

THE ROLE OF ZINC IN NITRILOTRIACETATE(NTA)- ASSOCIATED RENAL TUBULAR CELL TOXICITY

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Summary—Both the extent and the severity of renal tubular cell toxicity associated with constant systemic loads of nitrilotriacetate (NTA) were shown to be dependent upon the availability of zinc in the circulation. NTA ingestion at doses that produced plasma ultrafiltrate (UF) NTA concentrations $>20 \mu\text{M}$ caused an increase in UF zinc. The majority of the increased zinc but not the NTA was resorbed during renal clearance of NTA and the extent and severity of tubular cell lesions was proportional to the amount of zinc resorbed. Intravenous infusion studies demonstrated that both zinc salts and ZnNaNTA produced nephrotoxicity that is very similar to heavy metal toxicity. The toxicity of iv ZnNaNTA was accompanied by renal tissue accumulation of zinc but not of NTA and the increased tissue zinc was not removed by saline infusion. These studies have led to the development of a model for NTA-associated nephrotoxicity that demonstrates that the initiation, propagation and extent of renal tubular cell toxicity are all dependent upon NTA-induced changes in zinc metabolism. This model negates the use of any mathematical models that estimate low dose ($\text{UF}_{\text{NTA}} < 20 \mu\text{M}$) effects from toxicity data obtained at doses that exceed the threshold ($\text{UF}_{\text{NTA}} > 20 \mu\text{M}$).

Introduction

Nitrilotriacetate (NTA) is a divalent metal chelating agent which has been shown to be functional in laundry detergents. Reviews of the mammalian toxicity and environmental effects and fate of NTA have been published (Foley, Becking, Muller, Goyer, Falk & Chernoff, 1977; Shapiro, Chapman, Dick, Dillon, O'Melia, Spacie & Leduc, 1978; Thayer & Kensler, 1973). NTA is readily absorbed from the gut and is excreted only via the urine (Michael & Wakim, 1971). In transit through mammals there is no alteration in the structure of the NTA molecule (Thayer & Kensler, 1973) and renal clearance of NTA is accomplished by filtration with no evidence for the existence of a tubular cell transport system for either secretion or resorption (A. Licht, N. S. Bricker, R. E. Papendick & R. L. Anderson, unpublished data, 1980).

NTA ingestion, processing and excretion results in changes in divalent cation (M^{2+}) disposition (Anderson & Kanerva, 1978a; Michael & Wakim, 1973). The divalent cations most sensitive to NTA are magnesium (Mg) and zinc (Zn). NTA increases bone and urinary Zn and decreases faecal Zn (Anderson & Kanerva, 1978a; Nixon, Buchler & Niewenhuys, 1972). In contrast NTA increases faecal Mg and reduces plasma, bone and urinary Mg. Doses of NTA that alter Zn and Mg disposition (0.5% $\text{Na}_2\text{NTA} \cdot \text{H}_2\text{O}$ in diet) do not affect calcium (Ca) but higher doses of NTA ($\geq 0.75\%$ in the diet) increase urinary Ca excretion (Anderson & Kanerva, 1978b; Anderson & Kanerva, 1979; Michael & Wakim, 1973).

Acute (Merski, 1980), subchronic (Alden & Kanerva, 1979; Mahaffey & Goyer, 1972) and chronic (National Cancer Institute, 1977; Nixon *et al.* 1972) ingestion studies show that the renal tubules are a

primary site of NTA-associated toxicity. At dietary NTA doses of up to $18 \mu\text{mol/g}$ diet (0.5% $\text{Na}_2\text{NTA} \cdot \text{H}_2\text{O}$ in the diet) the toxic response noted after 24 months of NTA ingestion has been described as hydropic degeneration (Nixon *et al.* 1972). Higher doses of NTA are associated with renal tubular cell adenoma and adenocarcinoma (National Cancer Institute, 1977). Study of the natural history of NTA-associated tubular cell toxicity from acute, subchronic and chronic studies had led to the conclusion that the process is a continuum, which begins with proximal convoluted tubule (PCT) vacuolization, progresses to simple and nodular hyperplasia with vacuoles to adenomatous hyperplasia, and subsequently appears as tubular cell tumours (Alden, Kanerva, Anderson & Adkins, 1981). In addition to this specific sequence of responses, NTA ingestion also exacerbates the spontaneous nephrosis noted in ageing rats (Alden & Kanerva, 1980). The PCT toxicity, even at the nodular hyperplasia stage is reversible if NTA is removed from the diet (M. C. Myers, R. L. Kanerva, C. L. Alden & R. L. Anderson, unpublished data, 1980).

