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MOLECULAR MECHANISMS OF THE EPITHELIAL TRANSPORT OF TOXIC METAL IONS,
PARTICULARLY MERCURY, CADMIUM, LEAD, ARSENIC, ZINC AND COPPER

Progress Report for Period

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SUMMARY STATEMENT

Investigations were continued to elucidate the mode of transepithelial transport of potentially toxic metal ions across the gastrointestinal tract, as well as their interactions with biological processes and other metal ions. All experimental details either published, submitted for publication or in press during this report period are included in the Appendix.

Primary attention for this report has been given to lead, cadmium and zinc. Details of these experiments are attached in the enclosed Progress Report and highlights are presented here, as follows:

1. Lead is rapidly and extensively taken up by the intestinal mucosa and slowly transferred to the body, whereas less Ca is retained by the tissue and the transfer of calcium is many times as effective as that for lead.
2. Lead and calcium appear to respond in a similar manner to a low calcium intake and vitamin D treatment. Certain differences were, however, observed in the absorptive process of these two elements.
3. Increasing luminal lead concentration from 0.01 to 1.00 mM Pb significantly diminished the percentage of radiolead absorbed, but did not affect the absorption of calcium.
4. Vitamin D enhanced the transfer of plasma ^{47}Ca with the intestinal lumen, but did not affect the transfer of plasma ^{203}Pb .
5. Intravenous administration of $1,25-(\text{OH})_2\text{-D}_3$ to rachitic chicks enhanced both calcium and lead absorption but the time course differed indicating the possible involvement of more than one transport system.
6. It was concluded that lead is transported across the intestinal epithelial wall by the process of passive diffusion and that this process is influenced by vitamin D in a manner similar to that for the diffusional component of calcium transport.

7. Studies employing organ-cultured embryonic chick duodena showed that Cd^{2+} , in concentrations as low as 10^{-7}M , inhibits calcium-binding protein synthesis. Cadmium-binding activity (presumably metallothionein) was stimulated at Cd^{+2} concentrations of only 10^{-8}M .
8. Zn^{2+} at 10^{-4}M stimulated metallothionein synthesis at 4 hrs after the start of culture while Cd^{+2} ($5 \times 10^{-5}\text{M}$) was effective in this regard after 9 hrs.
9. The biosynthesis of metallothionein, whether Cd^{2+} - or Zn^{2+} -induced, appeared to be controlled at the translational level (cycloheximide, but neither actinomycin D nor α -amanitin inhibited).
10. Cd^{2+} or Zn^{2+} , while stimulating metallothionein production, also enhanced ^{109}Cd influx at the mucosal surface, a response which was diminished by $1,25-(\text{OH})_2\text{-D}_3$.

Effect of Vitamin D and Other Variables
on the Intestinal Absorption of Lead (^{203}Pb)

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Abstract

The purpose of the present studies was to elucidate the mechanism of lead transport, and especially the particular similarities or dissimilarities between lead and calcium in this process. The absorption of these metals was determined in 3-week old White Leghorn cockerels, raised on a commercial diet or on a specified diet, using in vivo ligated loop procedure. The dose administered into the loop contained 0.5 μCi ^{203}Pb (and/or 0.1 μCi ^{47}Ca), and 0.01 mM lead acetate (and/or 1 mM CaCl_2) in 0.5 ml 0.15 M NaCl, pH 6.5. It was shown that lead is rapidly taken up by the mucosal tissue, and slowly transferred into the body, whereas less calcium is retained by the tissue and the transfer of calcium is many times as effective as that for lead. They appear to respond in a similar manner to a low calcium intake and vitamin D treatment. Certain differences were, however, observed in the absorption process.

Increasing luminal stable lead concentration from 0.01 to 1.00 mM Pb, significantly reduced the percentage of radiolead absorbed, but did not affect the absorption of calcium. Also, vitamin D enhanced the transfer of plasma ^{47}Ca into the lumen but did not affect the transfer of plasma ^{203}Pb . Intravenous administration of $1,25(\text{OH})_2\text{CC}$ to rachitic chicks enhanced calcium and lead absorption, but the maximal absorption of these metals occurred at slightly different times after administering this metabolite, and the effect on calcium outlasted that on lead, indicating that two different transport systems may be involved. It was concluded that lead is transported across the epithelial wall by a passive diffusion and this process is affected by vitamin D in a similar manner as this vitamin affects the diffusional component of calcium transport.

Introduction

Lead has been recognized for years as being a highly toxic substance and as a significant pollutant of the environment. The degree of toxicity is dependent upon the concentration of lead in "sensitive" tissues and organs and a number of dietary and physiological factors are known to affect lead toxicity (9). Many of these factors undoubtedly act by determining the amount of ingested lead that is absorbed by the gastrointestinal tract and the rate of loss of already accumulated lead from the body. Significant factors are the calcium and phosphorus content of the diet, as was shown several years ago by Sobel and colleagues (27). Since that time, several studies clearly showed that calcium deficiency increased the retention of dietary lead and its toxicity, and conversely, that a high calcium intake decreased the retention and its toxicity (1, 16, 18, 20, 21, 25).

Sobel et al. (27) also showed that vitamin D affects lead retention and, in a recent study, Smith et al. (26) directly demonstrated that vitamin D markedly enhances lead absorption in rats, with the major response to the sterol occurring in the lower part of the small intestine of that species. This is somewhat surprising since vitamin D enhances the absorption of calcium to a greater degree in the duodenum, followed by the jejunum and lastly the ileum. Another interesting difference between calcium and lead is the mechanism of their translocation across the epithelial membrane. Whereas an active transport system exists for calcium (22, 30), the main mode of transport of lead appears to be by diffusion (3, 11). Since vitamin D is known to increase both the active transport and the diffusional permeability of calcium by the intestine (13, 23, 31), it might be speculated that vitamin D only influences the diffusion of lead across the intestine. It is thus

apparent that the role of vitamin D on lead metabolism requires clarification, and the exact manner by which calcium and lead interact in the intestine also requires definition.

The present studies were designed to investigate aspects of the problem of lead absorption, with emphasis on the effect of vitamin D, dietary calcium and phosphorus, and the concentration of lead itself on the degree of lead absorption.

Methods

Chicks used for absorption studies were 3-week old White Leghorn cockerels (Babcock Industries, Inc., Ithaca, NY) raised after hatching either on a commercial diet (Chick Starter Diet, Agway Inc., Ithaca, NY) or on experimental diets to be specified subsequently. The absorption of ^{203}Pb was determined by the ligated loop procedure, described in detail by Morrissey and Wasserman (19). The following indices of absorption were obtained: 'duodenal absorption' which refers to the amount of ^{203}Pb (% dose) which left the lumen and is calculated as $100 - (\% ^{203}\text{Pb in the loop} - \% ^{203}\text{Pb in the intestinal wall})$; 'mucosal retention' which is the amount of ^{203}Pb (% dose) in the intestinal wall; 'tibia uptake' which is the amount of ^{203}Pb (% dose) in the tibia; 'transfer to body' which refers to the amount of ^{203}Pb (% dose) in the whole body without the intestinal loop. The latter was determined by using a whole body counter or by calculation from the equation $100 - \% ^{203}\text{Pb retained in the duodenal loop}$. The effect of the following factors on ^{203}Pb absorption were assessed: Length of absorption period, luminal concentration of stable lead, pH of the dosing solution, site of absorption, body burden of lead, and age.

Expt. 1. Absorption of ^{203}Pb as a function of time of absorption. Prefasted chicks raised on the commercial diet were dosed with one ml of dosing solution containing 0.01 mM lead acetate, 0.001 M sodium acetate, 0.15 M NaCl and 0.5 μCi ^{203}Pb (as acetate), pH 6.5. At intervals of 10, 20, 30, 60 and 120 minutes after ^{203}Pb administration, six chicks at each time period were killed and the degree of absorption of ^{203}Pb was determined.

