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Intraduodenal delivery of intrinsically and extrinsically labelled $CaCO_3$ in the rat: effect of solubilization on calcium bioavailability*

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Abstract—The dissolution of $CaCO_3$ before intraduodenal administration was found to be an important factor determining calcium (Ca) bioavailability. Extrinsically and intrinsically labelled $^{47}CaCO_3$ preparations were sequentially dissolved by serial additions of HCl. Aliquots of these preparations were collected before (no HCl added) and during the solubilization process and administered intraduodenally to rats. Whole body ^{47}Ca retention 72 h post-dose was used as a measure of Ca bioavailability. Although dissolution of $CaCO_3$ significantly increased Ca bioavailability ($P < 0.001$), Ca from both intrinsically and extrinsically labelled $CaCO_3$ was absorbed and retained to some extent without prior acid dissolution. Due to a disproportionately high concentration of ^{47}Ca on the particle surface, extrinsically labelled $^{47}CaCO_3$ overestimated bioavailability when unsolubilized or partially solubilized $CaCO_3$ preparations were used ($P < 0.05$). These data indicate that dissolution is a determining factor for Ca bioavailability from $CaCO_3$. Incomplete dissolution will significantly limit but not completely prevent Ca bioavailability. The disintegration and dissolution characteristics of commercial $CaCO_3$ preparations, which vary widely, may produce important differences in Ca absorption.

Calcium carbonate is the most common type of calcium supplement. Commercial calcium carbonate preparations are known to vary widely in their disintegration and dissolution characteristics, which in turn are believed to affect the subsequent absorption of calcium in the small intestine. The significance of tablet disintegration as a determining factor in calcium bioavailability is clear (Carr & Shangraw 1987). However, even when calcium preparations are completely dissolved in the stomach, it is likely that recomplexation and precipitation will occur in the neutral environment of the small intestine where bicarbonate is a major buffer. The relationship between gastric dissolution and calcium bioavailability is, therefore, not entirely clear. To assess the effect of gastric dissolution, we measured calcium bioavailability from radiolabelled $CaCO_3$ samples which were partially dissolved and then directly introduced into the duodenum. The same technique was also used to assess the difference in apparent bioavailability of calcium from extrinsically and intrinsically labelled $CaCO_3$.

Materials and methods

Preparation of radiolabelled $CaCO_3$. $CaCO_3$ was prepared by adding a molar excess of carbonate (as Na_2CO_3) to a $CaCl_2$ solution and stirred at room temperature (21°) for a minimum of 2 h. The precipitate was collected by vacuum filtration, resuspended in water, refiltered and oven dried overnight at 32°C. The $CaCO_3$ preparations were radiolabelled with $^{45}CaCl_2$ (sp. act. 0·059 μCi mg^{-1} Ca^{2+}) for in-vitro experiments or $^{47}CaCl_2$ (sp. act. 0·25 μCi mg^{-1} Ca^{2+}) for in-vivo experiments (Amersham Corp., UK). Extrinsically labelled $CaCO_3$ was made by adding the radioisotope to a $CaCO_3$ suspension. This mixture was stirred overnight at room temperature (21°C). Intrinsically labelled $CaCO_3$ was made by adding radioisotope to the $CaCl_2$ solution before the addition of Na_2CO_3.

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Animals. Young adult male Sprague-Dawley rats, 200–220 g, were purchased from Charles River Breeding Laboratories (Portage, MI). Stringent animal care and use policies, conforming to the guidelines of the Committee on the Care and Use of Laboratory Animals (1985), were employed during all phases of the study. Upon receipt, the animals were individually housed in suspended stainless steel cages in an environmentally controlled room (22±2°C; relative humidity 30–60%; 12 h light-dark cycle). Food and water were freely available during acclimation and after administration of aqueous calcium carbonate suspensions. All rats were acclimatized for at least 5 days before treatment.

Release of ^{45}Ca from extrinsically and intrinsically labelled $CaCO_3$ during acid solubilization. Triplicate samples of intrinsically and extrinsically labelled $^{45}CaCO_3$ were suspended in distilled water to provide a final concentration of 5 mg Ca mL^{-1}. All suspensions were sequentially treated with eight equal portions of 12 M HCl. The total amount of HCl added was sufficient for complete $CaCO_3$ dissolution. Each preparation was maintained with constant vigorous stirring to ensure contact between reactants, as well as homogeneity of the subsequent sampling. Samples (1·5 mL) were removed 1 min after each addition of the acid and filtered through a 0·45 μm Acrodisc filter (Gelman Sciences, Inc., Ann Arbor, MI); the filtrate was analysed for ^{45}Ca by liquid scintillation spectrophotometry. The radioactivity of the filtrate was compared with an unfiltered sample to determine the percent solubilization of ^{45}Ca.