Extensive testing has shown that NTA is not mutagenic or teratogenic (Foley *et al.* 1977). This finding strongly implies that the tumorigenicity associated with the ingestion of high doses of NTA is an epigenetic phenomenon. Thus the primary concern is how a molecule that is devoid of genotoxicity, is not metabolized to a genotoxic moiety, is not transported across PCT cells, and the only significant chemical activity of which is its propensity to form stable complexes with M^{2+} , can be responsible for the PCT toxicity (including tumours) noted after chronic exposure at high doses.

A comparison of the dose-response curves showed a very close similarity between PCT toxicity and increased Zn excretion in the urine. For example, 24 months of ingesting a diet containing $1.1 \mu\text{mol}$ NTA/g

(0.03% $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$) caused no alteration in renal morphology or urinary Zn excretion. Dietary NTA at 5.5 $\mu\text{mol/g}$ (0.15% $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$) increased urinary Zn excretion and was associated with PCT vacuoles and hyperplasia, changes described as hydropic degeneration (Nixon *et al.* 1972). Both urinary Zn excretion and PCT lesions were enhanced when dietary NTA was increased to 18 $\mu\text{mol/g}$ (0.5% $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ and 0.5% CaNaNTA).

This report describes the results of a series of experiments in which the relationship between NTA-induced changes in Zn disposition and PCT toxicity was more extensively examined. The results of these experiments show that the initiation of PCT toxicity is dependent upon blood NTA attaining concentrations high enough to increase the plasma ultrafiltrate Zn (UF_{Zn}) levels. A large proportion of the increased UF_{Zn} is resorbed by the PCT during renal clearance of NTA, and by some undefined mechanism this alters the endocytotic-lysosomal system to induce vacuoles and subsequently tubular cell hyperplasia. This response has demonstrable no-effect levels which negate the validity of mathematical extrapolations of responses (including tumours) noted at high doses to possible incidences at low doses.

Experimental

All of the experiments reported used Sprague-Dawley-derived rats obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA. The animals were housed individually in stainless-steel metabolism cages and kept at controlled temperature ($70 \pm 5^\circ\text{F}$) and humidity (40–60%) with a 12-hr light/dark cycle. In most of the experiments the diet was ground Purina Laboratory Chow (Ralston Purina Co., St Louis, MO) and the animals were given feed and distilled water *ad lib.* For one study the rats were trained to consume their daily intake of feed in a 1-hr period (9–10 a.m.). Meal training was accomplished by reducing the time during which the diet was available over a 2-wk period. In the study in which the dietary Zn level was varied the animals were fed a semi-purified diet containing 8, 14, 21 or 52 ppm Zn. The diet was composed of 20% casein, 20% lard, 60% sucrose and all of the vitamins and minerals (except Zn) at greater than the recommended daily allowance (National Academy of Sciences/National Research Council, 1972) for the rat. In all instances NTA was added to the diets on a w/w basis. Weekly weight gains and twice-weekly feed consumption records were maintained on all animals. Urine samples for mineral and NTA analyses were total 24-hr collections obtained in cages with a separator which retained the faeces and allowed urine to be collected in tared bottles. The separators were not rinsed.

For iv infusions, 200–250 g male rats were fitted with catheters of Silastic 602-135 tubing (Dow Corning Corp., Medical Products, Midland, MI) in the jugular vein as described by Upton (1975). The catheters were connected to a Harvard 975 Infusion Pump (Harvard Apparatus, Millis, MA) set to deliver 10 μl /min. The animals were restrained throughout the infusion period and were provided with chow pellets (Ralston Purina Co.) and distilled water *ad lib.* During infusion the voided faeces were collected on a

nylon screen over a funnel and the urine was collected in tared vials.