Expt. 2. Effect of varying concentrations of stable lead in the dosing solution on ^{203}Pb absorption. Prefasted chicks raised on the commercial diet were divided into 6 groups of 6 chicks each. Each group received 1 ml of one solution containing 0.5 μCi ^{203}Pb (as acetate) in 0.15 M NaCl and 0.1 M sodium acetate, pH 6.5, and one of the following concentrations of stable lead: 0, 0.01, 0.03, 0.10, 0.30, or 1.00 mM lead acetate. To determine whether sodium acetate affects the absorption of ^{203}Pb , another group was injected with a dose containing 0.5 μCi ^{203}Pb (as acetate), 0.01 mM lead acetate in 0.15 M NaCl and 0.001 M sodium acetate, pH 6.5. Sixty minutes after dosing, the degree of absorption of ^{203}Pb was assessed.

Expt. 3. Effect of pH on the absorption of radiolead. The degree of ^{203}Pb absorption at different pH values was determined as outlined above in three-week old chicks raised on the commercial diet. The 1 ml dose contained 0.01 mM lead acetate, 0.001 M sodium acetate, 0.15 M NaCl and 0.5 μCi ^{203}Pb (as acetate), adjusted with NaOH or HCl to one of the following pH values: 4, 6, or 7. The absorption period was 60 min.

Expt. 4. The effect of various concentrations of dietary lead on the absorption of ^{203}Pb . One-day old cockerels were divided into 5 groups of 6 birds each. The groups were fed the commercial diet containing 0, 3, 10, 100 or 1000 ppm lead as lead acetate. After receiving the appropriate diets

for 3 weeks, the chicks were fasted for 24 hrs, and the absorption of ^{203}Pb from the in situ duodenal loop was determined as in the first experiment, except that the volume injected into the loop was 0.5 ml instead 1.0 ml. The smaller volume prevented the occasional fluid retention observed in some of the chicks after 60 min. when the 1.0 ml dose was used.

Expt. 5. Comparative absorption of ^{203}Pb from different segments of the small intestine in rachitic and vitamin D-repleted chicks. One-day old cockerels were fed a rachitogenic diet (33) based on the formula of Scott et al. (24) with vitamin D omitted. The composition of this diet is shown in Table 1. At three weeks of age, half of the chicks received 500 IU cholecalciferol in 0.2 ml of propylene glycol by intramuscular injection 72 hrs prior to the absorption study. The absorption of ^{203}Pb from different segments of the small intestine was determined in the manner described in Expt. 4. The jejunum was identified as the segment proximal to the yolk sac and distal to the duodenum, and the ileum was localized distal to the yolk sac and proximal to the ileocaecal junction.

Expt. 6. The effect of age on lead absorption. Chicks were fed a commercial diet and the absorption of ^{203}Pb was determined by the ligated duodenal loop procedure at 1, 3 and 5 weeks of age. The dose was as in the previous experiment.

Expt. 7. The effect of low calcium and low phosphorus diets on ^{203}Pb absorption. Two-week old cockerels raised on the commercial chick starter diet were assigned to one of the following dietary groups of 6 birds each:

- a) control diet (containing 1.22% Ca and 1.05% P), b) low calcium diet (0.12% Ca and 1.05% P), c) low phosphorus diet (1.22% Ca and 0.33% P), d) low calcium and low phosphorus diet (0.12% Ca and 0.33% P). The

experimental diets were prepared by adding calcium and phosphorus to the semisynthetic basal diet as calcium carbonate and mono-basic potassium phosphate. The composition of the basal diet was as in Table 1 with calcium and phosphorus omitted. Vitamin D₃ was also added to yield a concentration of 1200 IU cholecalciferol per kg. After maintaining the chicks on the appropriate diets for one week, the absorption of ²⁰³Pb was determined by the procedure previously outlined.

Expt. 8. The effect of dietary and parenteral cholecalciferol on lead absorption. Thirty one-day old chicks were divided into 5 groups of 6 chicks each. The groups were fed the rachitogenic diet (Table 1) supplemented with 0, 50, 150, 300 or 600 IU cholecalciferol per kg of the diet for 3 weeks at which time the absorption of ²⁰³Pb and ⁴⁷Ca was determined.

In another experiment, the 3-week old chicks raised on the rachitogenic diet received an intramuscular injection of a single dose containing 0, 10, 100, 1000 or 5000 IU cholecalciferol in 0.2 ml of propylene glycol, and the duodenal absorption of ²⁰³Pb and ⁴⁷Ca was determined 24 hr. after the administration of the vitamin.

In a third experiment, the absorption of ²⁰³Pb and ⁴⁷Ca at different time intervals after the administration of a single dose of cholecalciferol (100 IU in 0.2 ml of propylene glycol, intramuscularly) was determined. In these three experiments the dose injected into the ligated loop contained 0.5 µCi ²⁰³Pb and 0.1 µCi ⁴⁷Ca in 0.5 ml of 0.15 M NaCl, 0.01 mM lead acetate and 1 mM CaCl₂ (pH 6.5).

Expt. 9. Effect of 1,25(OH)₂D₃ on the duodenal absorption of ²⁰³Pb. The effect of 1,25(OH)₂CC on the duodenal absorption of lead was determined in the following manner: At various intervals before the absorption study,

6 birds were injected (i.v.) with 1 μ g of 1,25(OH)₂CC (gift of Dr. Uskoković, Hoffmann-LaRoche, Nutley, NJ) in 0.1 ml of propylene glycol. The absorption of ²⁰³Pb and ⁴⁷Ca was measured, using a dose of 0.5 μ Ci ²⁰³Pb and 0.1 μ Ci ⁴⁷Ca in 0.5 ml 0.15 M NaCl containing 0.01 mM lead acetate and 1 mM CaCl₂, pH 6.5.

Expt. 10. Transfer of plasma ²⁰³Pb and ⁴⁷Ca to the duodenal lumen. The transfer of plasma ²⁰³Pb and ⁴⁷Ca into a ligated duodenal loop (backflux) was determined, as follows: Prefasted rachitic or vitamin D-repleted chicks of 3 weeks of age were injected intravenously with a dose containing 1.0 μ Ci ²⁰³Pb and 0.5 μ Ci ⁴⁷Ca in 0.1 ml of 0.15 M NaCl (pH 7.0), containing 0.01 mM lead acetate and 1 mM CaCl₂ and the appearance of the isotopes in the luminal fluid was determined. Before the intravenous administration of the isotopes, the duodenum was ligated and 0.5 ml of nonlabeled solution containing 0.01 mM lead acetate, 1 mM CaCl₂ and 0.15 M NaCl (pH 6.5) was injected into the duodenal lumen. Two and one-half minutes after intravenous dosing with the radiotracers, blood samples were taken by cardiac puncture for analysis of ⁴⁷Ca and stable calcium, and the chicks were killed with an overdose of pentobarbital. The duodenal segments were rinsed with 2 x 20 ml of ice-cold saline into the counting vials for determining the ²⁰³Pb and ⁴⁷Ca activities in the luminal fluid. The rinsed segment and one tibia from each animal were also counted for ²⁰³Pb and ⁴⁷Ca.

Chemical assays. Serum was obtained from blood samples collected by cardiac puncture and assayed for calcium concentration by atomic absorption spectrophotometry (Perkin-Elmer Atomic Absorption Spectrophotometer 360, Perkin-Elmer, Co., Norwalk, Conn.) and for inorganic phosphorus, using an automated procedure (Autoanalyzer II-04, Technicon Instruments Corp., Tarrytown, NY). For measurement of calcium-binding protein (CaBP), the

mucosal tissue was scraped from the segment and homogenized (1:4 w/v) in Tris buffer (0.0137 M tris (hydroxy-methyl) aminomethane, 0.12 M NaCl, 4.74 mM KCl adjusted to pH 7.4 with HCl). The homogenate was centrifuged at 38,000 x g for 20 min. in a refrigerated centrifuge. The CaBP content of the supernatant fluid was determined by the radial immunodiffusion method (6). The supernatant protein was measured according to the method of Lowry (15).

Activities of ^{203}Pb and ^{47}Ca in the samples were determined, using a well-type gamma counter (Beckman 300, Beckman Instruments Inc., Irvine, CA), and the counts were expressed as % of the standard activity. The radioactivity in the whole body without the loop was determined, using a whole body counter (Tobor, Nuclear Chicago, Des Plaines, IL).

Carrier-free [^{203}Pb] acetate in 0.1 M acetic acid was obtained from New England Nuclear (Boston, MA), and [^{47}Ca] chloride with the specific activity ranging from 1574 to 2557 mCi/g in 1 N HCl was from Union Carbide Corp. (Oak Ridge, TN).

