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**Original article**

# Mannitol enhances intracellular calcium diffusion in the rat ileum – a hypothesis

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**Abstract** — The addition of 92 or 136 mM mannitol to a modified saline solution that contained 1.25 mM $Ca^{2+}$ led to a mannitol concentration-dependent increase in the amount of calcium absorbed in 1 h from 8 cm long ileal loops prepared from fasted male Sprague-Dawley rats, with body weights of 190 ± 10 g. It is argued that this mannitol-enhanced movement of calcium out of the loop cannot have utilized the paracellular pathway, inasmuch as the luminal calcium concentration of the mannitol instillate decreased during the experiment, with a negative calcium gradient between luminal and body fluids. Instead it is proposed that uncomplexed mannitol and the uncharged calcium complex of mannitol entered the ileal cells. The uncomplexed intracellular mannitol would bind additional calcium that had crossed the brush border down its gradient. The increase in total intracellular calcium will raise the effective intracellular gradient and thereby amplify intracellular calcium diffusion. This in turn increases calcium absorption.

**intestine / mannitol / paracellular calcium movement / transcellular calcium diffusion**

**Résumé — Comment le mannitol augmente-t-il la diffusion transcellulaire du calcium ? Une proposition.** L'addition de 92 ou 136 mmol·$L^{-1}$ de mannitol à une solution saline modifiée contenant 1,25 mmol·$L^{-1}$ de $CaCl_2$ entraîne une augmentation de la quantité de calcium absorbée en 1 h au niveau d'une anse iléale ligaturée (8 cm) chez des rats mâles Sprague Dawley (poids 190 ± 10 g) à jeun. L'augmentation de l'absorption de calcium est dépendante de la concentration de mannitol. L'entrée du calcium induite par la présence de mannitol dans la lumière ne peut avoir utilisé la voie paracellulaire car la concentration luminale du calcium diminue au cours du test et le gradient devient négatif entre lumière et milieu intérieur. Nous proposons que le complexe mannitol-Ca non chargé et le mannitol non complexé pénètrent dans la cellule iléale où ce dernier peut également lier le

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calcium qui a traversé la bordure en brosse. L'élévation de la concentration cellulaire du calcium amplifie la diffusion cellulaire et assure l'augmentation de l'absorption.

**intestin / mannitol / absorption paracellulaire / diffusion transcellulaire du calcium**

## 1. INTRODUCTION

Intestinal calcium absorption is mediated by two processes: a transcellular, vitamin D-dependent active movement and a paracellular, passive movement [5–7, 30, 36]. The active transport process involves entry down the concentration gradient across the brush border of the duodenal cell, diffusion across the cell interior which is enhanced by the cytosolic, vitamin D-dependent calbindin $D_{9k}$, and extrusion of the ion from the cell via the CaATPase at the basolateral cell pole. In the absence of calbindin $D_{9k}$, self-diffusion of the calcium ion is so slow that the transcellular component is essentially nil and absorption is only mediated by the paracellular process, dominant in both jejunum and ileum. However, because the time spent by chyme in the ileum accounts for three-fourths of the 3 h that the chyme sojourns in the small intestine, the ileum is the principal site of calcium absorption [11, 25].

It has been known for many years that sugars like lactose, or polyols like sorbitol or mannitol, when added to the regimen in sufficient quantities (> 20%), significantly enhance calcium absorption [12, 29, 33, 35]. When, in intestinal loop experiments, lactose or polyols are added to an instillate that contains relatively high calcium concentrations, calcium absorption can be doubled [13, 14, 21, 23, 34]. Although not universally agreed to [34], one explanation for this enhancement of calcium absorption by the intestine is that the presence of sugars or polyols in the lumen causes endogenous water to move into the lumen. As a result, the intestine swells and the tight junctions are widened. In turn this permits more calcium to move down its chemical gradient via the paracellular route [3, 4].

