and fasted
	Upper jejunum	Fasted 120 (95–160) Fed 240 (160–350)		

MATERIALS AND METHODS

Materials

Calcium citrate tetrahydrate $[Ca_3(C_6H_5O_7)_2 \bullet 4H_2O]$ (Lot 12202TA), calcium chloride dihydrate $[CaCl_2 \bullet 2H_2O]$ (Lot 39H0085), potassium chloride [KCl] (Lot 033K0166), D-glucose (Lot 58H012), sodium bicarbonate [$NaHCO_3$] (Lot 043K0090), *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid [HEPES] (Lot 113K5401) and 2-(*N*-morpholino) ethanesulfonic acid [Mes] (Lot 032K5446) were purchased from Sigma-Aldrich (St. Louis, MO). Hydrochloric acid, sodium hydroxide and lanthanum solution were purchased from Fisher Scientific (Fair Lawn, NJ). Carbon dioxide (5% in air) was obtained from Airgas, Inc (Stratford, CT). Dulbecco's modified eagle medium [DMEM], penicillin/streptomycin, non-essential amino acids, L-glutamine, and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Radiolabeled calcium chloride [^{45}CaCl] (Lot NEZ013) and Optiphase Supermix scintillation cocktail were purchased from Perkin Elmer (Boston, MA). Human colorectal adenocarcinoma (Caco-2) cells were purchased from American Type Culture Collection (Rockville, MD).

In Vitro Simulation of Gastric Dissolution and Intestinal Bicarbonate Secretion

Simulated gastric dissolution/intestinal bicarbonate secretion experiments were performed in a 250 ml jacketed beaker at 37°C in triplicate. The schematic of the experimental apparatus is shown in Fig. 1. Calcium citrate $\bullet 4H_2O$ was added to 150 ml of 0.1 N HCl and stirred for 60 min to simulate the "gastric phase". A 5 ml sample was withdrawn and filtered through a 0.2 μm filter at 60 min which represented the initial time point for the "intestinal phase". At that time, sodium bicarbonate (1 N) was added at a rate of 1.2 ml/min to simulate intestinal bicarbonate secretion. As the pH reached each integral value, a 5 ml sample was withdrawn until the pH reached a value of 7. At each integral pH value (pH meter, Model 710A, Orion, Beverly, MA), the time was recorded and P_{CO2} was measured (CO_2 monitor, Biovision 8500, YSI, Inc., Yellow Springs, OH).

One set of experiments was performed with the calcium solution open to the atmosphere (0.3 mmHg). The second set of experiments was performed to simulate an arterial P_{CO2} (38 mmHg CO_2) with 5% CO_2 flowing over the solution (Fig. 1). For both sets, the calcium dose was 250 mg elemental calcium.

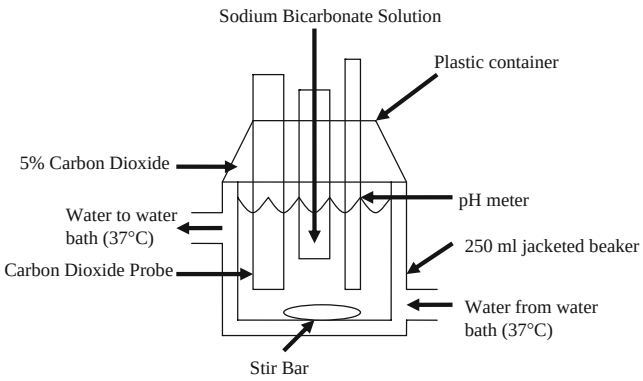

Fig. 1. Schematic of experimental apparatus.

Filtered samples were diluted in 0.1 N HCl for analysis of total soluble calcium by flame atomic absorption (AA) spectrometry (Varian Spectra AA 200, Walnut Creek, CA) with air/acetylene flame (13.02:2.00 l/min), Ca/Mg lamp wavelength of 422.7 nm and current of 10 mA. Lanthanum solution (5%) was added to each sample to minimize chemical interference common to AA analysis. A calibration curve was generated prior to each analysis.

Three calcium doses (250, 475, 630 mg elemental calcium as calcium citrate $\bullet 4H_2O$) were neutralized using a HCO_3^- secretion rate of 0.12 mEq/min. The 250 mg and 630 mg doses were also neutralized at a HCO_3^- secretion rate of 1.2 mEq/min. The volume of bicarbonate solution added was approximately 35 ml, which is 23% of the starting volume. However, with five to six aliquot withdrawals of 5 ml, the total volume remained constant. Any dilutional effects were accounted for in calculations.