The significance of all treatment differences was determined by Duncan's Multiple Range Test (28) or by Student's t-test.

Results

The time course of ^{203}Pb absorption in 3-week old chicks raised on commercial diet is shown in Fig. 1. Lead was rapidly taken up by the intestinal tissue, with more than 50% of the dose bound to the tissue within 10 min. after administering the dose. This percentage uptake was then maintained unchanged until a slight but significant decrease occurred at 2 hrs after administration of the dose. This decrease may have been due to possible damage to the tissue; as reflected by the retention of fluid within

some of the loops at the 1 or 2 hr absorption periods. The amount of ^{203}Pb transferred to body increased slowly throughout the experimental period, with 14% of the dose in the whole body at 2 hrs.

In the next experiment, the effect of varying concentrations of stable lead on duodenal lead absorption was determined. The percentage of the dose absorbed was similar in the groups of birds receiving less than 0.10 mM lead in the dosing solution (Table 2). In chicks receiving the dose containing 0.10 mM lead or more, the percentage of ^{203}Pb absorbed decreased significantly. However, the total amount of lead absorbed increased with increasing stable lead concentrations; a 3-fold increase in stable lead was accompanied by a 2-fold increase in the amount of lead absorbed. Similarly the percentage of Pb transferred into the body decreased with increasing stable lead concentration in the dosing solution, and the total amount of lead transferred to body increased up to 0.30 mM of lead acetate in the dosing solution. However, unlike the mucosal retention, the total transfer of lead plateaued in chicks receiving 0.30 mM lead or more, indicating saturation of this process.

Acetate concentration, as such, did not affect the absorption of ^{203}Pb since the values for groups of animals receiving 0.01 mM lead acetate with either 0.001 M or 0.1 M Na acetate were not different (Table 2).

In contrast to lead, calcium absorption under the present conditions did not appear to be affected by the concentration of lead in the dosing solution when administered to rachitic or cholecalciferol-repleted chicks (Table 3).

Because of the low solubility of lead salts under physiological conditions, an experiment was done to determine the effect of pH on lead absorption. The results indicate that the pH does not significantly affect the absorption or mucosal retention of ^{203}Pb in the chick duodenum (Table 4).

Feeding high concentrations of lead in the diet for 3 weeks did not appear to affect the duodenal absorption of this metal (Table 5). The highest concentration used in this experiment, 1000 ppm Pb, significantly reduced the growth of the birds and caused a reduction in the mucosal retention of ^{203}Pb , accompanied by an increase in the amount of ^{203}Pb in the bone.

Table 6 summarizes the absorption of ^{203}Pb from different segments of the small intestine. It appears that the absorption of lead (expressed as % ^{203}Pb of the dose) was similar in all segments studied in vitamin D-repleted animals but, in rachitic chicks, less lead was absorbed from the duodenum than from jejunum or ileum. When the results are presented on a dry weight basis of the tissue, it becomes more evident that the distal parts of the small intestine retained more lead than the duodenum, and that the enhancing effect of vitamin D on lead absorption is most prominent in the duodenal segment (Table 7).

It has been shown that the absorption of lead, similar to calcium and other divalent cations, is greater in the younger than in the older individual (7, 29). The duodenal absorption of ^{203}Pb was determined in normal chicks of varying ages, and the results clearly showed that lead absorption decreases with increasing age (Table 8). The reduction in ^{203}Pb absorption with age was not related to the amount of calcium-binding protein in the mucosa, as the values for mucosal CaBP were similar in the age groups studied.

To determine the effect of dietary calcium and phosphorus on lead absorption, the chicks were raised on the commercial diet for two weeks after hatching and then assigned to one of the following groups: a) control, b) low Ca, c) low P, and d) low Ca-low P. After one week on the experimental diets, the absorption of ^{203}Pb was determined. The results indicated that

the duodenal absorption and the mucosal retention of ^{203}Pb were not significantly affected by these treatments (Table 9). However, diets low in calcium significantly enhanced the uptake of ^{203}Pb by the tibia. The low phosphorus and low calcium-low phosphorus diets did not significantly increase the uptake of lead by the bone, although their mean values were greater than that in the control.

The results presented above indicate that the duodenal absorption of lead is affected by the vitamin D status of the animal (Table 6 and 7). Additional experiments were done to obtain further information on the response to cholecalciferol. In the first of these, the effect of various amounts of cholecalciferol in the diet on the duodenal absorption of lead and calcium was determined. It appeared that the addition of cholecalciferol to the diet did not affect the duodenal absorption or mucosal retention of ^{203}Pb , but did significantly enhance the absorption of ^{47}Ca and decreased the amount of ^{47}Ca bound by the intestinal tissue (Table 10). There was, however, an increased tibia uptake of both isotopes in the groups receiving cholecalciferol as compared to the rachitic control group. This increase was not directly related to the cholecalciferol content of the diet, but was accompanied by a similar increase in the mucosal calcium-binding protein.

Table 11 shows that the duodenal absorption of radiolead was enhanced in a dose-dependent fashion by the administration of varying amounts of cholecalciferol given as a single intramuscular injection to rachitic chicks and a similar pattern was observed for the absorption of ^{47}Ca . The percentage of ^{47}Ca transferred to body was, however, about 4-5 times that of lead-203, whereas more ^{203}Pb was bound to the intestinal tissue than ^{47}Ca .

The results of the time-response experiment are shown in Fig. 2. There

was a significant increase in the amount of ^{203}Pb transferred to body 15 hrs after administration of the vitamin, whereas ^{47}Ca transfer was increased already at 8 hrs. There was a similar decrease in the amount of both radio-tracers bound to the intestinal wall, indicating that the effect of vitamin D is one of facilitating the transfer of both metals from mucosa to body. Vitamin D elicited a greater effect on the process of calcium transport than that of lead and less calcium than lead remained in the tissue, as already observed in the above experiments.

Presumably the hormonal form of vitamin D in calcium absorption is $1,25(\text{OH})_2$ -cholecalciferol. The following experiment was done with the purpose of comparing the effect of this metabolite on lead and calcium absorption. Fig. 3 summarizes the results of this experiment. The duodenal absorption of both tracers was significantly increased by this metabolite. The maximal

Pb absorption occurred by 8 hrs after the administration of $1,25(\text{OH})_2\text{CC}$, whereas the peak for ^{47}Ca absorption was seen at 16 hrs. Moreover, 32 hrs after the intravenous injection of this metabolite, absorption of radiocalcium was still significantly higher than in the control group whereas, at that time, the absorption of radiolead was similar to the controls. Intravenous injection of $1,25(\text{OH})_2\text{CC}$ did not affect the amount of ^{203}Pb bound to the intestinal tissue, but did significantly reduce the amount of ^{47}Ca retained by the tissue (Fig. 3).

It has been shown that vitamin D enhances the transfer of plasma calcium to the intestinal lumen in chicks and rats (31). An experiment was done to see if a similar increase occurs in the transfer of lead from plasma to lumen. A significantly greater amount of calcium-47 penetrated across the duodenal wall in vitamin D-repleted chicks as compared to the vitamin D-deficient chicks (Table 12). However, vitamin D did not influence the amount of ^{203}Pb transferred to the luminal fluid.

Discussion

The present studies indicated that the absorption of lead from the intestine occurs in two distinct phases. First there is a rapid uptake of lead by the intestinal wall, with more than 50% of the administered dose bound by the mucosal tissue within 10 minutes after dosage. The nature of this interaction is not known, but it may be due to binding of lead by some components of the external surface of the mucosal membrane. A predominately covalent bonding of lead by phosphate has been suggested by Blair and coworkers (3). This step appears to be dependent on the concentration of lead in the lumen, and an increase in the luminal lead was accompanied by a decrease in the percentage of lead accumulated by tissue (Table 2). However, the total amount of lead bound increased with larger doses of stable lead. These results are in accordance with those reported by Blair and coworkers (3), using everted segments of rat small intestine, and with the results of Conrad and Barton (5), using an in vivo ligated loop procedure with rats.

