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Early Cellular Effects of Circulating Cadmium-Thionein on Kidney Proximal Tubules

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Circulating cadmium-thionein (Cd-MT) is cleared from the mammalian circulatory system by filtration through the kidney glomerulus with subsequent reabsorption by kidney proximal tubules. Damage to the tubules results following uptake of Cd-MT, which is dependent upon time and the dose level of cadmium administered. Intravenous administration of ^{109}Cd-MT at doses of 0.017 and 0.17 mg Cd/kg body weight with examination of total renal uptake of ^{109}Cd at 0.5, 3, and 24 hr disclosed that the rate of clearance from the blood and uptake by the kidney was significantly more rapid at the 0.017 mg Cd/kg dose. Ultrastructural changes resulting from intravenous injection of either form A or B of Cd-MT were characterized by increased numbers of pinocytotic vesicles and small, dense lysosomal structures. There was no evidence of mitochondrial swelling or cell death at either 3 or 6 hr after injection. The subcellular distribution of cadmium in kidney tissue at various times after administration of Cd-MT was determined by using differential centrifugation techniques with ^{109}Cd and *in situ* by using x-ray microanalysis. At 30 min after injection of Cd-MT, significant amounts of cadmium were present in lysosomal fractions indicating an interaction between the tubular lysosome system and Cd-MT prior to the onset of overt cellular toxicity. Results suggest that Cd-MT is reabsorbed and broken down by kidney tubule cells in a physiological manner with possible subsequent release of the toxic cadmium ion.

Introduction

Studies of the involvement of metallothionein (MT) in the metabolism of cadmium have shown that this protein plays a major role in determining the pharmacokinetics and toxicity of the cadmium ion in mammalian systems (*1*). Attention has recently turned to studies of the metabolism of the cadmium-thionein (Cd-MT) protein complex itself, however, because of the nephrotoxic properties of this compound (*2–4*). Following injection or oral exposure to Cd^{+2}, cadmium in plasma is primarily bound to the high molecular weight proteins alpha-2-macroglobulin and albumin (*5*). The ion is preferentially cleared from the blood stream by the liver (*3*). Plasma cadmium bound to the low molecular weight (6000-7000) thionein molecule, however, is reabsorbed by the kidney (*6*) leading to damage to the proximal tubules (*4*).

Although Cd-MT normally accumulates in liver and kidney tissues as an intracellular metalloprotein, recent evidence suggests that Cd-MT might also be present in blood under certain circumstances. Data reported by Ridlington and co-workers (*7*) suggest that cadmium is slowly translocated from the liver to the kidney over a 6 month period following short-term exposure to cadmium. Release of cadmium from the liver with transport to the kidney is thought to occur via circulating Cd-MT (*1, 8*). In support of this, Hirayama and Shaikh (*9*) have demonstrated the presence of Cd-MT in the circulatory system at 4 weeks after the start of continuous cadmium exposure. Cherian and co-workers (*10*) have suggested a second possible source of circulating Cd-MT to be the intestinal lumen following dietary exposure to Cd-MT.

This study was undertaken to examine the early morphological effects of intravenously injected

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Cd-MT on renal proximal tubules and the interaction of cadmium with subcellular organelles in order to study the mechanism by which circulating cadmium-thionein is reabsorbed by the kidney proximal tubule and its relationship to Cd-MT-induced renal toxicity.

Methods

Preparation of Cd-MT

Male Sprague-Dawley rats (CD strain, obtained from Charles River Laboratories) weighing 250-300 g were injected subcutaneously with 0.5 mg Cd as $CdCl_2$ in 0.9% (w/v) NaCl daily for 5 days and were sacrificed two days after the last injection. Hepatic Cd-MT was prepared by Sephadex G-75 gel filtration of liver-soluble proteins. For certain experiments, the MT fractions were further purified by DE52 cellulose anion exchange chromatography to obtain components MT-A and MT-B (*11*). Preparations of MT were lyophilized following dialysis against deionized water for 18 hr.

When ^{109}Cd-labeled Cd-MT was prepared, rats were injected as above with 0.1 mg Cd as $CdCl_2$ and 0.25 μCi ^{109}Cd per injection.

Cadmium concentrations were determined by using a Jarrel Ash 850 atomic absorption spectrophotometer. ^{109}Cd activity was measured in a Beckman Biogamma II gamma ray spectrophotometer.

Analysis of Cd-MT-Induced Morphological Changes in Kidney Proximal Tubule Cells

Male Sprague-Dawley rats weighing 100-120 g were injected with Cd-MT intravenously using the saphenous vein. Groups of five rats each were injected with either 0.18 mg Cd as Cd-MT-A or 0.17 mg Cd as Cd-MT-B per kg body weight in a volume of 0.1 ml and were sacrificed after 3 or 6 hr. Control rats were injected with saline. Immediately following sacrifice by exsanguination, slices of kidney cortex were fixed and processed for electron microscopy as previously described (*12*). Kidney cadmium concentrations from the same animals were determined by atomic absorption spectrometry of samples prepared by dry ashing at 450°C for 24 hr.

