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Calcium–cadmium interaction on L-threonine intestinal transport

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Summary — Cadmium is a highly toxic metal that can damage a number of organs including the gastrointestinal tract. It has been shown that cadmium partially reduces L-threonine intestinal absorption probably by binding to membrane proteins which pertain to active transport systems or are functionally related to them. Calcium, however, is an essential element in a wide variety of cellular activities. The aim of the present work was to study whether the inhibitory cadmium effect on L-threonine absorption across rabbit jejunum could be modified by calcium. In media with Ca^{2+}, cadmium significantly reduces the L-threonine absorption. In Ca^{2+}-free media, where calcium chloride was omitted and replaced isotonically with choline chloride, this amino acid transport was not modified by cadmium but it was inhibited when calcium chloride was replaced isotonically with magnesium chloride. Verapamil (blocking mainly Ca^{2+} transport) did not modify the inhibitory effect of cadmium on L-threonine transport. When A 23187 (Ca^{2+} specific ionophore) was added in media with/without Ca^{2+}, cadmium produced no change on L-threonine transport. These results suggest that calcium and cadmium could have an affinity for the same chemical groups on the enterocyte membrane. This property could affect the intestinal absorption of amino acids.

cadmium / calcium / L-threonine intestinal transport / verapamil / A 23187

Résumé — Interaction du calcium et du cadmium sur le transport de L-thréonine à travers le jéjunum de lapin. *Le cadmium est un ion métallique très toxique qui peut endommager de nombreux organes, y compris le tractus gastro-intestinal. Il a été montré que le cadmium provoque une diminution de l'absorption intestinale de L-thréonine. Cet effet est probablement dû à sa capacité de liaison aux protéines membranaires impliquées directement ou indirectement dans les systèmes de transport actif. Le but de ce travail était d'étudier si l'effet inhibiteur du cadmium sur l'absorption de L-thréonine dans le jéjunum de lapin pouvait être modifié par le calcium, un oligo-élément indispensable à de nombreuses fonctions cellulaires. En présence de Ca^{2+}, le cadmium diminue significativement l'absorption de L-thréonine. Dans les milieux dépourvus de Ca^{2+}, où le chlorure de calcium a été remplacé par une concentration isotonique de chlorure de choline, le cadmium n'a pas affecté le transport de cet*

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acide aminé. Cependant cette réduction de l'inhibition n'est pas observée lors de la substitution du chlorure de calcium par du chlorure de magnésium à la même concentration. Le vérapamil (inhibiteur du transport du Ca^{2+}) ne modifie pas l'effet du cadmium sur le transport de L-thréonine. En présence d'A23187 (ionophore spécifique de Ca^{2+}) et, en présence ou en absence de calcium dans les milieux, le cadmium n'affecte pas le transport de L-thréonine. Ces résultats suggèrent que le calcium et le cadmium intéragissent de manière compétitive avec les mêmes groupements chimiques de la membrane entérocytaire, modifiant ainsi le flux transmembranaire des acides aminés.

cadmium / calcium / absorption intestinale de L-thréonine / verapamil / A23187

INTRODUCTION

Cadmium is a heavy metal that is a significant environmental pollutant. Its high toxicity causes damage to a number of organs including the gastrointestinal tract. This latter organ is one of the main routes of cadmium absorption. The toxic effects of cadmium upon cell membrane structure and function have been well documented (Sorensen *et al,* 1985; Kunimoto *et al,* 1986). This metal readily reacts with thiol groups of proteins (Jacobson and Turner, 1980; Tuker and Matte, 1980; Templeton, 1990). In agreement with earlier observations reported in the literature, we have observed that the cadmium effect is due to impairment of the active transport mechanism, which could be altered by interaction with sulphydryl groups linked to the transport systems and (Na^+/K^+)-ATPase (Rodriguez-Yoldi *et al,* 1989, 1992, 1993, 1994; Mesonero *et al,* 1993a,b, 1994).

Calcium, however, is involved in vital cellular processes. Intracellular Ca^{2+} concentration plays a key role in the regulation of many cellular processes, acting as an intracellular second messenger. Cellular calcium homeostasis is often safeguarded by the ATP-dependent Ca^{2+} pump, a high affinity Ca^{2+}-ATPase (Van Os, 1987).