The next step, transfer of mucosal lead to body, progresses more slowly than mucosal uptake, and a noticeable saturation of this process was observed with larger amounts of luminal lead. Interestingly, this step in lead absorption is most affected by various experimental treatments, such as feeding diets low in calcium and phosphorus, the administration of vitamin D to rachitic chicks, and the response to an intravenous injection of $1,25(\text{OH})_2$ -cholecalciferol. In contrast to the results presented here, Blair and coworkers (3) demonstrated that the passage of lead into the serosal side of the everted gut sac preparation does not display saturation kinetics, but follows the passive movement of water. The stable lead concentrations in their study ranged from 10^{-7} to 10^{-5} M, whereas in the present study

using in vivo ligated loop procedure luminal lead concentrations varied from 10^{-5} to 10^{-3} M, which might explain the difference in results.

Chronic exposure to dietary lead could affect the intestinal absorption of lead by increasing the luminal and/or mucosal-bound lead or via some feedback mechanism. The present study showed clearly that feeding various amounts of lead in the diet for three weeks prior to the absorption experiment did not appear to affect the intestinal absorption or mucosal retention of radiolead. This is in agreement with the results reported by Conrad and Barton (5) and clearly indicates that there is no similar feedback mechanism for lead absorption as it has been reported for the absorption of calcium (32). With respect to the observed enhancement of ^{203}Pb transfer in the group of animals receiving 1000 ppm lead in the diet, it is interesting to note that doses of lead acetate greater than 200 μg per day decreased the transfer of calcium across the duodenal wall in rats (10). Interference of calcium assimilation by high lead intake may have caused, in a manner similar to a low calcium intake, the increased transfer of lead into the body.

Conflicting results have been reported on the absorption of lead from different parts of the small intestine. Studies using the everted gut sac technique imply that there is no significant difference in the amount of lead transported across different regions of the intestine (3, 11, 26). However, under in vivo conditions, maximal absorption has been reported to occur in the jejunum (8) or in duodenum (5). The present study, using in vivo ligated loop procedure, indicates that the absorption of lead, expressed as % ^{203}Pb of the dose, is not much affected by the segment in the vitamin D-repleted chicks (Table 6), but tends to be greater in the more distal segments of rachitic chicks. This presentation of the data may, however, not account

for the variability in the size of the loops from various segments. When the results are expressed on a tissue dry weight basis, the greater degree of lead absorption in distal segments of the small intestine becomes more evident, with the differences due mainly to the amount of lead bound by the tissue (Table 7). These results are in agreement with those reported by Cikrt (4) who also expressed his data per mg of tissue dry weight. Contrary to the findings of Smith et al. (26), the effect of vitamin D on lead absorption in chicks appears most prominent in the duodenum. The latter group used the everted gut sac technique in rats, whereas the present study was done using in vivo ligated loop procedure in the chick.

With increasing awareness of the consequences of environmental exposure, especially in children from urban slum areas, there has been a renewed interest in the interaction of dietary calcium with lead. The site of this interaction has been proposed to be the gastrointestinal tract, since these dietary treatments do not affect the retention of lead administered intraperitoneally (14, 20). The present results indicate that a low calcium intake affects lead absorption by facilitating the transfer of mucosal lead into the body. These results and those by Lederer and Bing (14) and Quarterman and Morrison (20) appear to be in conflict with the conclusions of Barton and coworkers (2), that low dietary calcium does not affect the absorption of lead in rats, but decreases the excretion of lead.

The enhanced transfer of the mucosal lead by low dietary calcium and phosphate in the present experiments may be associated with the mucosal calcium-binding protein. Low calcium and phosphorus intakes are known to increase the mucosal CaBP and the intestinal absorption of calcium (19, 32). Absorption of lead from the gastrointestinal tract appears to be governed

also by other physiological mechanisms regulating calcium metabolism. Vitamin D enhances lead absorption, if given in the diet (Table 10) or as a single intramuscular dose (Table 11, Fig. 2), in a similar manner as it increases calcium absorption. The differences observed appeared to be caused by different kinetics of transport which might reflect a difference in response to vitamin D. Stimulation of lead absorption by vitamin D has been reported before (26) and the functional form seems to be $1,25(\text{OH})_2\text{CC}$ (17).

Although absorption of lead responds to various dietary treatments in a manner similar to the absorption of calcium, these two metals may have different mechanisms of transport. Earlier peaking of ^{203}Pb absorption after an intravenous dose of $1,25(\text{OH})_2\text{CC}$ was observed in the present experiments, and the enhancing effect on lead absorption did not last as long as on calcium absorption, indicating that two possible transport systems may be involved. Vitamin D is known to increase both the active and passive (diffusional) component of calcium transport across the intestinal epithelium (23, 31) and it might be speculated that only the vitamin D-dependent diffusional path is available for lead. Secondly, plasma transfer of radiocalcium to intestinal lumen is increased by vitamin D (31), whereas no effect on plasma transfer of radiolead is seen in the present experiment (Table 12). This may be explained simply by the difference in the size between Ca and Pb ions, the ionic radii are .99 and 1.20 Å, respectively (12). The vitamin D may act by increasing the diameter of the pores in the basal membrane, or the size of the paracellular path but not enough to accommodate the larger lead ion.

The present study clearly indicates that the absorption of lead is a 2-step process, a rapid uptake by the mucosal tissue is followed by a slow, and probably saturable transfer of lead into the body. The slower rate of

transfer of lead than that of calcium may be due to the different ionic radii of the two cations, a difference in binding to a cellular component involved in the exit mechanism, difference in degree of binding to a cellular component that delays the exit of lead, or inhibitory effects. If difference in ionic radii were the explanation, the luminal concentration of lead would not affect the absorption of calcium, but the percentage of radiolead would decrease with increasing stable lead concentration, as was shown (Table 2 and 3). Furthermore, luminal calcium would decrease the absorption of lead, as it has already been reported (2, 18). The existence of a diffusional transport system for lead is further supported by the observation that the response of lead absorption to $1,25(\text{OH})_2\text{CC}$ is similar to the response of calcium absorption when the luminal calcium concentration is 25 mM, taken to indicate the diffusional component of calcium transport (34). The active component of calcium transport (1 mM Ca) clearly responds differently to $1,25(\text{OH})_2\text{CC}$ than that of lead, as shown in the present experiments.

Table 1. Composition of the rachitogenic (cholecalciferol-free) chick diet

<u>Ingredient</u>	<u>%</u>
Isolated soy protein	25.00
DL-methionine	.60
Glycine	.40
Corn oil	4.00
Cerelose (Dextrose)	60.27
Cellulose (Solka floc)	3.00
Choline chloride	.20
Vitamin premix ¹⁾	1.00
Mineral premix ²⁾	5.53

1) Supplies the following (mg/kg of the diet): thiamine HCl 15; riboflavin 15; nicotinic acid 50; folic acid 6; pyridoxine HCl 6; biotin 0.6; vitamin B₁₂ 0.02; Ca-pantothenate 20; menadione sodium bisulfite 1.5; BHT 100; vitamin E 50 IU/kg; vitamin A 4500 IU/kg, with 9.74 g glucose/kg.

2) Supplies the following (g/kg of the diet): CaHPO₄ 16.2; CaCO₃ 18.0; KH₂PO₄ 10.0; KCl 1.0; NaCl 6.0; MnSO₄·H₂O 0.35; FeSO₄·7 H₂O 0.5; MgSO₄ 3.0; KIO₃ 0.01; CuSO₄·5 H₂O 0.03; ZnCO₃ 0.15; CoCl₂ 0.0017; NaMoO₄·2 H₂O 0.0083; Na₂SeO₄ 0.002.

Table 2. Effect of stable lead concentration in the dosing solution on the duodenal absorption of ^{203}Pb in the normal chicks¹⁾

Lead in the dosing solution (mM)	Duodenal absorption	Mucosal retention ^{203}Pb (% dose)	Transferred to body
0	71.75 ± 2.87^a	61.32 ± 2.72^a	10.43 ± 1.35^a
0.01 ²⁾	66.11 ± 2.56^a	52.95 ± 2.75^b	$13.16 \pm 1.63^{a,b}$
0.01	72.12 ± 2.33^a	53.96 ± 2.02^b	16.71 ± 1.23^b
0.03	63.92 ± 1.47^a	47.45 ± 1.23^b	16.47 ± 1.18^b
0.10	49.35 ± 4.38^b	37.45 ± 4.09^c	$10.16 \pm 0.92^{a,c}$
0.30	33.97 ± 4.83^c	27.64 ± 2.59^d	6.33 ± 1.93^c
1.00	27.64 ± 1.26^c	25.67 ± 1.07^d	1.96 ± 0.80^d

All values are mean $\pm$ SEM of 5-6 chicks. Values in the same column with a common superscript are not significantly different ($p < .05$).