If, however, the calcium concentration in the instillate equals that of the body fluids, calcium will not move down a chemical gradient. Yet, experiments utilizing ileal segments in Ussing chambers, with calcium concentrations on the mucosal and serosal poles equal to 1.25 $mmol \cdot L^{-1}$, have shown that the presence of lactose [16] or of sorbitol and xylose [24] in the medium enhances calcium transport. Inasmuch as any transepithelial potential and the chemical gradient are adjusted to zero in the Ussing preparation, the transcellular pathway has been invoked as the route of transfer. The mechanism of this transfer is however not known. In the experiments reported here, we confirm an increase in transcellular transfer and offer a mechanistic explanation of how this might occur.

## 2. MATERIALS AND METHODS

Six weeks old male Sprague-Dawley rats (body weight: $190 \pm 10$ g), that had been on a semi-synthetic diet (UAR, Villemoisson-sur-Orge, France) containing 0.83% Ca, 0.59% P, and 1500 IU vitamin $D \cdot kg^{-1}$, were fasted for 48 h before they were anesthetized by intraperitoneal injection of 40 mg Na pentobarbital$\cdot kg^{-1}$ body weight, laparotomized and intestinal loops were prepared as previously described [10]. Ileal loops were 8 cm long, prepared 10 cm proximally from the ileocecal junction. For duodenal loops, 8 cm long, the hepatobiliary canal was tied off. Jejunal loops, 8 cm long, were from the proximal jejunum, the cecum was used as a whole for loop studies; loops from

the proximal colon were 4 cm long and loops from the transverse colon were 4 cm long, with the proximal tie 5 cm caudal from the cecum. Length was controlled using a tape-measure. No more than two loops were prepared from one animal.

Intestinal loops were rinsed by injecting and withdrawing an unlabeled control or test solution. One milliliter of either control or test solution was then instilled. At the end of 1 h (15 min in the case of the duodenum) the animals were killed, the loops removed, centrifuged and the supernate and the tissue were analyzed. Less than 5% of the calcium content in the instillate was found to have precipitated even in the most alkaline samples, the calcium content of the supernate was considered equal to that of the loop contents.

The control solution contained 130 mM NaCl, 10 mM $NaHCO_3$ (pH.8.2), 5.2 mM KCl, 5 $g \cdot L^{-1}$ polyethylene glycol 4000 (PEG) which contained 1.01 $kBq \cdot mL^{-1}$ $^3$HPEG (New England Nuclear, France), 1.25 mM $CaCl_2$ which contained 0.8 $kBq \cdot mL^{-1}$ $^{45}CaCl_2$ (New England Nuclear, Belgique). The $CaCl_2$ concentration was chosen to be below that of ionized calcium in body fluids. The test (or mannitol solution) contained either 92 mM or 136 mM mannitol, with the $Na^+$ concentration reduced to either 102 or 80 $mmol \cdot L^{-1}$, respectively. Note that sodium bicarbonate and $CaCl_2$ were added just before each experiment.

Water content was evaluated by weighing. Sodium and potassium contents were analyzed by flame photometry (Instrument Laboratory, Milan, Italy), chloride by coulometric titration (Eppendorf), bicarbonate by alkali-acid titration (Radiometer, Copenhagen) and total calcium by way of the Arsenazo III complex of calcium (pH 7.0) at 660 nm. $^{45}$Ca and $^3$H contents were analyzed by means of liquid scintillation spectrometry, the contamination of the tritium channel by $^{45}$Ca was calculated and substracted (Tricarb 1600 CA, Packard Instruments).

Tissue content of $^{45}$Ca was determined after the tissue was ashed (600 °C); the ash was dissolved in 1 mL concentrated HCl, diluted to 5 mL with $H_2O$ and aliquots of 0.5 mL were analyzed.

$^3$HPEG 4000 recovery from the luminal fluid was used as a control. When recovery fell below 70%, the results were excluded. While this recovery value may seem low, it is the result of some $^3$HPEG having been adsorbed on the intestinal mucus and removed from the supernate by centrifugation, with $^3$HPEG recovery determined on the supernate [26].