Calcium Permeation Across Caco-2 Cell Monolayers

The Caco-2 cells (Passage #29-37) were grown in DMEM with 1% penicillin/streptomycin, 1.1% non-essential amino acids, 1% L-glutamine, and 10% fetal bovine serum at 37°C and 38 mmHg CO_2. Confluent monolayers of cells were used in transport studies 21 to 26 days post-seeding on Costar Transwell™ 12-well plates (12 mm diameter, 0.4 μm pore size).

The transport media (apical and basolateral) used was Earle's buffered salt solution (117 mM NaCl, 5.33 mM KCl, 5.56 mM D-glucose and 26.2 mM $NaHCO_3$) with no phosphate present to minimize association with calcium. In addition, the apical media contained either 10 mM Mes buffer, adjusted to pH 5 or 6 using 0.1 N HCl, or 10 mM HEPES buffer, adjusted to pH 7 using 0.1 N HCl or 8 using 2 N NaOH. The basolateral media contained 10 mM HEPES, adjusted to pH 7.4 with 2 N NaOH. Trans-epithelial electrical resistance (Millipore Millicell®-ERS, Billerica, MA) values were recorded prior to and at the conclusion of each transport study to monitor monolayer integrity. Radiolabeled calcium [^{45}Ca]Cl at a concentration of 1 μCi/ml was added to calcium chloride solutions at concentrations of 15 and 50 mM in the apical media, previously adjusted to pH 5–8. Active calcium transporters were saturated at both concentrations, with approximately 1/2 calcium transported passively at 15 mM and 2/3 transported passively at 50 mM total soluble calcium (24).

The apical side of the monolayer contained 400 μl of the calcium solution (radiolabeled + "cold" solution), and 1,200 μl of the transport media (no calcium) was added to the basolateral side. During the transport study, the plates were incubated either at 38 mmHg CO_2 or within the range of 76–152 mmHg CO_2. The higher CO_2 atmosphere is expressed as a range because a specific pressure could not be precisely maintained; the P_{CO2} atmosphere will be expressed as its midpoint, 114 mmHg. At sample times of 15, 30, 60, and 90 min, 100 μl were withdrawn from the basolateral compartment and replaced with transport media. A scintilla-

Table II. Percentage of $CaHCO_3^+$ Relative to Total Soluble Calcium in Various pH and P_{co2} Environments

pH	38 mmHg CO_2	114 mmHg CO_2
5	0.10	0.42
6	1.0	4.0
7	9.5	30
8	51	81

tion cocktail (150 µl) was added to each sample which was then analyzed by liquid scintillation counting (1450 Microbeta, Perkin Elmer, Boston, MA).

A range of apical pH (pH 5–8) and CO_2 environments (38 and 114 mmHg) were used during the transport studies to obtain a wide range of $CaHCO_3^+$ and Ca^{2+} concentrations to determine any differences in their permeabilities (Table II). Apparent permeability coefficients (P_{app}) of total soluble calcium were calculated by the following:

$$P_{app} = \frac{dQ/dt}{A \times C_0} \quad (2)$$

where dQ/dt is the apical-to-basolateral flux of calcium over time, A is the area of the monolayer, and C_0 is the initial concentration of calcium on the apical side.

RESULTS AND DISCUSSION

The purpose of this work was to determine the extent of Ca^{2+} precipitation as $CaCO_3$ in a simulated intestinal environment during the secretion of HCO_3^- and whether $CaHCO_3^+$ that forms under some conditions has equivalent absorption to free calcium ion. The combination of *in vitro* neutralization profiles and Caco-2 cell transport under various *in vivo* conditions were used to determine the influence of bicarbonate on the availability of soluble calcium in the intestine.

Effect of CO_2 Atmosphere

The majority of bicarbonate in the GI tract is secreted to increase the pH of stomach contents when it enters the small intestine. Data from humans and dogs both indicate accumulation of significant levels of CO_2 in the various segments of the intestine (Table I), with values in the human duodenal lumen reaching 340 mmHg after a liquid meal. The concentration of dissolved CO_2 was previously reported to affect the concentration of carbonate species and the solubility of calcium (25).