Whole body ^{47}Ca retention from intraduodenally administered extrinsically and intrinsically labelled $CaCO_3$. Each animal received 5 mg of Ca^{2+} in a 0·5 mL dose volume. The dose suspensions or solutions were prepared by combining 1 mol extrinsic or intrinsic $^{47}CaCO_3$ with 0, 0·48, 1·44, 2·88, or 5·76 mol HCl in a 4 mL volume. The latter two HCl concentrations were adequate for complete calcium solubilization. The animals were deprived of food overnight and water for 2 h before experimentation and then anaesthetized by intraperitoneal sodium pentobarbitone injection. A small midline incision was made just posterior to the sternum, and the duodenum was exteriorized with a cotton-tipped swab. Test suspensions or solutions were administered by intraluminal injection (1 mL hypodermic syringe, 23 gauge needle) in the area immediately adjacent to the pyloric junction. Closing of the incision was accomplished with 4-0 silk sutures (abdominal wall) and 9 mm Clay Adams wound clips (skin). Access to food and water was made available 3 h post-dose.

The rats were placed in a small-animal whole-body gamma counter immediately after the the procedure and again 72 h later. All counts were corrected for background radioactivity and the 72 h count was corrected for isotopic decay. Percent whole-body ^{47}Ca retention was calculated as the 72 h count divided by the initial count × 100. In preliminary experiments we determined that 72 h was sufficient time to clear ^{47}Ca.

Statistics. Differences between treatment means were compared by Student's *t*-test.

Results and discussion

^{45}Ca was liberated in a linear manner from intrinsically labelled $CaCO_3$ during the in-vitro dissolution process (Fig. 1). In contrast, ^{45}Ca was released from extrinsically labelled $CaCO_3$ in a non-linear fashion during solubilization. Furthermore, there was a substantial amount of ^{45}Ca in a filtered sample of the extrinsic product before addition of HCl. At a $CaCO_3$:HCl molar ratio of 1:0·564, approximately 80% of the ^{45}Ca from extrinsically labelled $CaCO_3$ was solubilized compared with only 25% of the ^{45}Ca from intrinsically labelled $CaCO_3$.

The effects of $CaCO_3$ dissolution on whole-body ^{47}Ca retention are summarized in Table 1. Both intrinsically and extrinsically labelled $CaCO_3$ were absorbed and retained to some extent even without prior acid dissolution. Sequentially dissolving both salts with exogenous HCl before intraduodenal delivery, increased ^{47}Ca retention until the Ca^{2+} was completely solubilized ($CaCO_3$:HCl molar ratios above 1:2). Differences in ^{47}Ca retention between extrinsically and intrinsically labelled $^{47}CaCO_3$ were significant at $CaCO_3$:HCl molar ratios of 1:0 ($P=0·005$) and 1:0·48 ($P=0·006$), of borderline significance at a molar ratio of 1:1·44 ($P=0·08$) and not statistically different when ^{47}Ca was completely solubilized ($CaCO_3$:HCl molar ratios above 1:2).

Intraduodenal dosing deviates from the normal situation by bypassing the stomach and, thus, may not yield results equivalent to oral dosing. For example, although calcium retention from intraduodenally administered unsolubilized $CaCO_3$ was 39% lower than the completely solubilized sample (19 vs 58%), it is only 12% lower than the value previously obtained from a comparable oral dose (19 vs 31%) (Smith et al 1987). The two comparisons presumably reflect, respectively, potential and actual effects of gastric acid-related solubilization on absorption from a sparingly soluble source. The much smaller difference attributable to oral dosing suggests that the calcium may not be completely solubilized during its passage through the stomach. Alternatively, the slower delivery of an oral $CaCO_3$ dose to the intestinal environment would be more vulnerable to reprecipitation of calcium, which in turn, would limit bioavailability. Overall, however, these results do support widely held beliefs regarding the positive effect of gastric acid-related solubilization on calcium bioavailability from $CaCO_3$, but also demonstrate that a substantial amount will be absorbed even without the acid-related solubilization. Further data supporting the limited influence of solubilization before entry, on intestinal calcium absorption, and possible explanations to account for the substantial absorption which occurs, have been discussed by other investigators (Bo-Linn et al 1984; Sheikh et al 1987; Heaney et al 1990).