1) The dose contained 0.5 μCi ^{203}Pb in 1 ml of 0.15 M NaCl and various concentrations of lead acetate, pH 6.5.

2) Contains 0.001 M Na acetate, other groups received the dose containing 0.1 M Na acetate.

Table 3. Effect of stable lead concentration in the dosing solution on the duodenal absorption of ^{47}Ca in rachitic and vitamin D-repleted chicks¹⁾

Lead (mM)	Duodenal absorption	Mucosal retention ^{47}Ca (% dose)	Transfer to body
Rachitic			
0.01	42.97 $\pm$ 4.82	19.27 $\pm$ 2.23	23.70 $\pm$ 3.17
0.10	42.69 $\pm$ 2.81	20.91 $\pm$ 1.66	21.78 $\pm$ 2.34
1.00	37.72 $\pm$ 5.74	20.47 $\pm$ 3.76	17.25 $\pm$ 3.22
D - treated ²⁾			
0.01	96.26 $\pm$ 0.86	2.69 $\pm$ 0.43	93.51 $\pm$ 1.25
0.10	89.85 $\pm$ 4.89	4.10 $\pm$ 0.71	85.75 $\pm$ 5.58
1.00	95.13 $\pm$ 1.30	3.19 $\pm$ 0.44	91.94 $\pm$ 1.67

All values are mean $\pm$ SEM of 6 chicks. The differences between treatments were not significantly different ($p < .05$).

¹⁾ The dose contained 0.1 μCi ^{47}Ca in 0.5 ml of 0.15 M NaCl, 1 mM CaCl_2 , 0.1 M Na acetate and the concentrations of lead (as lead acetate) indicated above, pH 6.5. The length of absorption period was 20 min.

²⁾ 500 IU cholecalciferol, i.m., 72 hrs before the absorption study.

Table 4. Effect of the pH of the dosing solution on the duodenal absorption of ^{203}Pb in the normal chick¹⁾

pH	Duodenal absorption	Mucosal retention	Transferred to body ²⁾
		^{203}Pb (% dose)	
4	56.13 $\pm$ 5.01	46.29 $\pm$ 4.04	9.84 $\pm$ 1.25
6	59.16 $\pm$ 5.35	47.84 $\pm$ 4.15	11.31 $\pm$ 1.76
7	59.45 $\pm$ 3.72	48.97 $\pm$ 3.05	10.47 $\pm$ 1.95

All values are mean $\pm$ SEM of 6 chicks. There were no significant differences between the treatments ($p < .05$).

¹⁾ The dose contained 0.5 μCi ^{203}Pb in 1 ml. of 0.15 M NaCl and 0.01 mM lead acetate, the pH was adjusted with HCl or NaOH.

²⁾ Calculated as $100 - \% ^{203}\text{Pb}$ in the loop.

Table 5. Effect of feeding diets containing lead on the duodenal absorption of ^{203}Pb in chick¹⁾

Lead in the diet (ppm)	Body weight	Duodenal absorption	Mucosal retention	Tibia uptake
			^{203}Pb (% dose)	
0	181 ± 10^a	77.56 ± 0.49^a	54.23 ± 1.87^a	0.62 ± 0.07^a
3	177 ± 8^a	74.60 ± 1.89^a	53.86 ± 1.67^a	0.53 ± 0.09^a
10	183 ± 9^a	76.93 ± 3.03^a	54.38 ± 3.20^a	0.57 ± 0.06^a
100	186 ± 5^a	72.42 ± 1.91^a	52.56 ± 2.64^a	0.53 ± 0.04^a
1000	138 ± 3^b	70.25 ± 2.57^a	40.83 ± 0.99^b	0.93 ± 0.09^b

All values are mean $\pm$ SEM of 6 chicks. Values in the same column having a common superscript are not significantly different ($p < .05$).

¹⁾ The dose contained $0.5 \mu\text{Ci } ^{203}\text{Pb}$ in 0.5 ml of 0.15 M NaCl and 0.01 mM lead acetate, pH 6.5.

The diets were made by adding appropriate amounts of lead acetate to the commercial diet. These diets were fed for 3 weeks after hatching until the absorption study.

Table 6. Absorption of ^{203}Pb from different segments of the small intestine in rachitic and vitamin D-repleted chicks¹⁾

	Absorption	Mucosal retention ^{203}Pb (% dose)	Transferred to body ²⁾
Rachitic			
Duodenum	50.67 ± 2.96^a	43.19 ± 3.56^a	7.48 ± 1.26^a
Jejunum	63.60 ± 6.13^b	51.76 ± 4.67^a	11.84 ± 3.25^a
Ileum	63.45 ± 9.80^b	46.64 ± 7.24^a	$16.83 \pm 5.21^{a,c}$
D-treated ³⁾			
Duodenum	63.32 ± 3.16^b	33.72 ± 1.47^a	29.69 ± 3.14^b
Jejunum	69.50 ± 4.25^b	45.18 ± 3.38^a	24.32 ± 3.73^b
Ileum	67.85 ± 10.70^b	43.17 ± 7.08^a	$24.67 \pm 3.94^{b,c}$

All values are mean $\pm$ SEM of 5-6 chicks. The values in the same column having a common superscript are not significantly different ($p < .05$).

- 1) The dose contained 0.5 μCi ^{203}Pb in 0.5 ml of 0.15 M NaCl and 0.01 mM lead acetate, pH 6.5.
- 2) Refers to the amount of ^{203}Pb which left the intestinal region and is calculated as 100- % ^{203}Pb in the loop.
- 3) 500 IU cholecalciferol in 0.2 ml of propylene glycol (i.m.) 72 hrs. before the absorption study.

Table 7. Absorption of ^{203}Pb from different segments of the small intestine as determined by the percentage of the dose per mg of tissue dry weight¹⁾

	Absorption	Mucosal retention	Transferred to body ²⁾
	²⁰³ Pb (%/mg dry weight)		
Rachitic			
Duodenum	0.19 ± 0.01 ^a	0.16 ± 0.01 ^a	0.03 ± 0.004 ^a
Jejunum	0.33 ± 0.04 ^b	0.27 ± 0.04 ^b	0.06 ± 0.02 ^{a,b}
Ileum	0.39 ± 0.07 ^b	0.28 ± 0.05 ^b	0.11 ± 0.04 ^{b,c}
D-treated ³⁾			
Duodenum	0.26 ± 0.01 ^c	0.14 ± 0.003 ^a	0.12 ± 0.01 ^c
Jejunum	0.35 ± 0.03 ^b	0.22 ± 0.01 ^{a,b}	0.13 ± 0.02 ^c
Ileum	0.45 ± 0.08 ^b	0.28 ± 0.05 ^b	0.17 ± 0.03 ^c

All values are mean $\pm$ SEM of 5-6 chicks. The values in the same column having a common superscript are not significantly different ($p < .05$).

- 1) The dose contained $0.5 \mu\text{Ci } ^{203}\text{Pb}$ in 0.5 ml of 0.15 M NaCl and 0.01 mM lead acetate, pH 6.5.
- 2) Refers to the amount of ^{203}Pb which left the intestinal region and is calculated as $100 - \% ^{203}\text{Pb}$ in the loop.
- 3) 500 IU cholecalciferol in 0.2 ml of propylene glycol (i.m.) 72 hrs. before the absorption study.

Table 8. The effect of age on the duodenal absorption of ^{203}Pb in normal chicks¹⁾

Age (weeks)	Transfer to body (% dose) ^{47}Ca	Blood (% dose/ml)	Mucosal CaBP ($\mu\text{g}/\text{mg}$ protein)
1	19.23 ± 2.66^a	0.78 ± 0.12^a	5.47 ± 0.66^a
3	17.90 ± 2.07^a	0.34 ± 0.05^b	8.45 ± 0.96^a
5	9.49 ± 1.50^b	0.08 ± 0.01^c	5.72 ± 1.34^a

All values are mean $\pm$ SEM of 6 chicks. The values in the same column having a common superscript are not significantly different ($p < .05$).