Cadmium is not under homeostatic control (Cotzias *et al,* 1961), but there is evidence to show that the homeostatic transport mechanisms of some essential metals can accommodate intestinal cadmium transport. Several studies have shown than an important relationship exists between the toxic effects of cadmium and the level of calcium in the diet (Larsson and Piscator, 1971; Itokawa *et al,* 1973, 1974; Washko and Cousins, 1975, 1977).

The object of the present work was to study the calcium–cadmium interaction with reference to L-threonine intestinal absorption, in order to clarify the gastrointestinal action of cadmium.

MATERIALS AND METHODS

Materials

L-Threonine, A 23187 ionophore, verapamil hydrochloride and EGTA (ethyleneglycol tetraacetic acid) were obtained from Sigma (Saint-Louis, MO). $CdCl_2$ and $MgCl_2$ were obtained from Merck (Darmstadt). L-[U-^{14}C]Threonine and [^{14}C]polyethylene glycol were obtained from Amersham (Buckinghamshire). Liquid scintillation, formula 989 was obtained from Du Pont (Boston, MA).

Animals and incubation solutions

Male New Zealand rabbits weighing 1.5–2.0 kg were maintained at constant room temperature (24°C) with free access to water and standard rabbit fodder. The treatment of the animals, handling, equipment used and sacrifice were in accordance with the European Council legislation 86/609/EEC on experimental animal protection.

After being killed by a blow on the head, the proximal jejunum (5 cm distal to the ligament of Treitz) was removed and rinsed free of intestinal contents with ice-cold incubation solution. The tissue was then stripped of its serosal and external muscle layers. The incubation solution contained in mM: 127 NaCl; 10.18 KCl; 2.72 $CaCl_2$; and 15 Tris-HCl. It was continuously bubbled with O_2/CO_2 (19:1 v/v). In experiments where Ca^{2+}-free conditions were required, $CaCl_2$ was omitted and replaced isotonically with either choline chloride or $MgCl_2$. Cadmium was added as a chloride. After addition of the metal or other compounds, the pH was adjusted, if necessary, to 7.4.

L-Threonine was selected for study because it is a neutral amino acid that is essential to rabbits (Lebas, 1987; Moughan *et al,* 1988; Schultze *et al,* 1988).

Cell water determinations

Pieces of jejunum (about 100 mg) were incubated in the incubation solution at 37°C containing 0.02 μCi/ml [^{14}C]polyethylene glycol (mol wt 4 000, PEG 4 000, Amersham) for 20 min. The pieces of mucosa were then gently blotted on wet filter paper and weighed. They were then extracted in 0.5 ml 0.1 M HNO_3 overnight at 4°C. Aliquots of the extracts (200 μl) and aliquots of the bathing solutions (200 μl) were counted in 2 ml liquid scintillation fluid. Following extraction, the tissues were dried at 80°C for 12 h and then reweighed. The test agents (0.5 and 1 mM $CdCl_2$, 10^{-6} M A 23187 and verapamil, 5.44 mM choline chloride and 2.72 mM $MgCl_2$) were present in the rinsing solution from the start of incubation, and had no significant effect on the extracellular space, tissue water fraction or cell water fraction (data not shown).

Amino acid uptake measurements

The experiments were carried out with rings of rabbit everted jejunum weighing about 100 mg which were incubated at 37°C in 10 ml incubation solution containing 0.01 μCi/ml L-[U-^{14}C]threonine plus unlabelled 0.5 mM L-threonine for 3 or 20 min. In the short experiments (3 min), we studied the initial rate, but in the 20 min experiments the L-threonine absorption attained a steady state at the highest absorption (data not shown). At the end of the experiment the tissues were washed with 2 or 3 gentle shakes in ice-cold incubation solution and blotted carefully on both sides to remove excess moisture. The tissue was weighed wet and extracted by shaking for 15 h in 0.5 ml 0.1 M HNO_3 at 4°C. Samples were taken from the bathing solutions and from the extracts of the tissues for radioactivity counting. All the modifiers (cadmium, *etc*) were added to the incubation solution at the beginning of the incubation period. The cadmium concentrations used were 0.5 and 1 mM, determined according to the previous studies carried out in our laboratory (Rodríguez-Yoldi *et al,* 1989; Mesonero *et al,* 1993a,b, 1994). The results are expressed as μmol L-threonine/ml cell water, accumulated in 3 or 20 min, after correction for the extracellular space.