Before they were killed the animals were anaesthetized with ether, and then a mid-line incision was made and blood was collected from the vena cava in heparinized syringes. The blood cells were separated by centrifugation and when required, plasma ultrafiltrate (UF; MW < 10,000) was obtained by filtering a portion of the plasma through an Amicon PM-10 filter (Amicon Corp., Lexington, MA). Minerals were determined by atomic absorption spectrometry. Urine NTA analyses were by reverse isotope dilution when unlabelled NTA was fed and by direct count after 25:1 dilution with water when [^{14}C]NTA was used (Anderson & Kanerva, 1978b). Blood-cell, plasma and UF [^{14}C]NTA analyses were carried out after combustion of [^{14}C]NTA to $^{14}\text{CO}_2$. All ^{14}C levels were determined by scintillation counting.

The kidneys were processed using routine histological techniques followed at our laboratory. The tissue was placed in 10% neutral phosphate-buffered formalin and allowed to fix for 18 hr. The tissues were embedded in paraffin blocks according to standard histological methods, sectioned on a microtome at 5 μm , stained with haematoxylin and eosin and evaluated by light microscopy. [For further details see Alden *et al.* (1981) and Merski (1981).] Electron microscopy was carried out by Mr M. Myers of this laboratory.

All forms of unlabelled NTA were prepared from a commercial sample of $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ (Monsanto Co., Alvin, TX). H_3NTA was prepared by treating solutions of $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ with concentrated HCl which precipitates the H_3NTA . The divalent metal (M^{2+}) complexes of NTA were prepared by stirring equal molar amounts of the desired metal oxide and H_3NTA in distilled water for about 1 hr and then titrating to a pH of 7.4 with NaOH (NH_4OH was used for the Ca complex). The complexes were diluted to the desired concentrations with water. Each complex was assayed for its M^{2+} content to define the NTA complex concentration for infusion. The ZnNaNTA for feeding was prepared in the same way but was precipitated by addition of three volumes of 95% (v/v) ethanol and dried in a vacuum oven at 30°C . The [^{14}C]NTA used in all cases was labelled in the carboxyl groups of the acetate units. Periodic determinations of [^{14}C]NTA stability were made by isotope dilution with H_3NTA .

Results

The correlation between NTA-associated PCT toxicity and increased urinary Zn excretion suggested that ingestion of the Zn complex of NTA (ZnNaNTA) could result in increased NTA renal toxicity. To test this hypothesis, mature male rats were fed diets containing 0.5, 1.0, 1.5 or 2.0% ZnNaNTA for 3 wk. After 21 days of *ad lib.* feeding, the levels of Zn and NTA in a 24-hr urine collection were measured, and the kidney:body weight ratio was ascertained as an indicator of renal toxicity (Anderson & Kanerva, 1978a). The urinary NTA recovery was low and approximately constant at the three lowest ingestion rates but was increased at the highest intake rate (Fig. 1a). Urinary Zn displayed a linear increase with ingestion rate

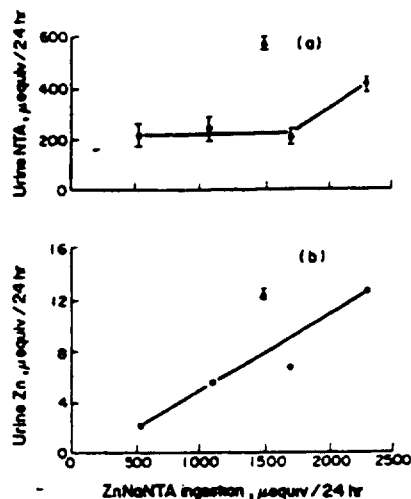


Fig. 1. Effect of ingestion of ZnNaNTA (●) at dietary levels of 0, 0.5, 1.0, 1.5 or 2.0% for 3 wk on (a) urinary NTA levels (mean $\pm$ SEM for five rats) and (b) urinary Zn levels (single analysis on pooled urine from five rats) in adult male rats. For comparison, the effect on the same parameters of $\text{Na}_2\text{NTA} \cdot \text{H}_2\text{O}$ given at 2.0% in the diet is also shown (Δ: mean $\pm$ SEM for five rats).

(Fig. 1b) but it did not approach the urinary NTA concentration. This clearly shows that the ZnNaNTA complex does not remain intact during passage through the body. The kidney:body weight ratio was not significantly influenced by any of the ZnNaNTA doses.