Simulated intestinal HCO_3^- secretion experiments were performed under a headspace of 0.3 mmHg CO_2 (i.e., open to atmosphere) and 38 mmHg CO_2 (corresponding to arterial P_{CO2}) to evaluate the effect of surrounding CO_2 on intestinal soluble calcium. At a dose of 250 mg of calcium as the citrate salt, all of the calcium dissolved in 150 ml of 0.1 N HCl to produce a pH of approximately 2. The concentration of total soluble calcium did not change significantly from initial to final time or pH (p value ≤0.05) during bicarbonate secretion at a rate of 0.12 mEq/min over 180 min as the pH increased from 2 to 7 (Fig. 2). In the open atmosphere at 0.3 mmHg, it was assumed that CO_2 would evolve freely into the atmosphere, producing a lower concentration of carbonate (CO_3^{2-}). Unexpectedly, the P_{CO2} in the solution accumulated to a maximum of approximately 250 mmHg, similar to the P_{CO2} of the closed atmosphere (Fig. 3a). This is attributed to the high concentration of dissolved CO_2 in both open and closed systems, which produces a large difference in the P_{CO2} across the air/water interface (ΔP_{CO2}). The relatively small difference in CO_2 in the surrounding atmosphere did not significantly change the ΔP_{CO2}, which is the driving force of CO_2 diffusion out of the solution. For example, the ΔP_{CO2} was 212 mmHg (i.e., 250–38) for the 38 mmHg CO_2 atmosphere *versus* 250 mmHg (250–0.3) for the "open" atmosphere. The relatively small difference (212 *versus* 250 mmHg CO_2) led to an insignificant difference in the driving force for the diffusion of CO_2 from the simulated intestinal environment. In both cases, the P_{CO2} in the solutions reached the levels reported in the fasted dog duodenum (Table I).

When the experiments were performed at the higher bicarbonate secretion rate of 1.2 mEq/min, there was even greater accumulation of CO_2, resulting in a maximum partial pressure of approximately 600 mmHg (Fig. 3b). The high P_{CO2}, similar to duodenal P_{CO2} in the fed dog (Table I), again resulted in no significant difference in CO_2 concentrations between the experimental atmospheres (38 and 0.3 mmHg), except for the final time point. Once it was determined that the surrounding CO_2 environment did not significantly affect the calcium profiles, the remaining experiments were performed open to the atmosphere. More importantly, the results

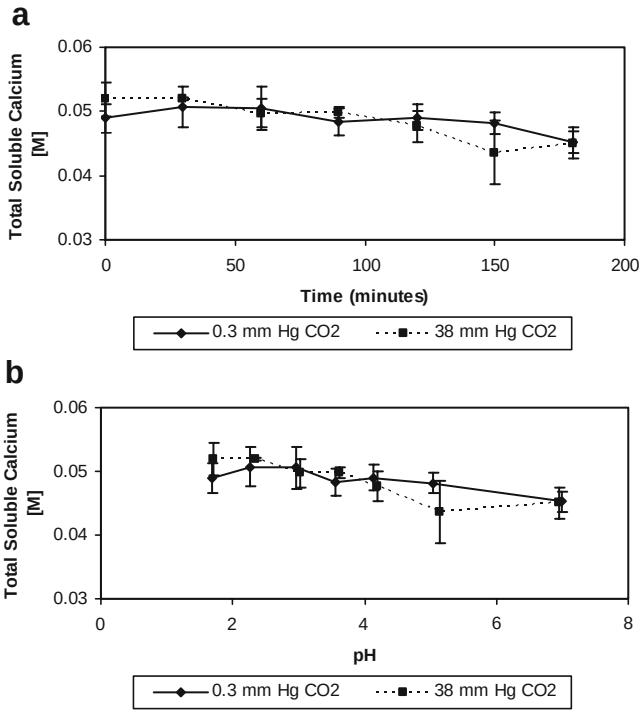

Fig. 2. Total soluble calcium *versus* **a** time and **b** pH (250 mg Ca^{2+}, 0.12 mEq/min HCO_3^- secretion rate) in an open and 38 mmHg CO_2 atmosphere.