Transepithelial flux measurements

The stripped mucosa was mounted as a flat sheet in Ussing-type chambers as described in previous studies (Rodríguez-Yoldi *et al,* 1992, 1993; Mesonero *et al,* 1993a). The bathing solutions on the mucosal and serosal surfaces of the tissue were maintained at 37°C using a circulating water bath. Both solutions contained L-threonine at the same concentration (10 mM). Mucosal to serosal amino acid fluxes (Jms) were measured by adding 0.04 μCi/ml L-[U-^{14}C]threonine on the mucosal side and serosal to mucosal fluxes (Jsm) by placing the L-[U-^{14}C]threonine on the serosal side. Samples were removed from the non-radioactively labelled side at 20 min intervals for 80 min, after a 30 min pre-incubation period. Only one sample was taken for counting from the radioactively labelled side. Samples of the radioactive solution were counted using a liquid scintillation counter.

The results are expressed as μmol L-threonine x cm^{-2} x $hour^{-1}$.

Statistics

All results are expressed as means ± SE. The comparison between means was evaluated by a 2-way (animal and treatment) analysis of variance (Anova) procedure as outlined by Steel and Torrie (1980). The Fisher's protected least significant difference test (PLSD) was used as a

multiple-comparison method to compare data among groups, and considered statistically significant when $p < 0.05$.

RESULTS

Effect of cadmium on intestinal L-threonine accumulation and transepithelial fluxes in calcium-free media

In the media containing calcium, the presence of 1 mM $CdCl_2$ in the solution, significantly inhibited the L-threonine accumulation, as reported in previous studies (Mesonero *et al,* 1993a,b, 1994). When calcium chloride was omitted from medium and replaced isotonically with choline chloride with or without 2 mM EGTA (calcium chelating agent), $CdCl_2$ produced no change in the amino acid accumulation (fig 1).

In the 20 min incubation period, the amino acid accumulation attained a steady state. For the purpose of studying the cadmium effect in media with or without calcium in a short time, at the initial rate, a group of experiments was carried out with incubations of 3 min. The results again showed that $CdCl_2$ produced no change in 0.5 mM L-threonine accumulation when Ca^{2+} was removed from the bath solution (fig 2).

When $CaCl_2$ was omitted and replaced isotonically with $MgCl_2$ in the incubation solution, cadmium did induce a change in the amino acid accumulation (fig 1). The L-threonine intestinal transport in the presence of $MgCl_2$ was greater than the control (media with $CaCl_2$). Magnesium has been demonstrated to be important as a regulator of the paracellular permeability (Hardwick *et al,* 1990).

With the intention of studying the cadmium effect in more detail, mucosal to serosal flux (*via* the brush-border surface) and serosal to mucosal flux (*via* the basolateral) were measured in media with or without calcium. Figure 3 shows that in media with calcium, the cadmium on the mucosal side at 1 mM concentration, significantly reduced mucosal to serosal L-threonine flux. In calcium-free media where $CaCl_2$ was omitted and replaced isotoni-

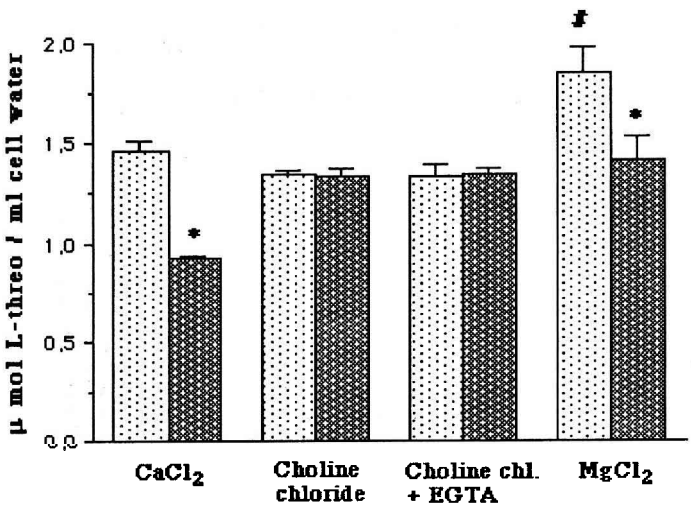

Fig 1. Effect of cadmium on the accumulation of 0.5 mM L-threonine in rabbit jejunum, in 20 min, when $CaCl_2$ was omitted from the bath solution and replaced isotonically with choline chloride or $MgCl_2$. The results are from 8 animals with 5 determinations/rabbit. * $p < 0.05$ compared with 0 mM cadmium; # $p < 0.05$ compared with 0 mM cadmium (calcium media). ▨ 0 mM Cd; ■ 1 mM Cd.