For comparative purposes the effect of the highest dietary level of NTA (2%) as its trisodium salt ($\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$) on the same parameters is included in Fig. 1. $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ ingestion resulted in about a three-fold greater urinary NTA load and a marked increase in kidney:body weight ratio (data not shown) and an even higher urinary Zn level at a much lower NTA intake level. This suggests that if NTA-induced renal toxicity is causally related to altered Zn metabolism the effects are due to NTA-associated alter-

ations in systemic Zn metabolism and are not a consequence of ingested ZnNaNTA absorption and excretion in the urine. This contention is supported by the results of a study of the effect of NTA on cation balance (Anderson & Kanerva, 1978a), which demonstrated that the increased urinary Zn excretion associated with NTA ingestion was compensated for by decreased faecal Zn excretion, so that NTA did not alter Zn balance compared to that of control rats.

Since increasing dietary Zn had the net effect of reducing NTA absorption, the renal toxicity associated with lowered dietary Zn at a constant dietary NTA level was ascertained. Male rats were fed semi-purified diets containing 1.2% $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ with either 52 (equivalent to chow), 21, 14, or 8 μg Zn/g diet. After 4 wk of ingestion of these diets *ad lib.*, plasma-Zn and urinary Zn and NTA levels were measured and the kidneys were evaluated histologically with emphasis on the specific NTA-associated histopathology (vacuoles and hyperplasia in the PCT). At the three lowest dietary Zn levels NTA administration resulted in a reduced plasma-Zn concentration which was overcome at the highest dietary Zn level (Fig. 2a). In contrast, at all dietary Zn levels, NTA ingestion resulted in an increase in urinary Zn excretion relative to control rats (Fig. 2b). This increase was dependent on dietary Zn concentration. The urinary NTA level was approximately constant at all four dietary Zn levels (Fig. 2b). Thus the aim of establishing approximately constant urinary NTA excretion with markedly different Zn excretion was achieved. In addition, these results show that when dietary Zn is limited, the increased urinary Zn excretion associated with NTA excretion results in decreased plasma-Zn levels.

Table 1 summarizes the results of the histopathology noted in the kidneys from the animals fed the two highest doses of Zn, expressed as the toxicity index, that is the product of incidence (kidneys having the specific lesion type/ten kidneys examined) and frequency (average number of the specific lesion/kidney section). The toxicity indices for the kidneys from rats fed the two lowest levels of Zn (8 and 14 ppm) are not included in Table 1 since in the 14 sections examined only vacuolated tubules and two tubules with

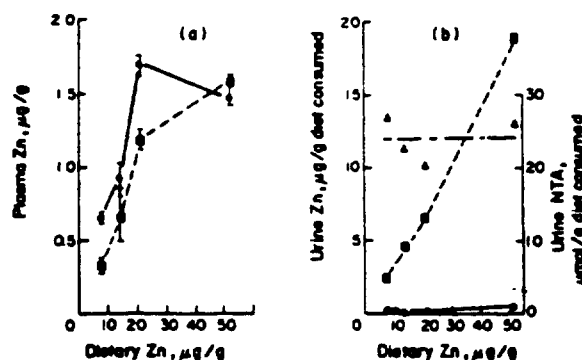


Fig. 2. Effects of different levels of dietary Zn (8, 14, 21 or 52 ppm) on (a) plasma Zn and (b) urine Zn in rats given either 0 (●—●) or 1.2% (Δ—Δ) $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ in the diet. Urinary NTA levels are also shown (Δ—Δ). Plasma values are means $\pm$ SEM for groups of five rats. The urine values were obtained on a pooled 24-hr urine sample from five rats per treatment.

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Table 1. Effect of dietary zinc concentration on renal tubular cell damage in male rats fed semi-purified diets containing $1.2\% \text{ Na}_2\text{NTA} \cdot \text{H}_2\text{O}$ for 4 wk

Tubular cell damage	Toxicity index* for rats given	
	21 ppm Zn	52 ppm Zn
Vacuolization and simple hyperplasia	15 (0)†	25 (0)
Vacuolization and nodular hyperplasia	3 (0)	50 (0)

*Toxicity index = incidence (number of kidney sections showing lesion/treatment group) $\times$ frequency (mean number of tubules showing lesion/kidney section). Ten kidneys were examined in each NTA-treated and control group.