Subcellular Distribution of Cadmium

Rats weighing 100–200 g were divided into six groups of three rats each and injected intravenously with either 0.17 mg Cd as ^{109}Cd-MT (0.02 μCi) or 0.017 mg Cd as ^{109}Cd-MT (0.002 μCi) per kg body weight. Groups of rats were sacrificed 0.5, 3, or 24 hr after injection. One entire kidney of each rat was quickly counted for total radioactivity in a gamma ray spectrophotometer. Kidney homogenates were prepared from both kidneys by using 3 ml of 0.25*M* sucrose–0.01*M* Tris/HCl buffer (pH 7.4) per gram of tissue with four strokes of a Potter-Elvejhem homogenizer. Homogenates were fractionated by centrifugation to obtain the following six fractions: 1500*g* × min pellet (fraction I); 7500*g* × min pellet (fraction II); 42,800*g* × min pellet (fraction III); 292,000*g* × min pellet (fraction IV); 3,600,000*g* × min pellet (fraction V); and the 3,60,000*g* × min supernatant fraction (fraction VI). All fractions were washed once with the homogenizing buffer and the wash combined with the corresponding supernatant fraction. Each pellet was resuspended in 2 ml of 0.01*M* Tris/HCl (pH 7.4) buffer, and the radioactivity measured in a Beckman gamma counter. Aliquots (2 ml) of homogenates and fraction VI were counted in a similar fashion. Acid phosphatase activity of the fractions was measured according to the procedure of Turnbull and Neil (*13*). Proteins were measured by the method of Lowry et al. (*14*) by using bovine serum albumin as a standard.

Energy-Dispersive X-Ray Microanalysis of Subcellular Cadmium

Three male rats weighing 175–200 g were injected intraperitoneally with 0.64 mg Cd as Cd-MT per kg body weight and sacrificed after 3 hr. Slices of kidney cortex were fixed and processed for x-ray microanalysis as previously described (*15*). The x-ray spectra were obtained over specific subcellular structures by using an EDAX energy-dispersive x-ray analysis system attached to a Phillips EM 300 using an accelerating voltage of 80 KeV.

Statistical Analysis

Analyses of significant differences were made by using a one-way analysis of variance test (*16*).

Results and Discussion

Experiments in this study were designed to evaluate the early effects of Cd-MT on rat kidney proximal tubule cells. Previous studies by a number of investigators (*2, 4, 17*) have demonstrated the renal toxicity of intravenously injected Cd-MT. Ultrastructural morphometric studies (*4*) of kidneys from rabbits injected with Cd-MT have shown that a dose of 0.4 mg/kg Cd as Cd-MT produced extensive