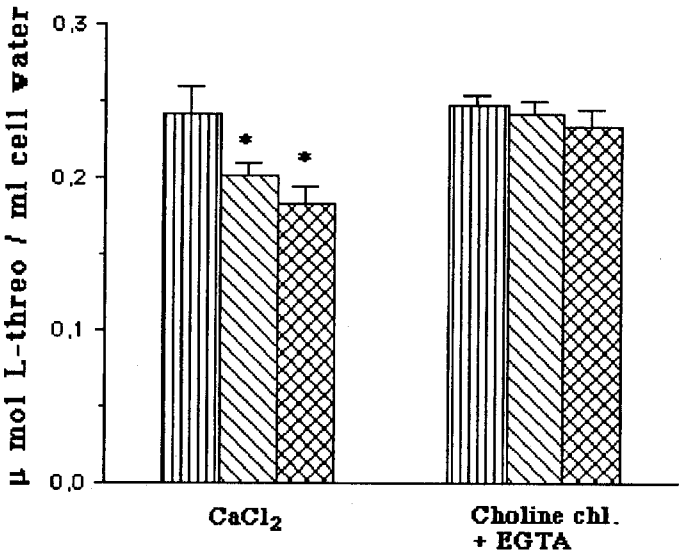

Fig 2. Effect of cadmium on the accumulation of 0.5 mM L-threonine in rabbit jejunum, in 3 min, when calcium chloride was removed from the bath solution and replaced isotonically with choline chloride and 2 mM EGTA. The results are from 7 animals with 5 determinations/rabbit. * $p < 0.05$ compared with 0 mM cadmium. ▥ 0 mM Cd; ▨ 0.5 mM Cd; ▩ 1 mM Cd.

cally with choline chloride, the cadmium produced no change in the amino acid flux, but if $CaCl_2$ was replaced isotonically with $MgCl_2$, the cadmium significantly reduced mucosal to serosal L-threonine flux. Moreover, the cadmium did not modify serosal to mucosal L-threonine flux in all the media tested (data not shown). When the cadmium was on the serosal side, the L-threonine flux (Jms or Jsm) was not modified in media with or without calcium (data not shown).

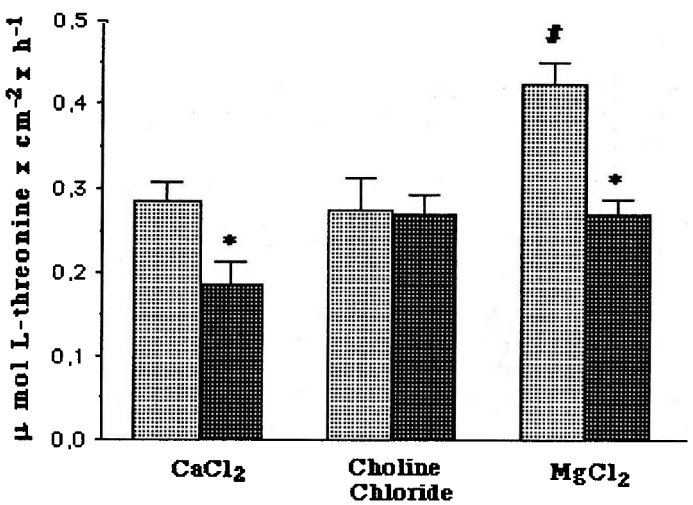

Fig 3. Effect of cadmium on mucosal to serosal (Jms) L-threonine flux across sheets of rabbit jejunum mucosa when $CaCl_2$ was omitted from the bath solution and replaced isotonically with choline chloride or $MgCl_2$. The cation was on the mucosal side. Amino acid concentration in the bathing solution was 10 mM. The results are from 8 animals. * $p < 0.05$ compared with 0 mM cadmium. # $p < 0.05$ compared with 0 mM cadmium (calcium media). ▥ 0 mM Cd; ▩ 1 mM Cd.