†Values in brackets are for control rats given diets that did not contain $\text{Na}_2\text{NTA} \cdot \text{H}_2\text{O}$.

vacuoles and simple hyperplasia were noted. Thus low dietary levels of Zn resulted in minimal PCT toxicity. The zero incidence for the two types of lesions in the control groups confirms that the lesions noted are specific responses to NTA. The PCT toxicity index in the rats fed the diet containing 52 ppm Zn was greater for both stages of toxicity development than in the rats fed the diet containing 21 ppm Zn. The effect of dietary Zn on the PCT toxicity was particularly evident with respect to the more severe lesion (PCTs with vacuoles and nodular hyperplasia) for which the toxicity index in the group ingesting the diet with 21 ppm Zn was only three compared to a value of 50 for the group given 52 ppm Zn.

These feeding studies demonstrate that the NTA-specific toxicity in the renal PCT, assessed after comparable levels of NTA have been excreted in the urine, is proportional to the amount of Zn but independent of the amount of NTA that has been excreted in the urine. This suggests that Zn availability in the circulation is a limiting factor in NTA-induced PCT toxicity. This hypothesis was further tested by ascertaining the effect of Zn administered by iv injection (ZnSO_4 at 0.3 mmol/kg given 30 min after NTA) on the response of PCTs to an acute oral dose of NTA (7.3 mmol/kg of $\text{Na}_2\text{NTA} \cdot \text{H}_2\text{O}$ titrated to pH 8.5 with HCl). The number of PCTs containing vacuoles was markedly increased in the animals given iv Zn after the NTA dose compared to those only given the oral NTA (Plate 1a,b). No PCT vacuoles were noted in kidneys from rats given iv Zn but no NTA. Thus both the acute (PCT vacuoles) and the subchronic nephrotoxicity (PCTs with vacuoles and hyperplasia) associated with a constant NTA dose are proportional to available Zn in the circulation.

The fact that renal clearance of NTA is via filtration with no evidence of a tubular cell transport system for NTA (Licht *et al.* 1980), coupled with the established relationship of PCT toxicity to Zn availability, prompted a detailed examination of the effects of NTA on Zn disposition and excretion. The effects of a nephrotoxic (73 $\mu\text{mol/g}$ diet), a non-nephrotoxic (0.73 $\mu\text{mol/g}$ diet) and an intermediate (7.3 $\mu\text{mol/g}$ diet) dose of [^{14}C]NTA on Zn metabolism were com-

pared in rats equilibrated with [^{14}C]NTA by 10 days of *ad lib.* ingestion. [^{14}C]NTA and Zn levels were measured in the plasma UF and a 24-hr voided urine sample to determine if renal clearance of NTA resulted in a change in the NTA:Zn ratio in the urine relative to that in its precursor, plasma UF. A lower NTA:Zn ratio in the urine relative to that in the UF would show an NTA-induced Zn secretion, whereas a higher ratio in the urine than in the UF would show specific Zn resorption. For reference, in control rats the majority of the filtered Zn is resorbed by tubular tissue (Yunice, King, Kraikitpanitch, Haygood & Lindeman, 1978). The two lower doses of NTA resulted in similar UF_{Zn} levels but the highest NTA dose produced a five-fold increase in UF_{Zn} levels (Table 2). At all of the NTA doses, the NTA:Zn ratio in the urine (product) was increased over the ratio in the UF (precursor) showing net renal resorption of the Zn. A constant proportion of the filtered Zn was resorbed since in each instance the NTA:Zn ratio in the urine was 2–3 times that in the UF. The mass of Zn resorbed (estimated from the UF NTA:Zn ratio and the urine total NTA and Zn levels) was only 0.4 or 1.7 $\mu\text{mol/day}$ following ingestion of 0.73 or 7.3 $\mu\text{mol NTA/g}$ diet, respectively, but there was a twelve-fold increase in Zn resorption (11.9 $\mu\text{mol/day}$) at the nephrotoxic dose of NTA (73 $\mu\text{mol/g}$ diet). This suggests that PCT toxicity is likely to be a consequence of tubular cell Zn resorption.