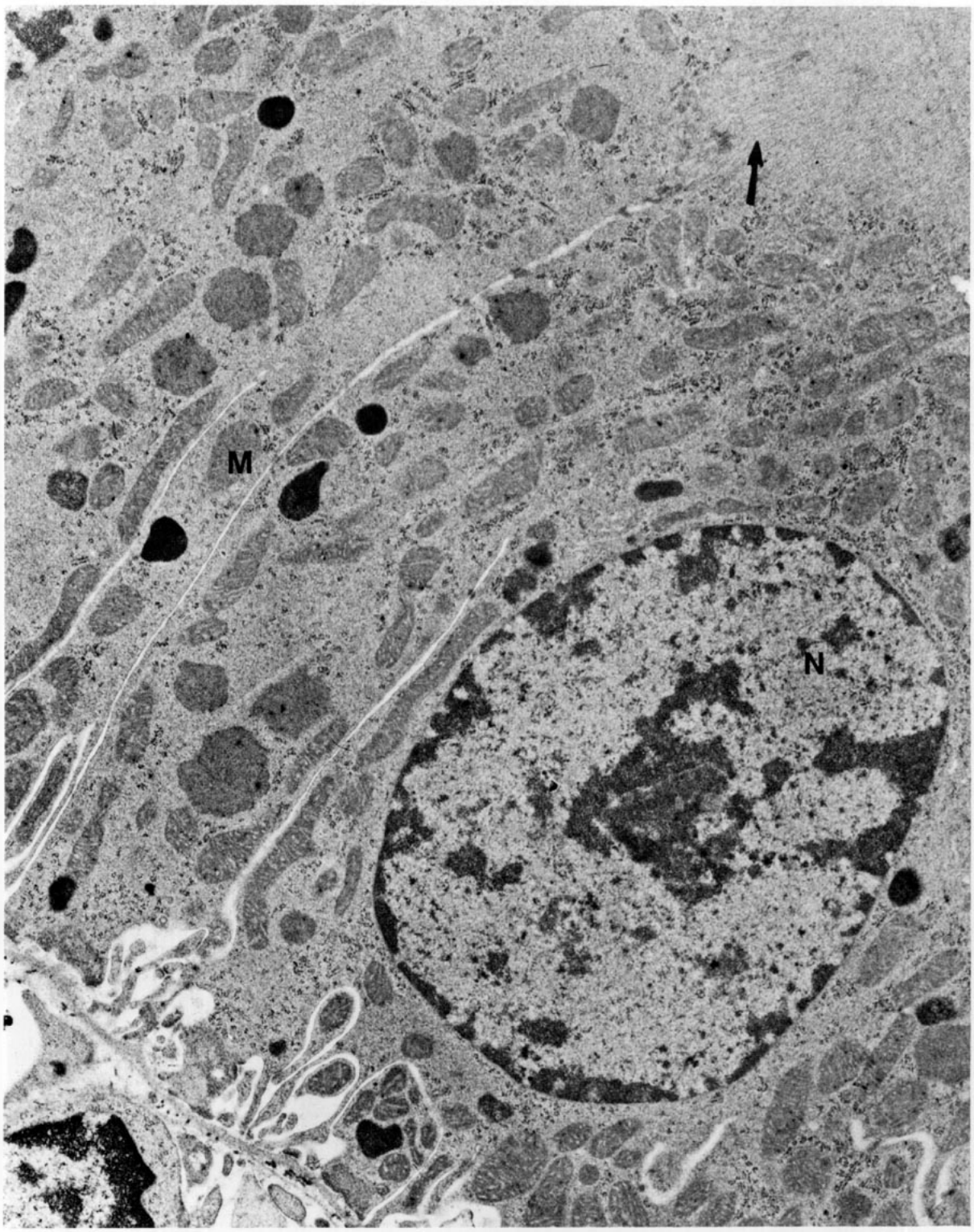

FIGURE 1. Electron micrograph of a proximal tubule cell from a control rat showing the nucleus (N), mitochondria (M), brush border (arrow) and normal complement of lysosomes and apical vacuoles. ×12,700.