Calbindin $D_{9k}$ (CaBP, $M_r \cong 9$ kD) content was determined from mucosal scrapings by the equilibrated chromatographic column procedure [8]. In brief, 100–400 mg mucosal scrapings were homogenized in 0.02 M ammonium acetate (pH: 7.3), centrifuged (100 000 *g*) for 60 min and the supernate was lyophilized. Eight milligrams of the lyophilizate was dissolved in 0.02 M ammonium acetate that contained 7 $\mu mol \cdot L^{-1}$ $CaCl_2$ and 1 $mBq \cdot L^{-1}$ $^{45}$Ca and applied to the chromatographic column (Sephadex G-50, fine) that had been equilibrated with the radioactive acetate buffer. All buffers contained 0.1 mM phenylmethylsulfonyl fluoride. Calbindin $D_{9k}$ content was determined from the amount of calcium bound to the protein and eluted in the second peak ($V_e/V_o \cong 1.4$) of radioactivity. The inner half of the peak area was considered equal to the total calcium bound by calbindin $D_{9k}$, with 2 $Ca_{bound}$ = 1 calbindin $D_{9k}$.

Comparison of means was by the Student t-test. Linear regressions were by least squares analysis.

Animal care and treatment followed the guidelines of the European Economic Community (J. Officiel des Communautés Européennes, L358, December 18, 1986), User No.: 0193.

## 3. RESULTS

Table I lists the initial and final values of volume, pH and ion contents of the luminal fluid of the ileum. As can be seen, one-third of the instilled control solution left the lumen in 60 min, but the amount of calcium lost was only about 10%. If calcium and fluid movement had been equal, the calcium content of the remaining luminal fluid should have been 0.84 µmol (1.25 × 0.67), whereas it was 1.09 ± 0.06 µmol (1.62 × 0.67). Instillation of the test solution caused a smaller loss of fluid from the loop, 0.19 mL rather than 0.33 mL, yet a significantly greater loss of calcium. The amount of calcium that should have been recovered from the luminal fluid 1 h after instillation of the test solution should have been 1.01 µmol (1.25 × 0.89), whereas it was 0.72 ± 0.04 µmol (0.81 × 0.89), significantly lower.

The calcium fluxes shown in Table I and referred to above are net fluxes. To measure the true absorptive transport, it is necessary to evaluate one-way movement out of the lumen. This was done with the aid of $^{45}Ca$ added to the instillate (see materials and methods). Table II lists the results of this approach. As can be seen, some 34% [100 (0.44–0.29)/0.44] of the calcium that had left the lumen still remained in the mucosal tissue 1 h after instillation of the control solution. Somewhat similar values apply to the tissue content after instillation of the test solution (34% and 25%, respectively). The presence of mannitol however caused a greater amount of calcium to be transported out of the lumen. Figure 1

**Table I.** Ion contents and net fluxes in ileal loops.

| | Concentration, mmol·L$^{-1}$ | | Flux, µmol |
|---|---|---|---|
| | Initial | Final | |
| Control solution ($n$ = 21) | | | |
| $Na^+$ | 148 | 143 ± 2 | 52 ± 5 |
| $HCO_3^-$ | 10 | 78 ± 3 | –42 ± 2 |
| $Cl^-$ | 146 | 72 ± 3 | 98 ± 3 |
| $K^+$ | 5.2 | 6.5 ±.0.1 | 0.9 ± 0.2 |
| $Ca^{2+}$ | 1.25 | 1.62 ± 0.05 | 0.17 ± 0.04 |
| pH | 8.2 | 8.17 | |
| Volume, mL | 1.00 | 0.67 ± 0.03 | 0.33 ± 0.03 |
| Test solution (136 mM mannitol; $n$ = 45) | | | |
| $Na^+$ | 80 | 54 ± 1 | 36 ± 2** |
| $HCO_3^-$ | 10 | 36 ± 1 | –19 ± 1*** |
| $Cl^-$ | 78 | 18 ± 1 | 63 ± 2*** |
| $K^+$ | 5.2 | 2.9 ± 0.1 | 2.8 ± 0.1*** |
| $Ca^{2+}$ | 1.25 | 0.89 ± 0.33 | 0.53 ± 0.03*** |
| pH | 8.20 | 7.85*** | |
| Volume, mL | 1.00 | 0.81 ± 0.03 | 0.19 ± 0.02*** |

One milliliter solution (for complete composition see text) was instilled in an ileal loop, 8 cm in length, and volume, pH, and ion composition were measured 1 h later. Flux was calculated by multiplying the final concentration with the final volume and subtracting that product from the instilled quantity.Values shown are means ± SEM; $n$ refers to the number of ileal loops. Starred values differ significantly from their respective initial values (** $P < 0.01$, *** $P < 0.001$). Calcium values refer to total calcium.