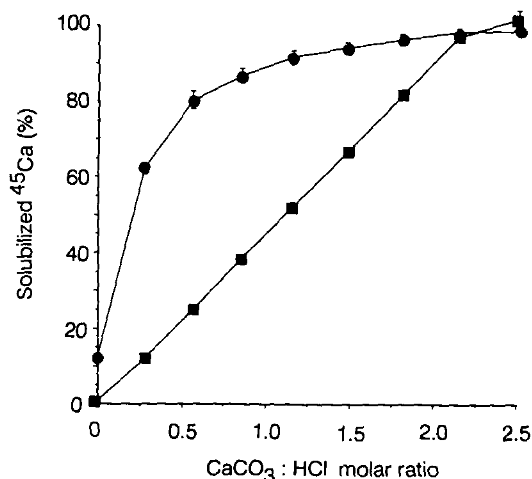

FIG. 1. Release of ^{45}Ca during sequential dissolution of intrinsically (■) and extrinsically (●) labelled $^{45}CaCO_3$. Each point is the mean ± s.d. for 3 determinations.

Table 1. Effect of $CaCO_3$ solubilization on whole body ^{47}Ca retention from intraduodenally delivered intrinsically and extrinsically labelled $^{47}CaCO_3$.

Molar ratio $CaCO_3$:HCl	% Whole-body ^{47}Ca retention Intrinsic	Extrinsic	P*
1:0	19·2 ± 8·9	33·5 ± 5·3	0·005
1:0·48	25·2 ± 6·4	42·5 ± 5·7	0·006
1:1·44	42·8 ± 5·3	49·2 ± 5·8	0·08
1:2·88	57·6 ± 7·4	55·3 ± 3·9	ns
1:5·76	58·0 ± 10·1	58·7 ± 11·3	ns

Each value is the mean ± s.d. for 6 determinations.
*Unpaired two-tailed *t*-test comparing intrinsic with extrinsic.

The passage of undissolved $CaCO_3$ particles into the intestine after ingestion of commerical preparations could occur for several reasons. Clearly, incomplete tablet disintegration will limit bioavailability and is an important formulation consideration. In addition, slow gastric dissolution, rapid gastric emptying, or reduced gastric acid production would also favour passage of intact $CaCO_3$ particles. Elderly individuals are known to experience an age-related decline in gastric acid secretion (Baron 1963; Christiansen 1968) and may, therefore, be prone to hypoabsorption of Ca^{2+} from $CaCO_3$. However, reduced bioavailability from poorly formulated $CaCO_3$ preparations has also been demonstrated in young healthy adult men (Sheikh & Fordtran 1990). Presumably, this occurs as a result of undissolved $CaCO_3$ entering the intestine. Thus, the impact of tablet formulation on Ca^{2+} absorption appears relevant to all age groups.

Results from both the in-vitro dissolution and whole-body retention studies indicate that extrinsically labelled $CaCO_3$ possesses an isotope-rich layer at the particle surface. This heterogeneous isotopic distribution could lead to an overestimation of calcium bioavailability when incomplete gastric dissolution occurs. Thus, when radioisotopic methods are used, intrinsically labelled materials will yield more precise estimates of Ca^{2+} absorption.

In summary, we conclude that passage of undissolved $CaCO_3$ particles from the stomach to the intestine will limit, although not prevent, the absorption of calcium. In addition, the use of intrinsically radiolabelled $CaCO_3$ preparations will assure more precise estimates of Ca^{2+} bioavailability. Differences in disintegration and dissolution of commercial $CaCO_3$ preparations, which are a function of tablet formation, and factors such as compressibility and coating are important considerations in selecting a $CaCO_3$ source for research, clinical, or personal needs.