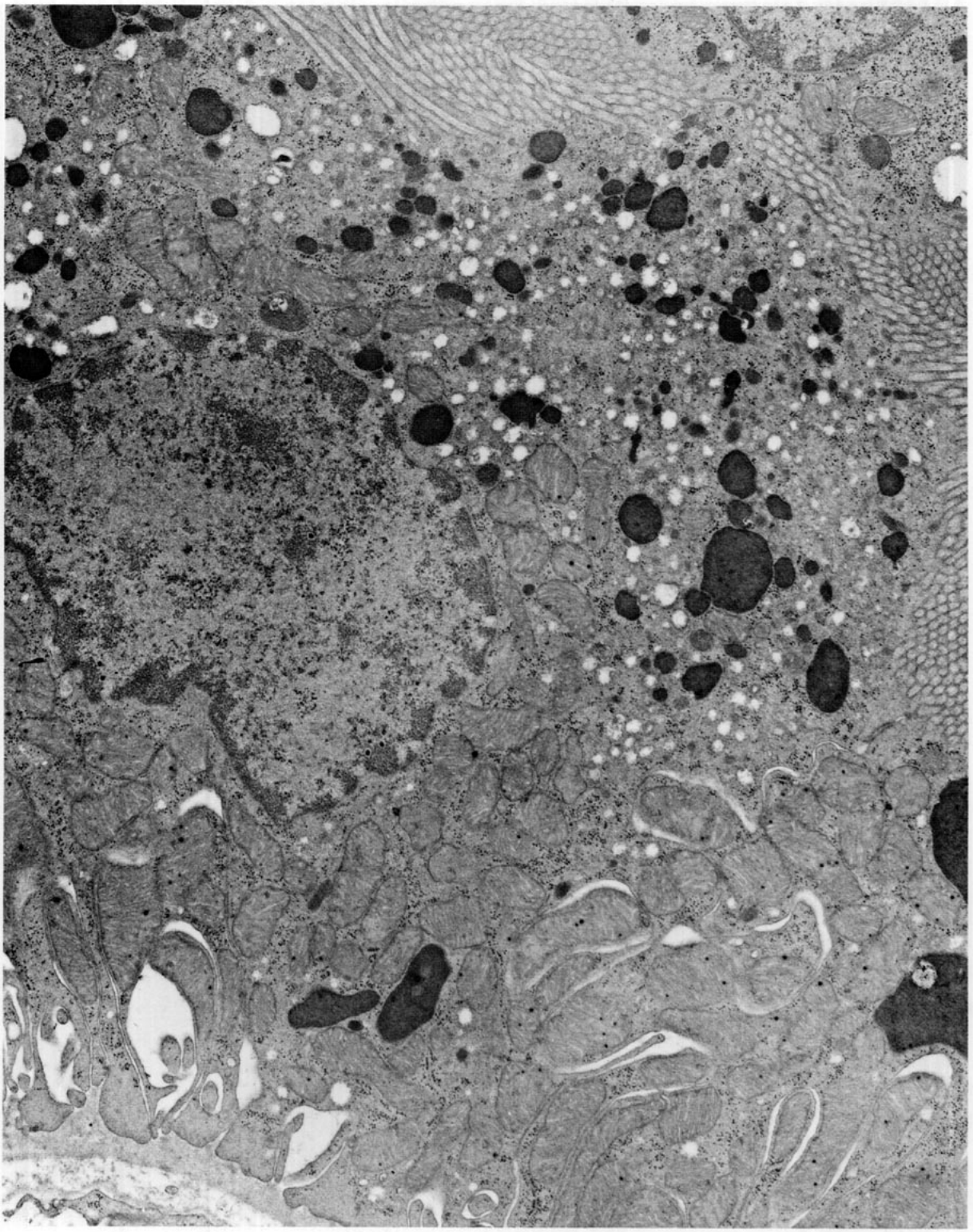

FIGURE 2. Electron micrograph of a proximal tubule cell from a rat injected with 0.18 mg Cd/kg body weight as Cd-MT-A 3 hr prior to sacrifice showing an increased number of pinocytotic vesicles and small dense lysosomal structures. ×16,320.

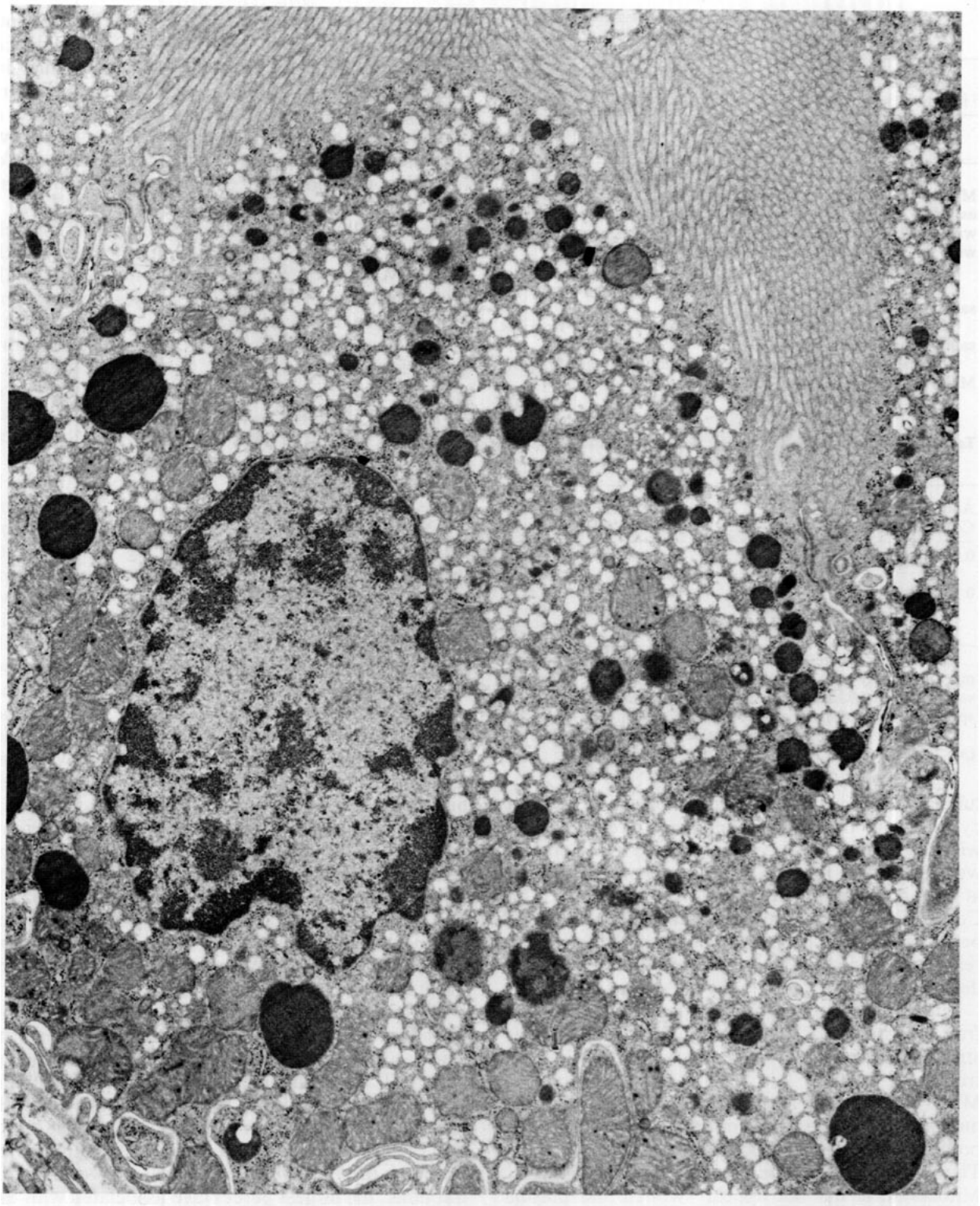

FIGURE 3. Electron micrograph of a kidney proximal tubule cell from a rat injected with 0.18 mg Cd/kg body weight as Cd-MT-A 6 prior to sacrifice showing an increased number of vesicles and dense lysosomal structures. ×16,375.