¹⁾ The dose contained $0.5 \mu\text{Ci } ^{203}\text{Pb}$ in $0.5 \text{ ml } 0.15 \text{ M NaCl}$ and 0.01 mM lead acetate, pH 6.5.

Table 9. Effect of dietary calcium and phosphorus on the duodenal absorption of ^{203}Pb in the chick¹⁾

Diet	Duodenal absorption	Mucosal retention	Tibia uptake
		^{203}Pb (% dose)	
Control	62.72 ± 4.94^a	47.82 ± 3.12^a	0.52 ± 0.04^a
Low Ca	69.17 ± 3.90^a	42.97 ± 1.31^a	0.81 ± 0.09^b
Low P	69.30 ± 5.44^a	43.63 ± 2.85^a	$0.67 \pm 0.09^{a,b}$
Low Ca-P	68.67 ± 2.65^a	44.22 ± 1.90^a	$0.60 \pm 0.08^{a,b}$

All values are mean $\pm$ SEM of 6 chicks. The values in the same column having a common superscript are not significantly different ($p < .05$).

1) The dose contained $0.5 \mu\text{Ci } ^{203}\text{Pb}$ in 0.5 ml of 0.15 M NaCl and 0.01 mM lead acetate, pH 6.5.

Table 10. Effect of varying levels of dietary cholecalciferol on the duodenal absorption of ^{203}Pb and ^{47}Ca in the chick¹⁾

Cholecalciferol in the diet (IU/kg)	Duodenal absorption		Mucosal retention (% dose)		Tibia uptake		Mucosal CaBP (µg/mg protein)
	²⁰³ Pb	⁴⁷ Ca	²⁰³ Pb	⁴⁷ Ca	²⁰³ Pb	⁴⁷ Ca	
0	60.12±4.77 ^a	47.34±3.56 ^a	56.99±5.02 ^a	26.44±2.53 ^a	0.03±0.02 ^a	0.71±0.09 ^a	0 ^a
50	62.43±5.82 ^a	73.11±6.50 ^b	57.75±3.37 ^a	15.96±2.45 ^b	0.16±0.05 ^b	2.26±0.24 ^{b,c}	8.16±1.47 ^b
150	70.81±4.71 ^a	74.24±5.43 ^b	61.79±4.06 ^a	17.03±1.64 ^b	0.16±0.03 ^b	1.94±0.24 ^b	7.73±1.23 ^b
300	67.76±4.54 ^a	86.35±2.49 ^b	59.30±2.99 ^a	11.96±1.62 ^b	0.16±0.05 ^b	2.82±0.14 ^c	11.12±0.56 ^b
600	70.02±5.37 ^a	78.38±6.36 ^b	62.27±4.62 ^a	18.00±2.18 ^b	0.13±0.04 ^b	2.30±0.41 ^{b,c}	7.87±1.50 ^b

All values are mean $\pm$ SEM of 6 chicks. The values in the same column having a common superscript are not significantly different ($p < .05$).

¹⁾ The dose contained 0.5 μCi ^{203}Pb and 0.1 μCi ^{47}Ca in 0.5 ml of 0.15 M NaCl, 0.01 mM lead acetate and 1 mM CaCl_2 , pH 6.5. The length of the absorption period was 20 min.

Table 11. Effect of varying amounts of cholecalciferol, administered as a single intramuscular dose, on the duodenal absorption of ^{203}Pb and ^{47}Ca in rachitic chicks¹⁾

Cholecalciferol (IU)	Duodenal absorption		Mucosal retention (% dose)		Transferred to body		Mucosal CaBP ($\mu\text{g}/\text{mg}$ protein)
	^{203}Pb	^{47}Ca	^{203}Pb	^{47}Ca	^{203}Pb	^{47}Ca	
0	56.73 $\pm$ 3.65 ^a	53.92 $\pm$ 4.79 ^a	55.24 $\pm$ 4.06 ^a	20.92 $\pm$ 4.85 ^a	5.72 $\pm$ 0.59 ^a	31.69 $\pm$ 1.81 ^a	0 ^a
10	72.35 $\pm$ 7.76 ^b	65.50 $\pm$ 8.80 ^a	64.91 $\pm$ 6.78 ^a	13.12 $\pm$ 2.82 ^b	7.58 $\pm$ 1.68 ^a	47.64 $\pm$ 7.30 ^a	0 ^a
100	71.85 $\pm$ 4.58 ^b	88.72 $\pm$ 3.67 ^b	57.26 $\pm$ 2.45 ^a	4.24 $\pm$ 0.77 ^c	16.88 $\pm$ 2.86 ^b	80.66 $\pm$ 4.48 ^c	3.25 $\pm$ 0.50 ^b
1000	82.15 $\pm$ 2.53 ^{b,c}	96.84 $\pm$ 0.82 ^b	57.72 $\pm$ 1.37 ^a	1.95 $\pm$ 0.24 ^d	21.07 $\pm$ 1.96 ^b	90.12 $\pm$ 0.62 ^c	9.05 $\pm$ 1.05 ^c
5000	86.79 $\pm$ 1.45 ^c	96.56 $\pm$ 1.07 ^b	52.72 $\pm$ 2.61 ^a	1.55 $\pm$ 0.40 ^d	28.62 $\pm$ 3.25 ^c	91.30 $\pm$ 1.54 ^c	9.40 $\pm$ 1.23 ^c

All values are mean $\pm$ SEM of 5-6 chicks. The values in the same column having a common superscript are not significantly different ($p < .05$).

¹⁾ The dose contained 0.5 μCi ^{203}Pb and 0.1 μCi ^{47}Ca in 0.5 ml of 0.15 M NaCl, 0.01 mM lead acetate and 1 mM CaCl_2 , pH 6.5. The length of the absorption period was 60 min.

Table 12. Effect of vitamin D status on the transfer of plasma ^{203}Pb and ^{47}Ca across the chick duodenum

	Rachitic	Vit. D-treated	
Luminal ^{203}Pb (% dose)	0.17 ± 0.01	0.16 ± 0.01	N.S.
Mucosal retention ^{203}Pb (% dose)	2.66 ± 0.21	2.06 ± 0.25	N.S.
Tibia uptake ^{203}Pb (% dose)	2.36 ± 0.25	2.08 ± 0.16	N.S.
Luminal ^{47}Ca (% dose)	0.19 ± 0.02	0.40 ± 0.05	P <.01
Mucosal retention ^{47}Ca (% dose)	0.96 ± 0.10	1.12 ± 0.05	N.S.
Tibia uptake ^{47}Ca (% dose)	2.55 ± 0.25	2.49 ± 0.09	N.S.
Plasma ^{47}Ca (%/ml)	1.51 ± 0.18	1.17 ± 0.08	N.S.
Plasma Ca (mM)	1.93 ± 0.10	2.67 ± 0.06	P <.001
Plasma SA (% ^{47}Ca /mmole Ca)	789 ± 101	449 ± 37	P <.01

All values are mean $\pm$ SEM of 6 chicks. The chicks were sacrificed 2.5 min after intravenous injection of the radioisotopes. The dose (0.1 ml) injected contained 1.0 μCi ^{203}Pb and 0.5 μCi ^{47}Ca , 0.01 mM lead acetate, 1 mM CaCl_2 and 0.15 M NaCl, pH 7.0. A nonlabeled solution, 0.5 ml, containing 0.15 M NaCl, 0.01 mM lead acetate and 1 mM CaCl_2 was injected into the ligated duodenal loop at zero time.

Statistical analysis by Student's t-test. NS=not significantly different.

Fig. 1. The effect of the length of the absorption period on the duodenal absorption of ^{203}Pb in 3-week old chicks.

Fig. 2. Response of the duodenal absorption of ^{203}Pb and ^{47}Ca to a single dose of vitamin D_3 (100 IU) administered at various intervals before the absorption study. Each value is mean $\pm$ SEM of 6 chicks.

Fig. 3. Effect of $1,25(\text{OH})_2\text{D}_3$ on the duodenal of ^{47}Ca and ^{203}Pb and appearance of calcium-binding protein in rachitic chicks.