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Anti-nociceptive activity of nitric oxide synthase inhibitors in the mouse: dissociation between the effect of L-NAME and L-NMMA

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Abstract—The anti-nociceptive effect of selective inhibitors of nitric oxide synthase has been assessed in a formalin-induced paw-licking model in mice. L-N^G-Nitro arginine methyl ester (L-NAME) but not L-N^G-monomethyl arginine (L-NMMA) exhibited anti-nociceptive activity in both the early and late phases of paw licking following intraperitoneal administration. The effect on the late phase response was more pronounced. L-NAME (0·1–100 μg) and L-N^G-nitro arginine base (L-NOARG; 10 μg) but not D-NAME (10 μg) were also anti-nociceptive following intracerebroventricular administration. L-NAME (10 μg) administered by this route did not influence locomotor activity. L-NMMA was inactive at doses up to 40 μg by this route. At higher doses (75–200 μg) L-NMMA produced a similar and non-dose related reduction in early/late phase paw-licking time. D-NMMA (100 μg) was inactive. The greater anti-nociceptive effect of L-NAME in this model accords with recently published biochemical data indicating that L-NAME is several orders of magnitude more potent than L-NMMA as an inhibitor of brain nitric oxide synthase. These data support the use of L-NAME as a selective tool to investigate the central pharmacological effects of nitric oxide.

Nitric oxide (NO) biosynthesis occurs in a number of non-neuronal, mammalian tissues including vascular endothelial cells, platelets, leucocytes, fibroblasts, Kuppfer cells and as yet unidentified cell types within the adrenal cortex and lung (Moncada et al 1989). Additionally, NO may serve as a neurotransmitter both in the peripheral (non-adrenergic, non-cholinergic, NANC) (Bult et al 1990; Gibson et al 1990; Tucker et al 1990) and central (Garthwaite et al 1988; Garthwaite 1991) nervous systems. Within the brain the NO synthase enzyme involved has been purified (Knowles et al 1989) and its regional distribution within different parts of the rat brain determined (Bredt et al 1990; Forstermann et al 1990).

Despite these not inconsiderable advances in our understanding of the biochemistry of the brain NO system, the functional significance of NO in the central nervous system remains to be established. For example, Garthwaite and colleagues have suggested that NO produced within cerebellar neurones under the influence of *N*-methyl-D-aspartate (NMDA) type glutamate receptors may play a part in the development of synaptic plasticity in this part of the immature rat brain (see Garthwaite 1991; Southam et al 1991). In addition, we have demonstrated that L-N^G-nitro arginine methyl ester (L-NAME), a selective inhibitor of NO synthase, exhibits a potent and long-lasting anti-nociceptive action in the mouse by what is apparently a naloxone-independent central mechanism (Moore et al 1991).

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Recent evidence has suggested that cerebellar NO synthase appears to be exquisitely sensitive to inhibition by L-NAME when compared with other inhibitors of this enzyme such as L-N^G-monomethyl arginine (L-NMMA) (East & Garthwaite 1990; Lambert et al 1991). For this reason we considered it worthwhile to compare the anti-nociceptive effect of L-NAME (and its base L-N^G-nitro arginine, L-NOARG) with that of L-NMMA in an attempt to determine whether the reported disparity in biochemical properties of the two inhibitors was correlated with differences in their biological activity in the brain. Some of these results have been communicated in preliminary form to the British Pharmacological Society (Babbedge et al 1991).

Materials and methods

The methods employed in this study have been fully discussed elsewhere (Moore et al 1991) and will thus be outlined only briefly. Male LACA mice (28–35 g) were used for all experiments. Animals were allowed free access to food and water until transported to the laboratory at least 1 h before the experiment. All experiments were conducted in the period between 1300 and 1700 h in normal room light and temperature (22 ± 2°C).

The anti-nociceptive effect of L-NAME, D-NAME, L-NOARG and L-NMMA was assessed using the formalin-induced paw licking procedure essentially as described by Hunskaar & Hole (1987). Animals were injected sub-plantar in one hindpaw with formalin (5%, 10 μL). The duration of paw-licking (an index of nociception) was measured 0–5 min and 15–30 min after formalin administration. L-NAME or L-NMMA (50 mg kg^{-1}) were administered intraperitoneally (i.p.) 15 min before formalin injection. In separate experiments, L-NAME (0·1–10 μg), D-NAME (10 μg), L-NOARG (10 μg), L-NMMA (10–200 μg) or D-NMMA (100 μg) was administered intracerebroventricularly (i.c.v.) as described by Oluyomi (1989) again 15 min before formalin injection. Control animals received an appropriate volume of 0·9% w/v NaCl (saline, 0·1 mL/10 g, i.p.; 5 μL, i.c.v.).

Locomotor activity was determined after i.c.v. administration of L-NAME (10 μg). Animals were injected with L-NAME or an