Action of verapamil on cadmium inhibitory effect in L-threonine intestinal transport

The above results showed that cadmium inhibited L-threonine intestinal absorption in media with calcium or magnesium (divalent cations). The action of 10^{-6} M verapamil (blocking mainly Ca^{2+} transport) and 1 mM cadmium on the accumulation of 0.5 L-threonine in 3 min was studied in order to explain why cadmium does not inhibit the amino acid transport in Ca^{2+}-free media. The results summarized in table I show that in media without calcium, cadmium produces no significant effect on L-threonine absorption in the presence or absence of verapamil.

Action of ionophore A 23187 on cadmium inhibitory effect in L-threonine intestinal transport

In order to study whether cadmium could interact with A 23187 (a calcium ionophore) and modify the L-threonine intestinal absorption, a group of experiments was carried out in media with and without calcium. The results showed that cadmium did not inhibit either the accumulation or the transepithelial flux of L-threonine when A 23187 was added to media (tables II and III).

DISCUSSION

The object of this experimental work was to study the calcium–cadmium interaction on L-threonine transport across the rabbit jejunum *in vitro* in order to increase understanding of cadmium action on intestinal absorption of nutrients.

The results showed that in calcium-free media, where $CaCl_2$ was omitted and replaced isotonically with choline chloride, cadmium produced no inhibitory effect in L-threonine intestinal transport when the absorption arrived at the steady state or when we worked at the initial rate. If $CaCl_2$ was omitted from media and replaced isotonically with $MgCl_2$, cadmium produced changes in the amino acid transport. Magnesium is a divalent cation similar to calcium (figs 1 and 2). We obtained the same results when we studied the transepithelial flux in media with and without calcium (fig 3).

The uptake of cadmium by intestinal mucosal cells has been shown to involve a

Table I. Effect of 1 mM cadmium on the accumulation of 0.5 mM L-threonine in rabbit jejunum in 20 min in media with or without calcium and when 10^{-6} M verapamil was added to the medium.

	L-Threonine µmol/ml cell water			
	0 mM Cd	*10^{-6} M verapamil*	*1 mM Cd*	*1 mM Cd + 10^{-6} M verapamil*
$CaCl_2$	1.36 ± 0.08 (8)	1.29 ± 0.06 (8)	0.92 ± 0.07* (8)	0.93 ± 0.07* (8)
Choline chloride	1.33 ± 0.10 (8)	1.32 ± 0.10 (6)	1.28 ± 0.06 (7)	1.30 ± 0.11 (6)

The values presented are the means ± SE. The figures in brackets indicate the number of animals with 5 determinations per rabbit. * $p < 0.05$ compared with 0 mM cadmium.

Table II. Effect of 1 mM cadmium on the accumulation of 0.5 mM L-threonine in rabbit jejunum in 20 min in media with or without calcium and when 10^{-6} M A 23187 was added to the medium.

	L-Threonine µmol/ml cell water			
	0 mM Cd	*10^{-6} M A 23187*	*1 mM Cd*	*1 mM Cd + 10^{-6} M A 23187*
$CaCl_2$	1.33 ± 0.02 (8)	1.30 ± 0.01 (8)	0.91 ± 0.01* (8)	1.31 ± 0.01 (8)
Choline chloride	1.32 ± 0.02 (8)	1.29 ± 0.01 (8)	1.31 ± 0.01 (8)	1.33 ± 0.01 (8)

The values presented are the means ± SE. The figures in brackets indicate the number of animals with 5 determinations per rabbit. * $p < 0.05$ compared with 0 mM cadmium.

Table III. Effect of cadmium on mucosal to serosal (Jms) L-threonine flux across sheets of rabbit jejunum in media with or without calcium and when 10^{-6} M A 23 187 was added to the medium; the cation was on the mucosal side; amino acid concentration in the bathing solution was 10 mM.

	L-Threonine µmol x cm^2 x h^{-1}			
	0 mM Cd	*10^{-6} M A 23187*	*1 mM Cd*	*1 mM Cd + 10^{-6} M A 23187*
$CaCl_2$	0.27 ± 0.02 (7)	0.28 ± 0.01 (8)	0.17 ± 0.01* (8)	0.28 ± 0.00 (7)
Choline chloride	0.20 ± 0.01 (7)	0.28 ± 0.02 (7)	0.28 ± 0.02 (8)	0.27 ± 0.01 (8)

The values presented are the means ± SE. The figures in brackets indicate the number of animals. * $p < 0.05$ compared with 0 mM cadmium.

non-specific electrostatic binding to anionic sites of the membrane (Foulkes, 1991). Cadmium is highly reactive with thiol groups of proteins (Jacobson and Turner, 1980; Tuker and Matte, 1980; Templeton, 1990).