Table 1. Kidney cadmium concentration following intravenous injection of Cd-MT or $CdCl_2$.[a]

Time after injection, hr	Cd, μg/g tissue[b]			
	MT-A	MT-B	$CdCl_2$	Saline
3	9.5[c, e] ± 1.5	10.8[c] ± 1.4	1.3[d] ± 0.3	0.9 ± 0.1
6	9.1[c, e] ± 0.1	1.08[c] ± 1.1	1.1[d] ± 0.1	0.8 ± 0.1

[a] Rats were injected intravenously with 0.18 mg Cd as Cd-MT-A or 0.17 mg Cd as Cd-MT-B or 0.20 mg Cd/kg body weight as $CdCl_2$ 3 or 6 hr prior to sacrifice. Control rats were injected with 0.9% (w/v) NaCl. Total kidney cadmium concentrations were determined as outlined in the Methods section.

[b] Means ± S.D.; $n = 5$.

[c] Indicates the number is significantly different from the saline-injected group ($p < 0.05$).

[d] Indicates the number is not significantly different from the saline-injected group ($p < 0.05$).

[e] Indicates the number is not significantly different from the MT-B-injected group ($p < 0.05$).

Table 2. Relationships between dose, time, and uptake of Cd-MT by kidney tissue.[a,b]

Administered Cd dose, mg/kg	Kidney cadmium		Time after injection, hr
	Total μg	% of dose	
0.017	3.2[c,d]	30.8	0.5
	6.3[c,d]	62.0	24
0.17	9.9[c,d]	9.6	0.5
	21.3[c]	20.6	3
	28.6[d]	27.7	24

[a] Rats were injected intravenously with 0.17 mg Cd or 0.017 mg Cd/kg body weight as ^{109}Cd-MT at 0.5, 3 or 24 hr prior to sacrifice. Total kidney cadmium was calculated by using the specific activity of the injected ^{109}Cd-MT.

[b] Numbers with the same superscript are significantly different ($p < 0.05$).

tubular necrosis, as evidenced by degeneration of cytoplasmic organelles, leading to renal failure within 7 days. In the present study, rat kidneys were examined following administration of Cd-MT at lower doses and at shorter time periods after Cd-MT injection to assess early changes which occur in kidney proximal tubule cells following reabsorption of Cd-MT but prior to the onset of cell degeneration.

The normal architecture of a proximal tubule cell from a control rat is illustrated in Figure 1. The most striking change observed in proximal tubule cells from rats injected with either Cd-MT-A or Cd-MT-B 3 hr prior to sacrifice was an increase in the number of small vesicles and small dense lysosomal bodies near the apical portion of the cell (Fig. 2). There was no evidence of cell death at this time. Mitochondria did not appear swollen and plasma and nuclear membranes appeared to be intact. The same morphological changes were observed following injection of either form of MT. Also, rats injected with either MT-A or MT-B showed no differences in the reabsorption of Cd-MT based on renal concentrations of Cd (Table 1). Such evidence indicates that the two forms of MT are similar in their interaction with the kidney and that the morphological changes observed are not due to a specific form of Cd-MT. Data in Table 1 also show that there was essentially no kidney uptake of Cd following injection of $CdCl_2$, which is consistent with work reported by other investigators (*2, 3*).

Figure 3 illustrates a kidney proximal tubule cell obtained from a rat injected 6 hr previously with Cd-MT-A. A further increase in the number of small dense lysosomes and small vesicles is apparent compared to the changes observed after 3 hr. Signs of cell necrosis are still not evident. These morphological changes suggest that Cd-MT interacts with the kidney lysosomal system following reabsorption of the protein. The following series of experiments were designed to study the uptake of Cd-MT by the proximal tubule cell.

Data in Table 2 show the relationship which exists between dose level and kidney uptake of Cd-MT. Following an intravenous injection of Cd-MT at a dose of 0.17 mg Cd/kg body weight, 9.6% of the injected dose is taken up by the kidneys after 30 min; by 3 hr after injection, 20.6% is recovered in the kidneys. The amount present at 24 hr is not significantly different than that at 3 hr, which suggests that the injected Cd-MT had been cleared from circulation by 3 hr. This is consistent with the data presented in Figure 4 which show that detectable levels of ^{109}Cd were present in serum at 30 min after injection, but by 3 hr serum ^{109}Cd activity had returned to background levels. In contrast to the high dose of Cd-MT, a dose of 0.017 mg Cd per kg body weight was reabsorbed more efficiently by the kidneys; by 24 hr a total of 62% had been reabsorbed. These data show that the Cd-MT reabsorption system is saturable, in agreement with data reported by Foulkes (*18*). Loss of Cd-MT in the urine, as reported by Cherian and co-workers (*3*), would be expected to occur when large amounts of Cd-MT are injected.