To determine the dose of NTA required to increase UF_{Zn} , rats trained to consume their daily intake of diet in a 1-hr period were used to measure UF_{NTA} and UF_{Zn} levels at the time of maximum blood-NTA levels (4 hr after the meal). Figure 3 shows that NTA doses that resulted in $\text{UF}_{\text{NTA}} \leq 21 \mu\text{M}$ did not alter the UF_{Zn} level but that the higher UF_{NTA} level (52 μM) was accompanied by an increased UF_{Zn} level. Thus ingestion of high doses of NTA induces an increase in plasma UF_{Zn} concentration that is largely resorbed during passage through the kidney. Further, the plasma UF_{NTA} level must exceed a threshold of about 20 μM in order to induce an elevation in UF_{Zn} levels.

Table 2. Effect of dietary NTA concentration on plasma ultrafiltrate (UF) and urinary concentrations of NTA and zinc

Dietary NTA ($\mu\text{mol/g}$ diet)	Concentration (nequiv ml) of		Ratio NTA : Zn
	NTA	Zn	
Plasma UF			
0.73	1.41 ± 0.10	0.79 ± 0.45	2
7.3	11.9 ± 0.6	0.77 ± 0.18	15
73	119 ± 11	3.8 ± 0.70	31
Urine			
0.73	0.30 ± 0.04	0.06 ± 0.01	5
7.3	2.77 ± 0.39	0.07 ± 0.01	40
73	27.2 ± 3.8	0.25 ± 0.05	109

Each value for UF and urinary Zn and NTA is the mean $\pm$ SEM for groups of 12 rats (6 males and 6 females). Three rats of each sex were fed the indicated dietary concentration of either $\text{H}_2[^{14}\text{C}]\text{NTA}$ or $\text{Na}_2[^{14}\text{C}]\text{NTA} \cdot \text{H}_2\text{O}$ *ad lib.* for 10 days. The urine values are based on a total 24-hr urine collection and the UF samples were obtained between 8 and 10 a.m.

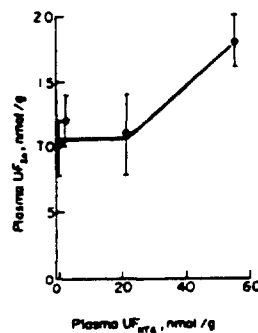


Fig. 3. Effect of plasma ultrafiltrate NTA levels (UF_{NTA}) on plasma ultrafiltrate Zn levels (UF_{Zn}) in rats ingesting their NTA dose in a 1-hr period. Blood was obtained 4 hr after the tenth NTA meal. Each value is the mean $\pm$ SEM for three samples.

In the experiments described, the total-plasma and plasma-UF concentrations of Ca and Mg were also measured. NTA ingestion did not alter total-plasma Ca or UF_{Ca} levels in any study but it did reduce total-plasma Mg and UF_{Mg} levels at the time of maximum levels of blood NTA (4 hr after the meal). The reduced Mg levels probably reflect changes in Mg absorption since NTA ingestion increases faecal Mg (Anderson & Kanerva, 1978a).

To examine further the role of Zn in the PCT toxicity associated with renal clearance of NTA, the effect of iv infusions of the forms of NTA most likely to be present in the plasma were compared. The iv dose chosen (6 mmol/kg/24 hr, except for ZnNaNTA) was equivalent to twice the systemic load of NTA in animals consuming diets containing 73 μ mol/g. Five forms of NTA were examined—the Zn, Ca and Mg complexes and the Na and K salts. The infusions with the two salts and the Ca and Mg complexes of NTA at 6 mmol/kg/24 hr for 3–7 days all induced some PCT vacuoles (Plate II). These infusion studies resulted in no more PCT toxicity than had been noted after 3 days of ingestion of a diet delivering approximately one-half the systemic dose of NTA (Plate III). In contrast, infusion of the ZnNaNTA at its limiting solubility of 3 mmol/kg/day was lethal to every animal in less than 48 hr. The animals displayed a very consistent response during ZnNaNTA infusion: for the 0–24-hr period the animals all voided urines containing glucose, protein and haemoglobin. At about 24 hr the animals ceased to urinate and soon became comatose. Kidneys removed after 48 hr of ZnNaNTA infusion at 3 mmol/kg/24 hr had a speckled surface and when sectioned showed a distinct white precipitate at the cortico-medullary junction. Histologically, the kidneys displayed massive renal coagulative necrosis and tubular cell sloughing. Electron-microscope evaluation of the material accumulated at the cortico-medullary junction showed sloughed tubular cells and intra- and intercellular crystalline material.