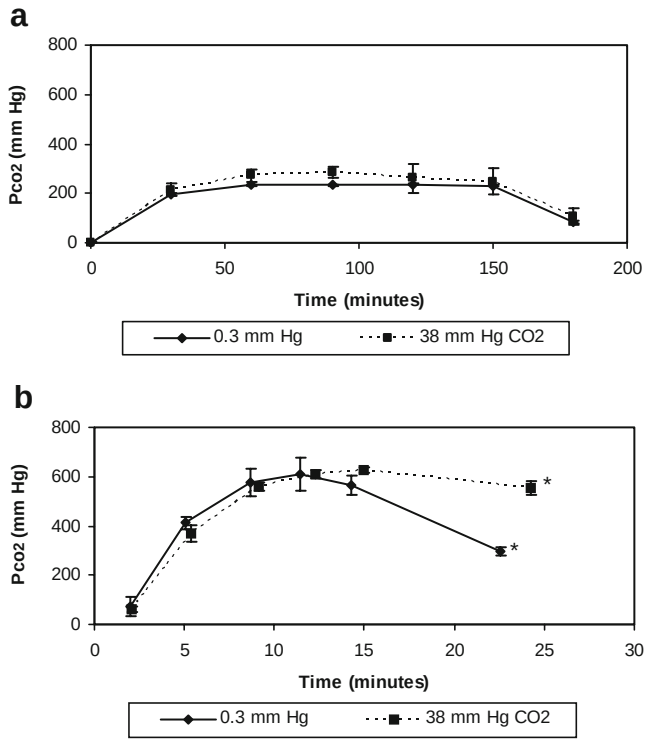

Fig. 3. P_{CO2} (mmHg) *versus* time at **a** 0.12 mEq/min HCO_3^- secretion rate and **b** 1.2 mEq/min HCO_3^- secretion rate (250 mg Ca^{2+} in an open and 38 mmHg CO_2 atmosphere) [*asterisk p* value ≤ 0.05].

indicate that the evolution of dissolved CO_2 from the solution into the surrounding atmosphere is rate-limiting. The intestinal P_{CO2} measured in humans and dogs (Table I), and the data in Fig. 3 suggest that CO_2 does not freely diffuse out of the intestinal lumen as previously suggested (26). Instead, it is abundant and could affect the fate of calcium.

Effect of Calcium Dose

Total soluble calcium, as measured in these experiments, represents the sum of the calcium ion (Ca^{2+}) and any other soluble calcium ions, such as calcium bicarbonate ($CaHCO_3^+$). The concentration of total soluble calcium is affected by calcium dose in the range of 250–630 mg elemental calcium, representing the variation in typical supplement doses (Fig. 4). Calcium citrate was added to 0.1 N HCl and allowed to dissolve for 60 min prior to the addition of bicarbonate. While the low dose (250 mg elemental calcium) completely dissolved in the 150 ml simulated "gastric phase", there was undissolved solid remaining at the higher doses (475 and 630 mg), resulting in the same higher initial concentration of total soluble calcium at the higher doses prior to bicarbonate secretion (Fig. 4a). The solutions with an amount of calcium per volume that exceeded solubility had the same higher pH values (approximately pH 3) before neutralization because more calcium citrate had dissolved as compared to the low dose that had a final pH of approximately 1.2 after dissolution in the "gastric phase" (Fig. 4b).

The low dose of 250 mg did not significantly precipitate during bicarbonate secretion, indicating that insufficient calcium and carbonate were present to reach the $CaCO_3$ solubility product (K_{sp}), regardless of pH. However, there was a dramatic reduction in total soluble calcium for the 630 mg dose between 15 and 150 min, corresponding to pH 3 and 5. When the dose was decreased to 475 mg, the same reduction in soluble calcium concentration was observed; however, the decrease did not occur until 100 min (pH 4). As pH increases, CO_3^{2-} concentration increases, allowing for greater potential of the CO_3^{2-} and Ca^{2+} product to reach K_{sp}. The 475 mg dose did not reach the limiting concentration of both species until pH 4.

At the dose of 250 mg, all of the calcium is in solution. However, at higher doses, calcium had reached its solubility and was in solution in the form of calcium bicarbonate ($CaHCO_3^+$), along with calcium (Ca^{2+}) and some calcium citrate species. Since HCO_3^- was associated with the calcium at low pH, it remained in solution rather than diffusing out slowly as carbon dioxide. As the pH of the solution was raised by neutralization with bicarbonate, the $CaHCO_3^+$ released additional bicarbonate thereby raising the concentration of carbonate (the predominant species at higher pH values) causing precipitation of calcium as calcium carbonate. At the lower dose of 250 mg, there is less $CaHCO_3^+$ in solution to maintain the bicarbonate concentration as high when the pH is raised. This behavior is seen more readily at the lower neutralization rates than at high neutralization rates. At the higher rates, the solution is nearly saturated with carbonate species.