**Table II.** Ileal calcium transport with or without mannitol.

| | $Ca^{2+}$ lost from lumen in 60 min, μmol | $Ca^{2+}$ transported out of tissue in 60 min, μmol |
|---|---|---|
| Control solution (1.25 mM $Ca^{2+}$; *n* = 21) | 0.44 ± 0.03 | 0.29 ± 0.04 |
| Test solution (1.25 mM $Ca^{2+}$, 92 mM mannitol; *n* = 9) | 0.73 ± 0.03 | 0.48 ± 0.05 |
| Test solution (1.25 mM $Ca^{2+}$, 136 mM mannitol; *n* = 45) | 0.76 ± 0.03 | 0.57 ± 0.05** |

One milliliter solution (for complete composition see legend, Tab. I and text) was instilled in an ileal loop, 8 cm in length. The amount of $Ca^{2+}$ lost from the loop was obtained by measuring the $^{45}Ca$ content in the luminal fluid remaining after 1 h and dividing by the specific activity of the instilled solution. The tissue content of $^{45}Ca$ was determined by counting the ashed solution of the tissue (see text) and dividing the count by the specific activity of the instilled solution. This value was then subtracted from the $Ca^{2+}$ lost to obtain the $Ca^{2+}$ transported out of the tissue. Values shown are means ± SEM; *n* refers to the number of ileal loops. Starred values differ significantly from their respective initial values (**$P < 0.01$).

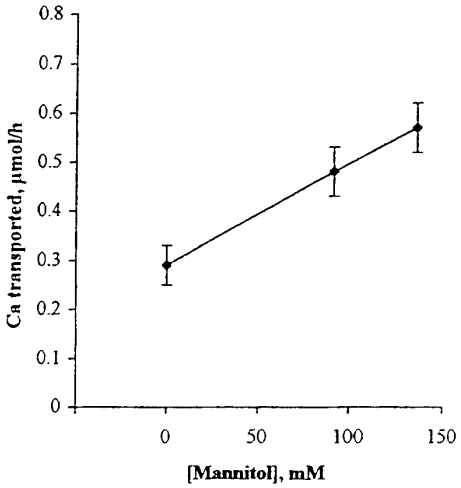


**Figure 1.** The relationship between luminal mannitol concentration and ileal calcium transport. One milliliter of a solution (for complete description see legend, Tab. I and text) that contained 0, 92, and 136 mM mannitol, 1.25 mmol·$L^{-1}$ calcium and $^{45}CaCl_2$ was instilled into ileal loops, 8 cm long, and the amount of calcium transported out of the loop into the body was calculated (Tab. II). The equation describing calcium transport as a function of mannitol concentration is:

$$Y = 0.29 + 0.002\ X$$

where Y = μmol calcium transported per hour and X = mmol·$L^{-1}$ mannitol in the luminal instillate. Values are shown as means ± SEM. For *n*, see Tab. II. The standard errors of the intercept and slope of the equation were less than 0.3%.

indicates that over the concentration range of mannitol studied, the increase in calcium transported out of the tissue was a function of the mannitol concentration, even though the calcium concentration was identical in all three instances.

Figure 2 shows that the amount of calcium transported out of the loops made from the small or large intestine was directly proportional to the tissue content of calbindin $D_{9k}$ when the instillate contained no mannitol.