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Studies on the Effects of Ca^{2+} (or Zn^{2+}) in the Organ-Cultured
Embryonic Chick Duodenum

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Introduction

It is now clear that the embryonic chick duodenum maintained in organ culture provides a useful system for the evaluation of toxic and other effects of heavy metal cations on intestinal transport mechanisms, particularly, the calcium absorptive mechanism. Work to date has shown that $3-5 \times 10^{-5}$ M Cd^{2+} in the culture medium, while not grossly toxic, inhibits $1\alpha,25-(\text{OH})_2\text{-D}_3$ -induction of CaBP and unidirectional ^{45}Ca influx at the intestinal mucosal border of the cultured duodenum (1). Zn^{2+} (10^{-4} - 10^{-5} M) has similar effects. Simultaneously, Cd^{2+} (or Zn^{2+}) induces the synthesis of a ^{109}Cd -binding protein (CdBP) as assessed by a competitive ion exchange assay, gel filtration and polyacrylamide gel electrophoresis (2). This CdBP migrates in the two aforementioned systems identically to chick liver cadmium metallothionein (CdMT). Inhibitor studies revealed that cycloheximide, but neither actinomycin D nor α -amanitin, interfere with Cd^{2+} (or Zn^{2+}) induction of the CdBP in the organ culture system. The ineffectiveness of these two latter transcriptional-level inhibitors on CdBP induction was observed at the same time that, as expected, either inhibited $1\alpha,25-(\text{OH})_2\text{-D}_3$ -induced CaBP. Thus, the inhibitor studies are unique in that one induction process known to be regulated at the transcriptional level, CaBP synthesis (3,4), serves as a control for the study of CdBP synthesis under various experimental conditions. These inhibitor studies, along with the metals stimulate biosynthesis by a mechanism operating at the translational level (2).

More recent studies have revealed some additional features of heavy metal (Cd^{2+} , Zn^{2+} or Cu^{2+}) action on the organ cultured duodenum which are described below.

Methods

Organ culture. Entire duodena from three to four 20-day old chick embryos

were slit-open and arranged mucosa-side-up on specially designed stainless steel grids inside petri dishes as previously described (5,6). The medium is Waymouth 752/1 (Gibco) as modified (6) with addition of 0.275 μ M hydrocortisone-22-Na-hemisuccinate (7). The duodena were incubated at 37.5°C in a humidified incubator continuously gassed with a CO₂:O₂:air mixture containing 5% CO₂ and 50% O₂ for up to 24 hr.

Additions. Cd²⁺ (as CdCl₂ · 2½ H₂O) or Zn²⁺ (as ZnSO₄ · H₂O) was added as a sterile H₂O solution to the medium. Crystalline synthetic 1 α ,25-(OH)₂-D₃ (courtesy of Dr. M. Uskoković, Hoffmann-LaRoche, Nutley, NJ) was added as an ethanolic solution to the medium. In some experiments ³⁵S-cystine (NEN, Boston) was added to the culture medium to label the Cd (or Zn)BP. L-cystine-³⁵S, of approximate specific activity, 500 mCi/mmole, was added as a sterile solution at a level of 1 μ Ci/ml and was present throughout the 24 hr culture period. In all cases equivalent volumes of water or ethanol were added to the control medium. The final concentration of ethanol in the medium never exceeded 0.2%.

Homogenization. Duodena cultured as described were homogenized by brief (0.25 min) sonication (Heat Systems Ultrasonics, Model H-1, equipped with a special microtip horn) in a Tris buffer solution (5). During sonication the sample was contained in a 1 ml autoanalyzer sample cup held in a specially-designed aluminum block cooled to ~-25°C on a cold plate (Thermoelectrics Unlimited, Inc., Wilmington, DE).

Assays

CaBP. Portions of the duodenal homogenates were centrifuged at 25,000 g for 15 min. The supernatants were heated at 60°C for 15 min in heat-blocks and recentrifuged. This step removes up to 50% of heat-labile proteins without affecting the heat-stable CaBP. The heat-treated supernatants were analyzed for CaBP content by a radial immunodiffusion technique (5).

CdBP. Portions of the heat-treated supernatants prepared as described above were diluted with Tris buffer and subjected to a competitive ^{109}Cd -binding assay using Chelex 100 resin (Bio-Rad, Richmond, CA) essentially as described for ^{45}Ca -binding assay (8). The percent of the total ^{109}Cd available which is bound by the supernatant (versus the resin), when corrected for "background" binding by Control supernatants, is a measure of the amount of CdBP present in the supernatant.

Total protein. Portions of the heat-treated supernatants were dissolved in 0.1N-NaOH and assayed for total protein by the Lowry procedure on a Technicon Autoanalyzer using crystalline bovine albumin as a standard.

^{45}Ca or ^{109}Cd Influx Measurement. Duodena from each treatment were subjected to either ^{45}Ca or ^{109}Cd influx at the mucosal surface by using the filter paper blotter technique exactly as described elsewhere (9).

Results

Unlike Cd^{2+} or Zn^{2+} , Cu^{2+} when added to the culture medium over the range 10^{-9} - 10^{-4} M had no effect on CaBP induction by $1\alpha,25\text{-(OH)}_2\text{-D}_3$ nor on ^{109}Cd binding activity of homogenate supernatants. At 10^{-3} M Cu^{2+} , the cultured duodena were grossly abnormal. No further studies with this cation are planned.

Induction of CdBP and Inhibition of CaBP as a function of Cd^{2+} concentration.

Earlier studies had indicated that Cd^{2+} , between $3\text{--}5 \times 10^{-5}$ M (or Zn^{2+} , between 10^{-6} - 10^{-4} M) induced CdBP and inhibited CaBP (2). Additional studies were conducted to further examine the range of effective concentrations of Cd^{2+} . Table 1 illustrates a small but statistically significant inhibition of CaBP at a Cd^{2+} concentration as low as 10^{-7} M. $^{109}\text{CdBA}$, and thus presumably metallothionein, was stimulated at a Cd^{2+} concentration as low as 10^{-8} Cd^{2+} . CaBP inhibition was progressively increased by increasing levels of Cd^{2+} until a maximum inhibition was reached at 2×10^{-5} M Cd^{2+} . However, $^{109}\text{CdBA}$ was maximal at 3×10^{-5} M Cd^{2+} .

Induction of CdBP and Inhibition of CaBP by Cd^{2+} or Zn^{2+} as a function of time.

Zn^{2+} at 10^{-4} M first stimulated $^{109}\text{CdBA}$ at 4 hrs after the start of culture. Cd^{2+} at 5×10^{-5} M first stimulated $^{109}\text{CdBA}$ at 9 hrs after the start of culture. In both cases these times coincided with the first times that a clear peak of ^{35}S -labeled protein activity migrating in the metallothionein region, appeared on polyacrylamide gel electrophoretograms (data not shown). This further suggests that the single ^{35}S -labeled peak is related to the $^{109}\text{CdBA}$ of duodenal supernatants and that the ^{35}S -labeled protein and the substance responsible for ^{109}Cd binding in the supernatant are one and the same protein, indistinguishable from metallothionein.

^{109}Cd or ^{45}Ca Influx at the mucosal surface: Influence of Cd^{2+} or Zn^{2+} and $1\alpha,25-(\text{OH})_2\text{-D}_3$.

Table 3 reveals several interesting features of ^{45}Ca and ^{109}Cd influx at the mucosal surface as influenced by stable Cd^{2+} or Zn^{2+} and $1\alpha,25-(\text{OH})_2\text{-D}_3$. First, $1\alpha,25-(\text{OH})_2\text{-D}_3$ stimulated ^{45}Ca influx as amply demonstrated before (9). The addition of stable Cd^{2+} or Zn^{2+} to the culture medium, subsequently elevated ^{45}Ca influx regardless of the presence of $1\alpha,25-(\text{OH})_2\text{-D}_3$. However, the $1\alpha,25-(\text{OH})_2\text{-D}_3$ effect itself was abolished as previously demonstrated (1). Second, ^{109}Cd influx was stimulated by either Cd^{2+} or Zn^{2+} regardless of the presence of $1\alpha,25-(\text{OH})_2\text{-D}_3$, but $1\alpha,25-(\text{OH})_2\text{-D}_3$ in all cases, regardless of addition of Cd^{2+} or Zn^{2+} , inhibited ^{109}Cd influx.