The pH range where total soluble calcium decreased in Fig. 4 is comparable to the pH in the duodenum, where

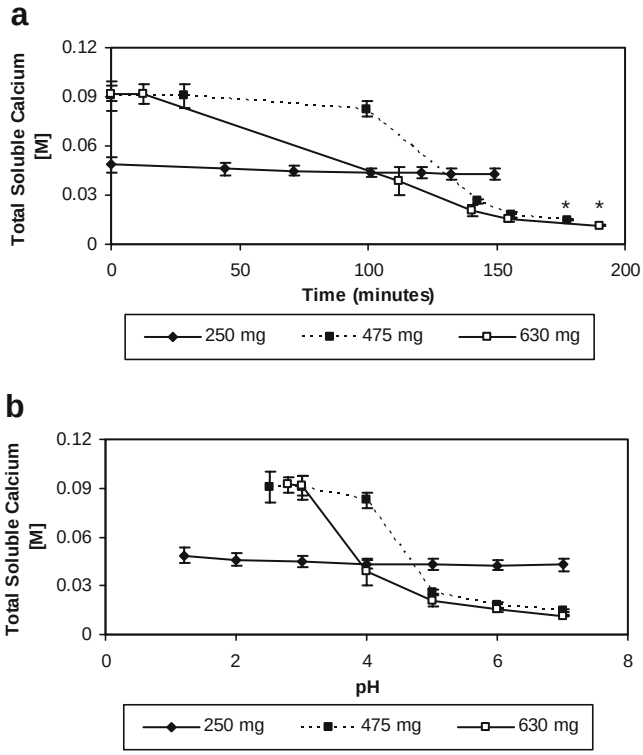

Fig. 4. Total soluble calcium *versus* **a** time and **b** pH (0.12 mEq /min HCO_3^- secretion rate) at three calcium doses [*asterisk p* value ≤ 0.05 from initial value].

contents are quickly neutralized to approximately pH 6 prior to entering the jejunum (27). The calcium profiles at the higher doses suggest that a calcium concentration of approximately 0.1 M entering the intestine could decrease an order of magnitude to 0.01 M in the jejunal/ileal segments of the small intestine due to precipitation of calcium carbonate, so the data can explain in part the low absorption [approximately 20% (13)] of calcium from supplement. The 250 mg dose would also be subject to precipitation if sufficient concentrations of Ca^{2+} and CO_3^- are present to reach K_{sp}. Under the experimental conditions, $CaCO_3$ did not precipitate at the 250 mg dose. However, clinical studies have shown that the fraction of calcium absorbed reaches 35% at approximately 250 mg dose (28), so a higher CO_3^- concentration or other precipitating counterions, such as phosphate, not represented in the current methodology may influence precipitation *in vivo*.

Effect of Bicarbonate Secretion Rate

The intestinal bicarbonate (HCO_3^-) secretion rate varies with GI conditions, such as the presence of food and the pH of the stomach contents entering the duodenum. The concentration of HCO_3^- due to secretion and local pH will determine the local concentration of CO_3^{2-}, and if sufficient, may alter total soluble calcium. Total soluble calcium concentrations remaining from doses of 250 and 630 mg elemental calcium were determined as a function of time at two HCO_3^- secretion rates, 0.12 mEq/min and 1.2 mEq/min, with neutralization times of approximately 180 and 30 min, respectively (Fig. 5a). The secretion rates were chosen based on the P_{CO2} profiles, ranging from approximately 250–600 mmHg (Fig. 3a and b), which are in the range of human and dog values (Table I).

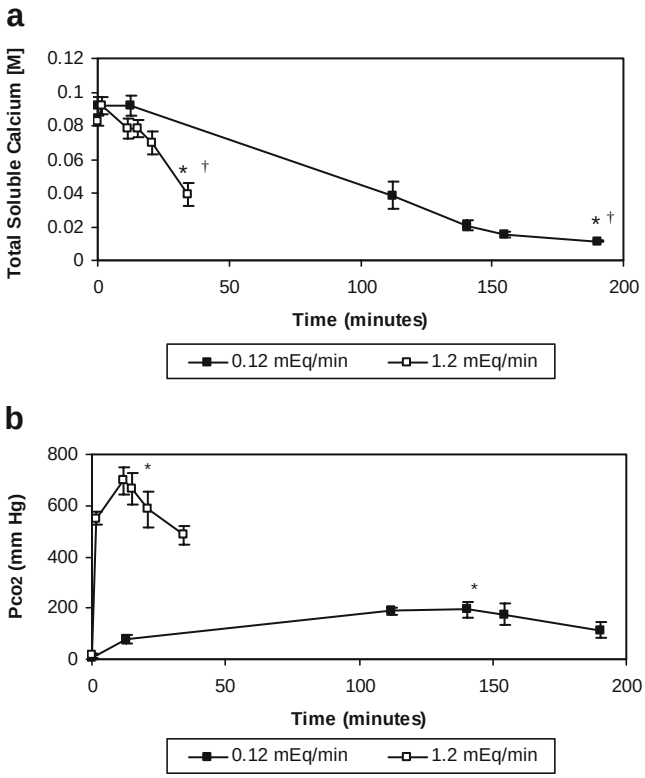

Fig. 5. **a** Total soluble calcium and **b** P_{CO2} *versus* time (0.12 and 1.2 mEq /min HCO_3^- secretion rate) at 630 mg elemental calcium [*asterisk* p value ≤ 0.05 from initial, *dagger* p value ≤ 0.05 between secretion rates].