To study the mechanism of uptake of Cd-MT by kidney tubule cells, the subcellular distribution of Cd was measured after intravenous injection of ^{109}Cd-MT. Data showing the association of ^{109}Cd with various subcellular fractions obtained by differential centrifugation at various times after injection of Cd-MT are given in Table 3. At 30 min after injection of ^{109}Cd-MT, ^{109}Cd was recovered in all fractions, the largest percentage associated with the crude nuclear fraction (fraction I). The three fractions composed predominantly of lysosomes and mitochondria (fractions II–IV) together accounted for about 38% of the total Cd present in the tissue.

By 3 hr after injection of MT, the relative amount of Cd associated with the lysosomal-mitochondrial fractions had decreased considerably. Fraction I Cd remained approximately the same, while the proportion of Cd present in the soluble fraction of the cell had increased from 25% to 62%. By 24 hr, there was a decrease in Cd associated with fraction I and a corresponding increase in soluble Cd. The lysosomal-mitochondrial fractions remained unchanged.

The column in Table 3 labeled "control" was obtained by addition of ^{109}Cd-MT to excised control rat kidneys prior to homogenization. The amount added was equal to that present in the 3 hr injected group. This control group measures the nonspecific binding of Cd-MT to cellular fractions following disruption of the cell. Although the majority of the Cd-MT was recovered in the soluble fraction, a significant proportion of cadmium (14%) was recovered in fraction I, due probably to absorption of the Cd-MT molecule to either plasma or nuclear membranes. Only small amounts of Cd were recovered in fractions II–V.

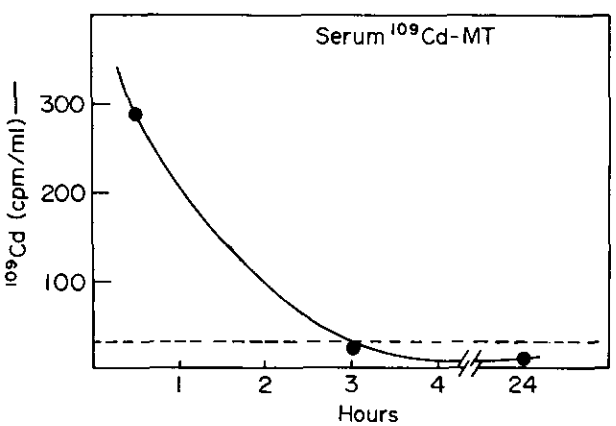

FIGURE 4. Serum ^{109}Cd activity at various times after intravenous injection of 0.17 mg Cd/kg body weight as ^{109}Cd-MT.

By using the control group as a baseline, it is evident that Cd does associate with the lysosomal-mitochondrial fractions of the cell after injection of Cd-MT. Table 4 shows the data expressed as total weight of Cd associated with each fraction. At all three time periods, 0.5, 3, and 24 hr, the actual amount of cadmium associated with fractions II–IV was greater than that which could be accounted for by the control group. The greatest association of Cd with the lysosomal-mitochondrial fractions occurred at 0.5 hr, the earliest time period measured after injection.

When the data are expressed on a concentration basis (as %Cd/% protein, Table 5), it is evident that after 30 min, the cadmium was most concentrated in fractions I and III. These two fractions also had relatively high acid phosphatase activities, an enzyme normally used as a marker for lysosomes. The fact that cadmium is concentrated in fraction III rather than the lighter lysosomal fraction would suggest that Cd-MT is present in heavier lysosomes possibly derived from the fusion of pinocytotic vesicles with lighter primary lysosomes.

It is difficult to interpret the significance of the cadmium present in fraction I. The relatively high concentration of acid phosphatase and cadmium in this fraction could be due to the presence of large

Table 3. Subcellular distribution of ^{109}Cd following intravenous injection of ^{109}Cd-MT.[a]

Subcellular fraction	Centrifugation speed, $g \times$ min	Cadmium content of fraction at various times following Cd-MT injection, % of injected dose[b] 0.5 hr	3 hr	24 hr	Control	Predominant composition
I	1,500	33.2 ± 0.6[c]	30.2 ± 3.2[c]	22.4 ± 2.4[c]	14.2 ± 0.5	Nuclei, large cytoplasmic bodies
II	7,500	14.3 ± 1.1[c]	2.7 ± 0.3[c]	2.7 ± 0.5	1.4 ± 0.3	Mitochondria, lysosomes
III	42,800	18.3 ± 0.8[c]	1.6 ± 0.2	1.7 ± 0.4	0.9 ± 0.1	Mitochondria, lysosomes
IV	292,000	6.0 ± 0.6[c]	1.9 ± 0.2[c]	1.7 ± 0.2[c]	0.6 ± 0.1	Mitochondria, lysosomes
V	3,600,000	2.5 ± 0.1[c]	1.7 ± 0.1[c]	1.0 ± 0.1	0.4 ± 0.2	Microsomes, Small mitochondria, lysosomes
VI	3,600,000	25.6 ± 1.8[c]	61.8 ± 5.4	70.4 ± 5.6	82.5 ± 1.4	Cytoplasmic sap