Discussion

Work to date with the organ culture system, including the data presented here, has established the following:

First, Cd^{2+} (or Zn^{2+}) inhibits intestinal responses to $1\alpha,25-(\text{OH})_2\text{-D}_3$, namely, CaBP induction and ^{45}Ca influx at the mucosal border, by a direct, apparently non-toxic action(s) on the intestine;

Second, the inhibitory actions of Cd^{2+} or Zn^{2+} start at concentrations as low as 10^{-7} and 10^{-6} M, respectively.

Third, Cd^{2+} or Zn^{2+} stimulates production of a $^{109}\text{CdBP}$ within 9 and 4 hrs, respectively, at high concentrations, and at 24 hrs at concentrations as low as 10^{-8} M and 10^{-6} M, respectively.

Fourth, the Cd^{2+} or Zn^{2+} -induced ^{109}Cd binding protein is extensively labeled by inclusion of ^{35}S -cystine in the culture medium. Polyacrylamide gel electrophoresis reveals that only one protein is so labeled and it migrates identically to chick liver metallothionein (2). The first appearance of this ^{35}S -labeled protein coincides exactly with the first elevation of $^{109}\text{CdBA}$ by duodenal supernatants which presumably reflects the appearance of the same CdBP.

Fifth, the biosynthesis of the CdBP, whether Cd^{2+} - or Zn^{2+} -induced, appears to be controlled at the translational level (cycloheximide, but neither actinomycin D nor α -amanitin, inhibits CdBP production, 2). Thus, while CaBP is synthesized in response to de novo induction of a specific CaBP-mRNA (4), i.e., at the transcriptional level, CdBP is apparently synthesized on pre-existing mRNA and control may be on the rate of mRNA translocation, mRNA processing, or some process within the cytoplasm, i.e., at the translational level. This provides a unique opportunity to study regulation of synthesis of two specific proteins, controlled at two different molecular levels, in the same tissue under in vitro conditions.

Sixth, Cd^{2+} or Zn^{2+} , while stimulating CdBP production, also enhance ^{109}Cd influx at the mucosal surface, but this response is substantially reduced by $1\alpha,25-(\text{OH})_2\text{-D}_3$. Thus, while $1\alpha,25-(\text{OH})_2\text{-D}_3$ stimulates ^{45}Ca influx, and Cd^{2+} inhibits this influx, Cd^{2+} stimulates ^{109}Cd influx and $1\alpha,25-(\text{OH})_2\text{-D}_3$ inhibits this influx. Although the $1\alpha,25-(\text{OH})_2\text{-D}_3$ -induced CaBP is involved in calcium influx at the mucosal border, there has been no previous demonstration that

metallothioneins may be involved in ^{109}Cd influx. This appears to be the case from the work reported here but no mechanism can currently be postulated. The $1\alpha,25-(\text{OH})_2\text{-D}_3$ -inhibition of Cd^{2+} -stimulated ^{109}Cd influx may be related to the structural modification of the mucosal border reported previously by us (9) and others. This latter observation may have implications for the reduction of cadmium absorption from the environment by increased vitamin D consumption.

Currently, work is underway to isolate, purify and characterize the CdBP's induced by Cd^{2+} and Zn^{2+} in the organ culture system. This will be greatly facilitated by the ability to easily and extensively label these proteins in vitro with ^{35}S -cystine.

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Table 1

CaBP and ^{109}Cd -Binding Activity ($^{109}\text{CdBA}$): Effect of Stable Cd^{2+} Concentration in the Culture Medium^a

Added Cd^{2+} (M)	CaBP ^b ($\mu\text{g}/\text{mg}$ solubule protein)	$^{109}\text{CdBA}$ ^c (% bound/0.1 mg soluble protein)
0	29.4 $\pm$ 2.2	5.5 $\pm$ 0.6
10^{-9}	25.4 $\pm$ 2.5	6.4 $\pm$ 0.5
10^{-8}	30.1 $\pm$ 0.7	7.7 $\pm$ 0.8
10^{-7}	23.2 $\pm$ 2.1 ^d	7.2 $\pm$ 0.2
10^{-6}	23.0 $\pm$ 1.8	11.8 $\pm$ 1.1
10^{-5}	21.3 $\pm$ 1.9	12.0 $\pm$ 0.8
3×10^{-5}	15.2 $\pm$ 1.8	14.9 $\pm$ 0.4
4×10^{-5}	11.9 $\pm$ 0.6	16.3 $\pm$ 1.0
5×10^{-5}	6.1 $\pm$ 1.9	16.1 $\pm$ 1.2

^a Values are the mean $\pm$ S.E.; 8 duodena/treatment.

^b Induced by 16 nM $1\alpha,25\text{-(OH)}_2\text{-D}_3$.

^c $^{109}\text{CdBA}$ reflects all ^{109}Cd -binding substances in the supernatant. To obtain an estimate of that amount of $^{109}\text{CdBA}$ specifically stimulated by stable Cd^{2+} , subtract control (zero Cd^{2+}) $^{109}\text{CdBA}$ from that stimulated by a given level of stable Cd^{2+} .

^d CaBP at this and higher levels of Cd^{2+} significantly less than control at the 5% level.

^e $^{109}\text{CdBA}$ at this and higher levels of Cd^{2+} significantly greater than control at the 5% level.

Table 2

^{109}Cd -Binding Activity ($^{109}\text{CdBA}$): Effect of Cd^{2+} or Zn^{2+} as a Function of Time^a

Time (hrs. of culture)	$^{109}\text{CdBA}$ (% dose/0.1 mg soluble protein) ^c	
	Zn^{2+} (10^{-4} M)	Cd^{2+} (5×10^{-5} M)
$\frac{1}{2}$	4.4 ± 0.5	5.7 ± 0.7
1	6.0 ± 0.8	7.1 ± 0.5
2	5.7 ± 0.4	4.5 ± 0.3
4	7.1 ± 0.6^d	5.6 ± 0.2
9	19.7 ± 1.1	16.4 ± 0.5^d
24	26.6 ± 1.3	21.8 ± 1.0

a,c Superscript meanings same as in Table 1.

d Time at which $^{109}\text{CdBA}$ first significantly higher than control at 5% level.

Table 3

Effect of $1\alpha,25-(\text{OH})_2\text{-D}_3$ and Zn^{2+} or Cd^{2+} on ^{109}Cd or ^{45}Ca Influx at the Mucosal Surface^a

Added Cd^{2+} (5×10^{-5} M)	Added Zn^{2+} (10^{-4} M)	$1\alpha,25-(\text{OH})_2\text{-D}_3$ (16 nM)	Influx at the mucosal surface	
			$^{45}\text{Ca}^b$	$^{109}\text{Cd}^b$
-	-	-	100 (controls)	100
-	-	+	153 ^c	57 ^c
+	-	-	179 ^{c,d}	172 ^{c,d}
+	-	+	160 ^{c,d}	122 ^{c,e}
-	+	-	148 ^{c,e}	180 ^{c,d}
-	+	+	129 ^{c,e}	117 ^{c,e}

^a Values are the mean $\pm$ S.E.; 8 duodena/treatment.

^b ^{45}Ca or ^{109}Cd influx at the mucosal surface was done using the filter paper blotter technique (9).

^c Significantly different from minus added cation and minus $1\alpha,25-(\text{OH})_2\text{-D}_3$ Controls.

^{d,e} Values in a column followed by the same superscript not significantly different from each other.

APPENDIX*
Progress Report
to the
DEPARTMENT OF ENERGY

*reprints & figures
removed*

Published and Submitted Manuscripts

- A-1. Koo, S. I., Fullmer, C. S. and Wasserman, R. H. Intestinal Absorption and Retention of ^{109}Cd : Effects of Cholecalciferol, Calcium Status and Other Variables. J. Nutr. 108: 1812-1822 (1978).
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- A-6. Corradino, R. A., Fullmer, C. S., Frelief, E. and Maxwell, S. Induction of Metallothionein(s) in Organ-Cultured Duodenum: Relationship to $1\alpha,25\text{-(OH)}_2\text{-D}_3$ -Induced CaBP Biosynthesis. Fourth Workshop on Vitamin D, Kongresshalle Berlin (West), Germany (1979).

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