When the HCO_3^- secretion rate was increased by an order of magnitude to 1.2 mEq/min (data not shown), the total soluble calcium profile for the 250 mg dose did not differ from the lower secretion rate (Fig. 4a). Therefore, increasing CO_3^{2-}, by increasing HCO_3^-, did not influence precipitation, indicating that this low calcium dose is insufficient to reach K_{sp} of calcium carbonate, regardless of HCO_3^- secretion rate. This suggests that the fraction of calcium absorbed from a 250 mg elemental calcium dose of calcium citrate administered with 100 ml should be very high *in vivo* in both fed and fasted states, assuming existing 50 ml of solution in the stomach. The soluble calcium remaining from a 630 mg dose decreased during bicarbonate secretion but the reduction (0.09 M to 0.04 M) was not as large as compared to that at the lower secretion rate (0.09 M to 0.01 M) when both solutions were neutralized to pH 7 (Fig. 5a). The difference in soluble calcium profiles of the 630 mg dose was due to the time scale differences in P_{CO2} profiles (Fig. 5b). There was an immediate dramatic increase in P_{CO2}, resulting in higher CO_3^{2-} with the high secretion rate, while the low secretion rate did not produce the same extent of CO_2 accumulation. When the precipitation is compared on the time scale, the higher HCO_3^- secretion rate caused more precipitation at a specific time point. However, at the end of each profile when pH 7 was reached, the lower HCO_3^- resulted in greater overall precipitation, indicating the importance of the concentration of HCO_3^- available to associate with calcium but still remain soluble. The kinetic aspect in reaching K_{sp} is mostly due to the dynamic profile of CO_2 accumulation that includes contribution from CO_3^{2-} and HCO_3^-, both which can interact with Ca^{2+} resulting in precipitation or the formation of a soluble ion.

The P_{CO2} profile would be expected to have significance *in vivo* due to the differences in HCO_3^- secretion rate in the fed and fasted states, as seen in the intestinal P_{CO2} values of the dog (Table I). It might be expected that the fed state, which has a higher P_{CO2} value would result in higher $CaCO_3$ precipitation. However, calcium absorption is enhanced when calcium is administered with a meal (29). This is most likely due to the slower gastric emptying with food [25% of liquid remains in stomach as opposed to 80% of solid remaining after 1 h (30)]. Therefore, a higher concentration of calcium enters the duodenum at one time in the fasted state, where high calcium concentrations can be precipitated by basal levels of carbonate (Fig. 4). The longer sojourn time in the intestine would also increase the exposure of calcium to active transporters, specifically in the duodenum, thereby increasing the efficiency of absorption.

Many different calcium species can exist along the intestinal tract, and concentrations can be estimated by using the relationship between the species at equilibrium. With total soluble calcium, $(Ca_{sol})_{total}$, consisting of at least the two ions, free calcium ion (Ca^{2+}) and calcium bicarbonate ($CaHCO_3^+$), the concentration of free calcium ion $[Ca^{2+}]$ can be written as