The recovery of [14 C]NTA in the urine following iv infusion of various [14 C]-labelled forms of NTA showed that with all NTA species examined, including ZnNaNTA, more than 90% of the infused

[14 C]NTA was recovered in the urine. The high and constant urinary recovery of NTA, regardless of NTA species infused, indicated that the nephrotoxicity induced by ZnNaNTA was a consequence of the Zn and not of increased retention of NTA. This contention was further investigated by comparing the renal toxicity of Zn salts and Zn ethylenediaminetetraacetic acid (ZnEDTA), a more stable complex than ZnNaNTA. When infused at 3 mmol/kg/day, the Zn salts produced the same *in vivo* response as ZnNaNTA but in a shorter time period, and also caused PCT necrosis. In contrast, ZnEDTA infused at 3 mmol/kg/day for 3 days produced only minimal renal damage (a few PCT vacuoles).

Comparison of urinary recovery of the Zn when given as an infusion of ZnNaNTA, ZnEDTA, Zn acetate or Zn gluconate (Fig. 4) showed that the renal toxicity induced was inversely proportional to the urinary recovery of the infused Zn and, therefore, proportional to the carcass retention of the infused Zn. The renal toxicity induced by iv Zn (necrosis and cell sloughing) was different from that associated with NTA ingestion (vacuoles and hyperplasia). However, when the iv dose of ZnNaNTA was reduced to c. 1 μ mol/rat/day, the PCT response was similar to that induced by dietary NTA (Plate IV).

In order to contrast the acute effects of ZnNaNTA infused iv with those of dietary NTA, the total-plasma, plasma UF and urinary [14 C]NTA:Zn ratios were determined after 72 hr of infusion with ZnNa[14 C]NTA. These parameters showed that at least after 48 hr of infusion the Zn and NTA were distributed differently in the blood pools than in the infusion solution (Table 3). The blood cells and plasma proteins contained more Zn than NTA. In contrast the plasma UF contained far more [14 C]NTA than Zn. This was accompanied by a urinary NTA:Zn ratio that was lower than the NTA:Zn ratio in the UF—a result opposite from that noted in rats fed NTA (Table 2). Thus ZnNaNTA infused iv dissociates in the circulation resulting in a marked elevation in plasma-protein and cellular Zn and Zn secretion into the urine during NTA clearance. This implies that the difference in renal lesions between ZnNaNTA infused iv and dietary NTA could be due to either or both of the following: (1) a difference in the route of Zn entry into the tubular cell—from

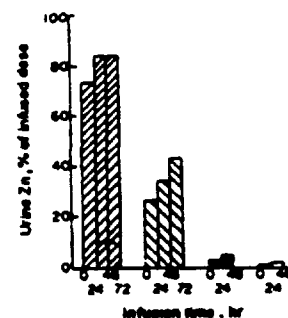


Fig. 4. Comparison of urinary recovery of Zn during iv infusion of ZnEDTA (▨), ZnNaNTA (■), Zn acetate (□) or Zn gluconate (◻) at levels to give 7.3, 7.6, 7.7 or 7.4 mg Zn/24 hr, respectively.

by 10 days levels were voided urine of NTA in the urine. A lower in the UF, whereas a would show control rats by tubular food & Lin-NTA resulted dose probable 2). At in the urine e UF (pre-Zn. A con-berd since urine was n resorbed d the urine r 1.7 μ mol/ol NTA/g ld increase ephrotoxic, that PCT ubular cell

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Table 3 Blood component and urine NTA and Zn levels after 48 hr of iv infusion of ZnNaNTA

Fluid or tissue	Conc (μM)		Ratio NTA:Zn
	NTA	Zn	
Rats given 4.2 μmol ZnNaNTA/hr			
Infusion fluid	7100	7000	1.01
Blood cells	3.2 ± 0.5	292 ± 25	0.011
Plasma	26 ± 3	159 ± 4	0.164
Plasma ultrafiltrate	19 ± 2	1.3 ± 0.7	14.6
Urine	2600 ± 300	1000 ± 200	2.5 ± 0.5
Rats given 12.6 μmol ZnNaNTA/hr			
Infusion fluid	21400	21000	1.02
Blood cells	20 ± 1	1159 ± 103	0.017
Plasma	85 ± 7	407 ± 20	0.209
Plasma ultrafiltrate	56 ± 5	7.4 ± 2.0	7.6
Urine	10200 ± 600	6400 ± 400	1.6 ± 0.1