Interaction between Cd^{2+} and Ca^{2+} on the plasma membrane could lead to reduced Cd^{2+} influx if both cations were present extracellularly. Theoretically, extracellular calcium would be capable of binding to the same sites to which cadmium was bound. Both divalent cations have the capacity to bind to single anionic binding sites on adjacent phosphatidylserine head groups, triggering phase separations and resulting in domain formation (Ohnishi and Ito, 1974; Jacobson and Papahadjopouos, 1975). Similar effects were reported for phosphatidic acid head groups (Trauble and Eibl, 1974; Hartmann *et al,* 1977). In hepatocytes, a reduction in the number of adherent Cd^{2+} ions, as a result of competition between the 2 divalent cations, could cause reduced cadmium influx in the presence of extracellular

calcium (Sorensen, 1988) and involvement of Ca^{2+} channels in Cd^{2+} uptake has also been suggested in these cells (Blazka and Shaikh, 1991). In muscle cells, it has been reported that Ca^{2+} has a higher affinity than Zn^{2+} or Cd^{2+} with the binding sites of the Ca^{2+} channel (Hagiwara and Byerly, 1981). For instance, Cd^{2+} and Ca^{2+} were reported to compete for voltage-sensitive Ca^{2+} channels in secretory cells from the pituitary (Hinkle *et al,* 1987). Van Os (1987) reported the absence of such Ca^{2+} channels in intestinal epithelium.

With the possibility that cadmium produces no inhibitory effect of L-threonine absorption in calcium-free media due to membrane crossing by specific calcium channels, we carried out a study in which these channels were blocked with verapamil. Previous studies have demonstrated that verapamil inhibits calcium transport in isolated intestinal segments. Furthermore, it appears that verapamil acts on the mucosal (apical) membrane to inhibit calcium transport (Wrobel and Michalska, 1977).

Our results showed that cadmium produced no significant effect on L-threonine intestinal transport in calcium-free incubation solution in the presence or absence of verapamil. The above hypothesis could, therefore, be rejected.

With the purpose of corroborating this result, a group of experiments was carried out with a carboxylic antibiotic named A 23187. It was identified as a divalent cation ionophore by Reed and Lardy in 1972. Their laboratory characterized the affinity of this ionophore with divalent cations as $Mn^{2+} >> Ca^{2+} = Mg^{2+} >> Sr^{2+} > Ba^{2+}$ (Pfeiffer *et al,* 1974). Ion size (Cotton and Wilkinson, 1988) seems to be important in ionophore binding, with smaller ions (Mn, Ca, Mg) binding better than larger ions (Sr, Ba). In the biological system, A 23187 is considered to be a Ca^{2+}-specific ionophore, probably because of the large gradients that are maintained between extracellular and cytoplasmic spaces and between intracellular compartments (endoplasmic reticulum and mitochondria) and the cytoplasm (Resendez *et al,* 1986; Xiong *et al,* 1992). The fact that Ca^{2+} and Cd^{2+} have similar ionic radio, 0.99 *vs* 0.97 Å, respectively (CRC, 1981), may allow the cadmium to be transported across the plasma membrane by AB23187.

The results obtained have shown that cadmium did not inhibit L-threonine accumulation when A 23187 was added to media. This occurrence could indicate that when the calcium channels are created by this ionophore, the bioavailability of some chemical groups on plasma membrane for cadmium is greater.

In summary, the results obtained in the present work and previous studies in our laboratory, show that cadmium inhibits the active intestinal transport of L-threonine in the presence of calcium and other divalent ions. When calcium is removed from the incubation solution, cadmium produces no changes in the amino acid transport. We propose the possibility of competition between cadmium and calcium for binding sites on mucosal membrane which do not pertain to carrier L-threonine. These chemical groups could pertain to the membrane calcium channels or be functionally related to them. In the absence of calcium in media, cadmium could interact with these Ca^{2+}-binding sites of enterocyte with greater affinity than with sulphydryl groups of amino acid carriers. This occurrence would produce no inhibitory effect on L-threonine intestinal transport.

Further experiments are required to verify which chemical groups in the mucosal membrane (besides sulphydryl groups) bind to cadmium.

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