$$\left[Ca^{2+}\right] = [Ca_{sol}]_{total} - \left[CaHCO_3^+\right] \quad (3)$$

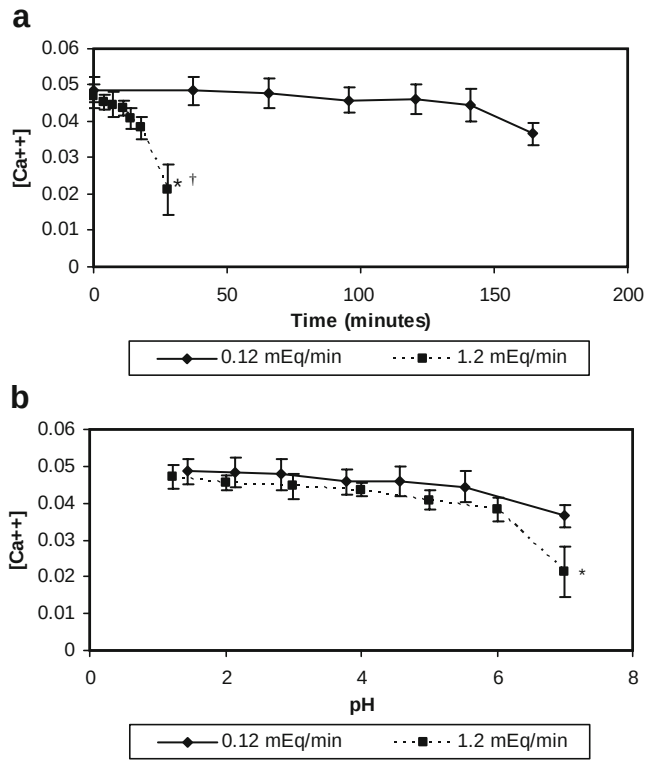

Fig. 6. Free calcium ion (no $CaHCO_3^+$) *versus* **a** time and **b** pH at a dose of 250 mg Ca^{2+} at different HCO_3^- secretion rates [*asterisk p* value ≤ 0.05 from initial, *dagger p* value ≤ 0.05 between secretion rates].

When pH and P_{CO2} are known (using experimentally determined values), the concentration of bicarbonate can be determined:

$$[CO_2] = K_H P_{CO2} \tag{4}$$

$$[HCO_3^-] = \frac{K_{a1}[CO_2]}{[H^+]} \tag{5}$$

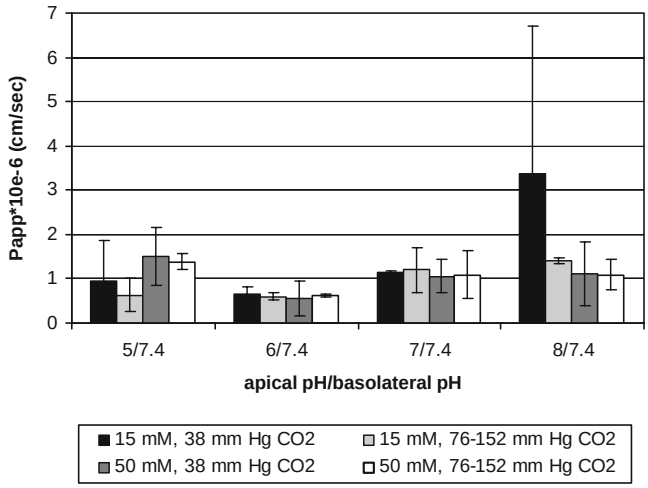

Fig. 7. Apparent permeability (P_{app}) of two calcium concentrations (15 and 50 mM) and two CO_2 environments (38, 76–162 mmHg) through Caco-2 cell monolayers.

where K_H is 2.6×10^{-2} and K_{a1} is 4.9×10^{-7} (at 35°C) (31). The concentration of calcium bicarbonate is calculated using the following equation:

$$[CaHCO_3^+] = K_{asn}[Ca^{2+}][HCO_3^-] \tag{6}$$

where K_{asn} is 16.6, the association constant of calcium and bicarbonate. Substitution of Eq. 3 into Eq. 6 yields the concentration of calcium bicarbonate ion in terms of the total soluble calcium:

$$[CaHCO_3^+] = K_{asn}([Ca_{sol}]_{total} - [CaHCO_3^+])[HCO_3^-] \tag{7}$$

Once $[CaHCO_3^+]$ is known, $[Ca^{2+}]$ can be calculated (Eq. 3). All constants (equilibrium and association) are adjusted for ionic strength according to the Bromley equation (32). Equations 1, 3–7 account for the concentration of each individual calcium species (both soluble and insoluble).

From calculations at different pH and P_{CO2} values reported *in vivo*, we find that intestinal $CaHCO_3^+$ is present in significant concentrations (Table II). For example, at 152 mmHg CO_2, the species $CaHCO_3^+$ exists at a level of 0.4–80% of total soluble calcium in the pH range of 5–8. While there was no change in total soluble calcium at the 250 mg dose (Fig. 4), the assay method used in the *in vitro* experiments could not distinguish changes in the proportion of each soluble calcium species. Since pH, P_{CO2} and total soluble calcium were experimentally determined, it was possible to estimate the proportions of Ca^{2+} and $CaHCO_3^+$ to total soluble calcium assuming no significant concentration of other soluble ions.