[a] Rats were injected intravenously with 0.17 mg Cd/kg body weight as ^{109}Cd-MT at 0.5, 3, or 24 hr prior to sacrifice. Control rats were injected with 0.9% (w/v) NaCl and ^{109}Cd-MT was added to the kidneys at the time of homogenization. Subcellular fractions were prepared as outlined in the Methods section. Assignment of the predominant composition of the fractions was made according to Maack et al. (*19*).

[b] S.E.M., $n = 3$.

[c] Indicates the number is significantly different ($p < 0.05$) than the control value for the same fraction.

Table 4. Total cadmium content of subcellular fractions of the kidney.[a]

Subcellular fraction	Cd at various times after injection, μg/fraction[b]			
	0.5 hr	3 hr	24 hr	Control
I	2.86 ± 0.05[b]	5.13 ± 0.55	4.29 ± 0.47	2.24 ± 0.08
II	1.24 ± 0.10	0.46 ± 0.04	0.53 ± 0.11	0.22 ± 0.04
III	1.58 ± 0.07	0.28 ± 0.04	0.32 ± 0.08	0.14 ± 0.02
IV	0.52 ± 0.05	0.33 ± 0.04	0.32 ± 0.04	0.10 ± 0.01
V	0.22 ± 0.01	0.29 ± 0.02	0.20 ± 0.02	0.07 ± 0.03
VI	2.22 ± 0.20	10.48 ± 0.92	13.47 ± 1.07	13.04 ± 0.23

[a] Rats were injected intravenously with 0.17 mg Cd/kg body weight as ^{109}Cd-MT at 0.5, 3, or 24 hr prior to sacrifice. Control rats were injected with 0.9% (w/v) NaCl, and ^{109}Cd-MT was added to the kidneys at the time of homogenization. Subcellular fractions were prepared as outlined in the Methods section.
[b] S.E.M.; $n = 3$.

Table 5. Relative content of cadmium in subcellular fractions of the kidney following intravenous injection of Cd-MT.[a]

Subcellular fraction	Cd concn at various times after injection, % Cd/% protein				Acid phosphatase, % activity/ % protein
	0.5 hr	3 hr	24 hr	Control	
I	2.24	2.04	1.51	0.96	1.29
II	0.64	0.12	0.12	0.06	0.90
III	1.44	0.13	0.13	0.07	1.42
IV	0.59	0.18	0.16	0.06	1.49
V	0.32	0.22	0.12	0.05	1.17
VI	0.93	2.26	2.56	3.01	0.71

[a] Rats were injected intravenously with 0.17 mg Cd/kg body weight as ^{109}Cd-MT at 0.5, 3, or 24 hr prior to sacrifice. Control rats were injected with 0.9% (w/v) NaCl, and ^{109}Cd-MT was added to the kidneys at the time of homogenization. Subcellular fractions were prepared as outlined in the Methods section.

lysosomal-type cytoplasmic bodies, as suggested by Maack and co-workers (*19*). However, adsorption of Cd-MT to components of this fraction and/or the presence of unbroken cells may give rise to contamination of this fraction. It is also possible that released Cd ions may have entered the nuclei (*20*). It should also be noted that cadmium present in the soluble fraction (fraction VI) could arise from a number of sources, such as disruption of subcellular organelles containing Cd-MT or from Cd-MT present in the tubular lumen or extracellular spaces at the time of homogenization. Therefore, a precise analysis of the subcellular localization of Cd *in situ* cannot be obtained from this type of experiment alone.

The x-ray microanalysis of kidney tissue for cadmium supported the suggestion that Cd-MT is taken up by lysosomes following reabsorption of the protein by the kidney proximal tubular cells. Figure 5 is a picture of the cathode ray display of the x-ray monitor unit obtained following analysis of a dense lysosomal structure in a proximal tubule cell from a rat injected with 0.64 mg Cd/kg body weight as Cd-MT 3 hr prior to sacrifice. After background subtraction (Fig. 6), a small but significant peak was left at the position of the Cd Lα line. Although we were working very close to the detection limits of the x-ray analysis unit on our electron microscope with these tissues, we did regularly obtain small peaks for Cd which remained following background subtraction when lysosomes were analyzed. Such *in situ* localization of cadmium within lysosomes supports the biochemical location of cadmium within this organelle after Cd-MT reabsorption. Because the cells did not show signs of necrosis at the short time intervals used in these experiments, the association of Cd with lysosomes should be indicative of the reabsorption mechanism for Cd-MT and cannot be attributed to an association of Cd resulting from the irreversible processes of cell death.