## 4. DISCUSSION

Table II shows that in 21 experiments using the control solution, instillation of 1.25 μmol Ca led to a positive calcium transport of 0.29 ± 0.04 μmol, even though the ileum contains no calbindin $D_{9k}$ (Fig. 2). This can be explained by recalling that water movement out of the ileum is rapid. As a result the luminal fluid will become concentrated. This increased the calcium concentration from 1.25 to 1.6 mmol·$L^{-1}$ by the end of the hour (Tab. I). It seems reasonable to postulate that calcium moves down its concentration gradient along the paracellular route whenever the luminal calcium concentration exceeds 1.25 mmol·$L^{-1}$.

The increase in calcium movement due to the presence of mannitol in the instillate (Tabs. I and II and Fig. 1) cannot be explained as having resulted from a chemical gradient, inasmuch as the calcium concentration in the final luminal fluid was lower than in the instillate (Tab. I). Moreover, the amount of calcium transported out of the loop increased with the mannitol concentration, even though the calcium concentration of the instillate was unchanged (Tab. II).

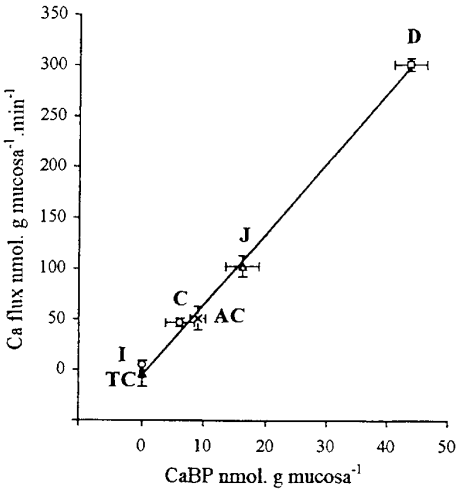


**Figure 2.** Correlation between calbindin $D_{9k}$ content (CaBP, nmol·g$^{-1}$ mucosa) and calcium absorption (nmol·min$^{-1}$.g$^{-1}$ mucosa) in six segments of the small and large intestine of rats. One milliliter of the control solution (1.25 mM Ca + $^{45}CaCl_2$, for complete composition see Tab. I and text) was instilled in 8 cm loops of duodenum (**D**), jejunum (**J**) and ileum (**I**), in 3 cm loops of the colon (**AC**, ascending colon; **TC**, transverse colon) and in the caecum (**C**) as a whole. $^{45}Ca$ loss from the loop was determined after 15 min in duodenum and jejunum and at the end of 60 min in all other loops, with absorption calculated on the basis of the initial specific activity, corrected for the measured tissue content (Tab. II). To express calcium transport on the basis of the mucosal weight, the ratio of mucosal weight/tissue weight that had been determined for analysis of CaBP (see materials and methods) was applied to the transport measurement. The equation describing transport as a function of CaBP concentration was derived from the mean values by a least squares procedure:

$$Y = -2.00\ (\pm\ 4.7) + 6.88\ (\pm\ 0.24)\ \text{CaBP}$$

where Y = nmol calcium transported min$^{-1}$·g$^{-1}$ mucosa, CaBP = nmol·g$^{-1}$ mucosa ($r$ = 0.98). The values in parentheses are the SEM.

How can one explain the mannitol-dependent increase in calcium absorption? Calbindin $D_{9k}$ is thought to act by augmenting the rate of intracellular diffusion of calcium [5, 7]. It does so by increasing the intracellular calcium concentration and the intracellular calcium gradient, thereby increasing the rate of calcium diffusion [7, 17], even though the rate of self-diffusion of the calcium-calbindin $D_{9k}$ molecule is only about 1/15 that of calcium[1].

Mannitol is largely absorbed by the extracellular route, but it does enter intestinal cells, the mannitol content plateaus in cells some 20–30 min after exposure to the polyol [18]. Moreover, polyols bind calcium, forming both charged and uncharged complexes [22, 31].