At the low HCO_3^- secretion rate (0.12 mEq/min), the calculated concentration of Ca^{2+} gradually declined with time (Fig. 6a) between pH 6 and 7 (Fig. 6b). At the high HCO_3^- secretion rate (1.2 mEq/min), $[Ca^{2+}]$ also declined until pH 6 was reached at 20 min. When neutralization was completed at 30 min, $[Ca^{2+}]$ decreased by more than 50% from initial. The reduction in free calcium concentration was due to the higher P_{CO2} at the higher secretion rate (Fig. 3a and b), thereby generating more $CaHCO_3^+$ at pH 7 than at the lower secretion rate. If Ca^{2+} and $CaHCO_3^+$ have different rates of absorption, the distinction between the two ions, which are both in solution, is an important one.

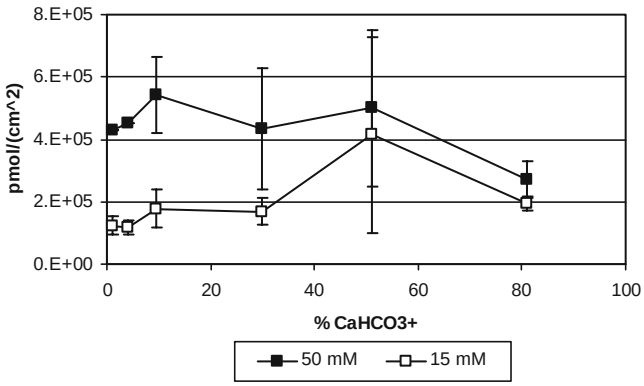

Fig. 8. Calcium transport at 120 min *versus* % $CaHCO_3^+$ through Caco-2 cell monolayers.

Permeation of Ca^{2+} and $CaHCO_3^+$ Across an Intestinal Cell Monolayer

Calcium permeation across Caco-2 cell monolayers was determined under different pH and CO_2 environments to ascertain any difference is absorption of Ca^{2+} and $CaHCO_3^+$. The apparent calcium permeability did not change with pH and P_{CO2} (Fig. 7), representing a wide range of Ca^{2+} and $CaHCO_3^+$concentrations (Table II), suggesting no differences in absorption of Ca^{2+} and $CaHCO_3^+$. When the transport of total calcium is compared across the range of % $CaHCO_3^+$ established in Table II, there is no difference at either the 15 mM or 50 mM concentration (Fig. 8). Although there was a decrease in $[Ca^{2+}]$ with bicarbonate secretion in the dissolution/secretion simulation experiments (Fig. 6), it would not be expected to affect absorption since the presence of $CaHCO_3^+$ did not alter the transport of calcium. It should be noted that the pH throughout the cellular membrane in the intestine is above pH 7 (33), which may have affected the variability of calcium species transported across the membrane.

CONCLUSIONS

The results suggest the potential of calcium carbonate precipitation in the intestine as a barrier to absorption of large calcium supplement doses. Therefore, administration of high elemental calcium doses should not result in comparable increases in absorption and is supported by clinical data (13). Other soluble calcium ions may need to be accounted for in refinements of this simulated intestinal model. Since the concentration of bicarbonate is secreted for intestinal neutralization, it would be a major source of ionic association with calcium. Bicarbonate secretion, and therefore $CaCO_3$ precipitation, would be expected to decrease when stomach contents were more neutral.

The data illustrate the extent of precipitation under "normal" conditions. If the precipitation could be minimized, the fraction of calcium absorption would increase, as previously reported in achlorhydric patients (22). It may be critical to patients administered proton pump inhibiting drugs that have been shown to absorb dangerously low fractions of calcium (3.5%) (6). This is an area of clinical study that may yield an *in vitro/in vivo* correlation between human calcium absorption and the methodology presented in this paper. Understanding the profiles of calcium precipitation may increase calcium absorption through formulation optimization. For example, administering calcium as a solution to a neutral stomach would bypass the solubility barrier in the stomach while reducing bicarbonate secretion and the intestinal calcium precipitation that accompanies it.

Other components in the GI tract, besides calcium dose, pH and P_{CO2} may effect intestinal $CaCO_3$ precipitation. Intestinal bile salts could maintain solubility of calcium but whether the complex is absorbable is unknown. Phosphate ions present in the GI tract are another source of calcium precipitation, depending on the K_{sp} of many possible calcium phosphate species. In the present work, only carbonate species are evaluated because bicarbonate is the physiologic buffer used to increase intestinal pH, and the concentrations of carbonate species are far higher than the other species to be considered. In addition, the presence of food may impact precipitation in various ways. First, sojourn time in each segment of the intestine could change, allowing more time for duodenal absorption with more active transporters, relative to other segments. Second, the dietary components, such as protein and amino acids may affect precipitation. The extent of these effects is the basis for future work.

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