Data from these studies suggest that circulating Cd-MT is reabsorbed by kidney proximal tubules by the same mechanisms involved in reabsorption of other low molecular weight plasma proteins (*21*). The increase in pinocytotic vesicles and small lysosomes and the association of Cd with lysosomal fractions suggests that Cd-MT is reabsorbed by the kidney via pinocytosis and is broken down by lysosomes following fusion of the vesicles with primary lysosomal bodies. Such a process is consistent with data reported by Cherian and Shaikh (*6*) on the degradation of injected ^{35}S-labeled Cd-MT. Of the soluble reabsorbed thionein, 60% was degraded by 3 hr after injection. This rate of degradation is considerably greater than that reported by Oh et al. (*22*) for the normal turnover of Cd-MT in the kidney which was approximately 3.5 days. This difference in rates of degradation would suggest that Cd-MT reabsorbed by the kidney cell is more rapidly associated with the degradative lysosomal system than Cd-MT synthesized within the cell itself. Degradation of the protein following reabsorption is also consistent with what is presently known about the uptake of proteins by kidney proximal tubular cells in general (*23–25*). There is little convincing evidence to date to suggest that any proteins are reabsorbed and transported across the cell in their

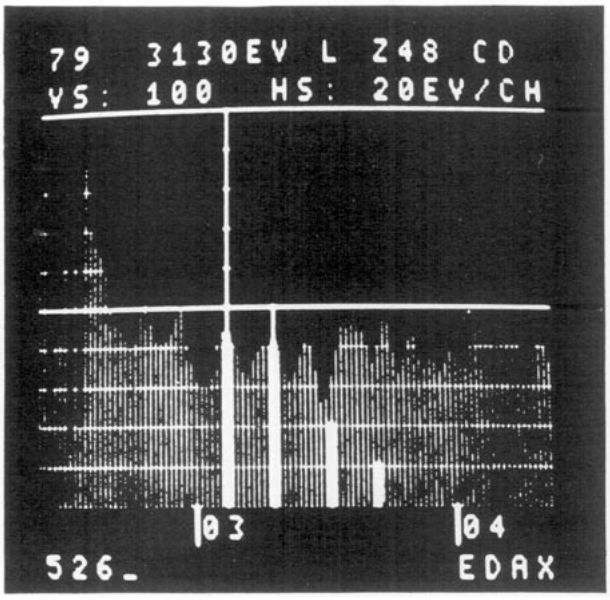

FIGURE 5. Energy-dispersive x-ray spectra from a lysosomal structure in a proximal tubule cell of a rat injected IP 3 hr prior to sacrifice with 0.64 mg Cd/kg body weight as Cd-MT. The marked line is the Cd Lα peak (3.13 KeV).

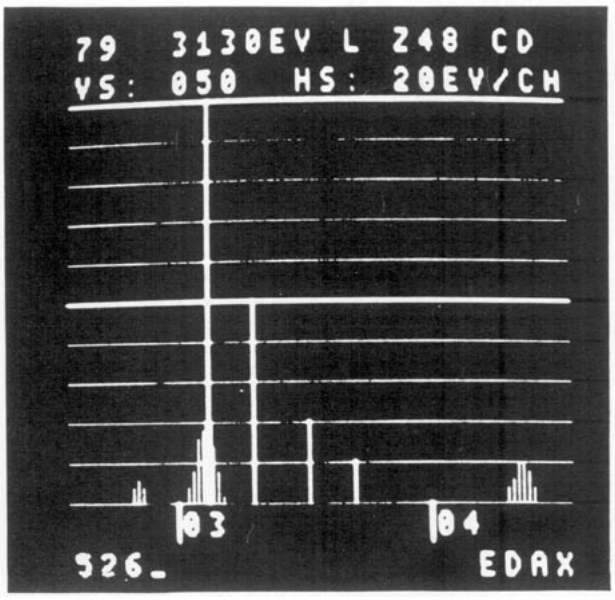

FIGURE 6. Energy dispersive x-ray spectra following computer background subtraction from a lysosome in a renal proximal tubule cell of a rat injected with 0.64 mg Cd/kg body weight as Cd-MT. The Cd Lα peak in significant above background with a 2 σ confidence interval.

intact form (*25*).

Although the mechanism of the toxic action of Cd-MT on the kidney is not well understood, results of this study suggest that Cd ions released in or from lysosomes following degradation of Cd-MT are responsible for the development of tubular necrosis. Fowler and Nordberg (*4*) have previously suggested that toxic Cd ions are released from lysosomes in excess of the amount of apothionein present in proximal tubule cells available to resequester the ions, thus leading to cell death. Mego and Cain (*26*) have reported that Cd inhibits proteolysis in heterolysosomes *in vivo*. It is possible, therefore, that Cd ions remaining within the lysosomes might inhibit lysosomal function and thereby lead to cell death via a mechanism involving the inability of the cell to degrade other reabsorbed proteins such as albumin with a resultant loss of control over intracellular osmotic conditions.

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