To transport an excess of 0.28 µmol Ca·h$^{-1}$ (0.57–0.29, Tab. II) would require a minimum transcellular flow of 0.65 µmol mannitol·h$^{-1}$, on the assumption that 1 molecule of mannitol binds 1 molecule of calcium and that the mannitol-calcium complex diffuses at 43% [$(40/220)^{1/2}$] of the rate of self-diffusion of the calcium ion. Inasmuch as the total flow of mannitol out of the loop is at least 13.6 µmol·h$^{-1}$ [19], the amount of mannitol that needs to enter the mucosal cells of the ileal loop is 0.65/13.6 or 4.7% of the absorbed mannitol, but less than 0.5% of the mannitol in the lumen.

The preceding analysis implies that the increased calcium transport due to mannitol

[1] Calbindin $D_{9k}$ binds two molecules of calcium. One of the calcium molecules is, however, bound far more tightly than the other and may therefore not participate importantly in calcium transport. The ratio of the self-diffusion of calcium to that of calbindin is $(8900/40)^{1/2}$ = 14.9.

in the ileal lumen cannot be attributed to calcium moving down an electrical potential along the paracellular route. The reversion of polarity from mucosa$^{-}$/serosa$^{+}$ to mucosa$^{+}$/serosa$^{-}$ when test solutions contain carbohydrates or polyols has been attributed [16, 24] to the decrease of $Na^+$ concentration in the lumen (Tab. I). No increase in calcium transport occurred however in the jejunum, even though the test solution contained the same low 102 or 80 mM $Na^+$ concentration [15]. Moreover, experiments utilizing Ussing chambers, where the initial transepithelial potential is adjusted to zero, have shown comparable results for the ileum [16, 24].

When the instillate contains mannitol, the final luminal pH is lower than when the control solution is instilled. The reason for this is the activation of the $Na^+/H^+$ exchanger by a lower $Na^+$ concentration, as demonstrated with the aid of inhibitor studies [20]. It is unlikely that the drop in pH caused transcellular calcium transport, inasmuch as addition of mannitol or other polyols [15, 28] to jejunal loops which are more acidic (pH < 6.6) [11] had no effect on transcellular calcium transport.

Mannitol, an open-chain hexalcohol, functions as a tridentate ligand for cations. The oxygens of the hydroxy groups of carbons 2–4 are complexation sites, with a complexation formation constant of 0.7 $L \cdot mol^{-1}$ at 40 °C in $^2H_2O$; 10% of the mannitol is in a complex form [22]. The complexes are sufficiently stable to permit separation on a calcium column [2] and to migrate to the cathode [1].

In analogy with what has been described for EDTA [32], we propose that of the three forms of the mannitol-Ca complex, $(mannitol\text{-}Ca)^{++}$, $(mannitol\text{-}Ca)^{+}$ $(mannitol\text{-}Ca)^{0}$, the amount of the uncharged form will increase at the rate $1/[H]^2$ when the pH goes from 6.6 (jejunum) to 7.6 (ileum). Moreover, the uncharged form, even though least frequent, will dominate the transmembrane flux because the diffusion rate of a neutral molecule is much faster [27]. The existence of a non-polar form of calcium and lactose that moves into the intestinal cell has been argued by Charley and Saltman [9].

Mannitol may therefore induce transcellular calcium flux by two mechanisms: (a) by entering the cell as an uncomplexed molecule [18] and raising the intracellular calcium concentration in a calbindin $D_{9k}$-like fashion to permit calcium to diffuse through the cell at a rate faster than self-diffusion; (b) at the alkaline pH of the ileum, the uncharged calcium-mannitol complex crosses the brush border and as a result of the increase in intracellular calcium and a corresponding increase in the intracellular gradient of calcium, calcium is transported at a greater rate than self-diffusion.

The added amount of calcium transported transcellularly under our experimental conditions was quite small and therefore had minimal nutritional significance. To significantly increase the amount of absorbed calcium with the aid of mannitol or other polyols requires a considerable increase in their intestinal concentrations. This leads to a marked increase in paracellular calcium movement [3, 4] far in excess of the effect on transcellular movement. If, however, other molecules can be identified that bind more calcium than the polyols and that enter the intestinal cell, transcellular calcium transport can be increased at relatively low cellular calcium concentrations. Such a finding may have therapeutic significance.

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