Values are means $\pm$ SEM for groups of three male rats (c. 250 g) infused iv with the indicated dose of ZnNa[^{14}C]NTA for 48 hr. The infusion volume was c. 10 $\mu\text{l}/\text{min}$.

the circulation into tubular cells with infused ZnNaNTA and from the tubular lumen into PCT cells with dietary NTA; (2) differences in the Zn concentration obtained in the circulation—dietary NTA does not appreciably increase Zn levels in the total plasma but does shift the distribution from the plasma-protein pool (MW > 10,000) to the UF while iv ZnNaNTA causes a marked increase in Zn in the total-plasma pool, but most of the Zn is protein-bound. The fact that infusion of Zn salts produces a response similar to ZnNaNTA suggests that the renal tubular necrosis is associated with the increase in plasma-protein Zn levels since very little of the infused Zn from the salts enters the urine. Further, infusion of Zn gluconate results in a dose-dependent increase in renal-tissue Zn which is readily removed

by short-term infusion of saline after Zn gluconate infusion (Fig. 5).

The results with iv-infused Zn (both as the NTA complex and as salts) are indicative of acute heavy-metal toxicity (Hammond & Belites, 1980). This similarity was confirmed by showing that HgCl₂ infused iv (21 $\mu\text{mol}/\text{rat}$ for 4 hr) resulted in the same series of *in vivo* responses with iv Zn, and histological evaluation of the kidneys from these animals showed marked tubular cell necrosis (Plate V).

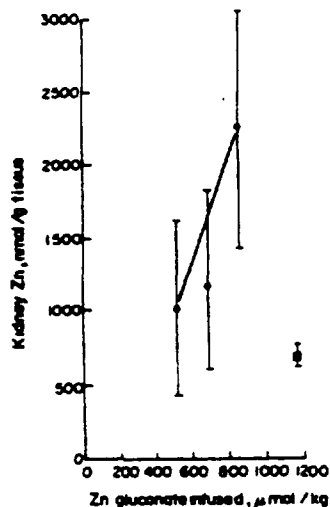


Fig. 5. Renal tissue Zn concentration as a function of the amount of Zn gluconate infused iv: with only Zn gluconate infusion (●); with Zn gluconate infusion followed by a 4-hr saline infusion (■). Values are means $\pm$ SEM for groups of three rats.

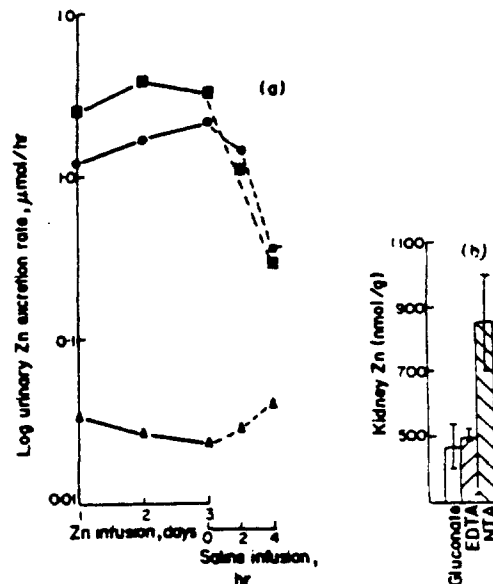


Fig. 6. (a) Urinary Zn excretion rate during 3 days of iv infusion (—) of Zn gluconate (▲), ZnNaNTA (●) or ZnEDTA (■) at c. 550 $\mu\text{mol}/\text{kg}/\text{day}$ and for two subsequent 2-hr periods of saline infusion (---). (b) Levels of Zn in kidney after 4 hr of saline infusion following Zn gluconate (□), ZnEDTA (■) or ZnNaNTA (●) infusion. Renal zinc values are means $\pm$ SEM for groups of two rats. Rats infused with ZnEDTA, ZnNaNTA and Zn gluconate excreted, respectively, 72, 37 and <1% of the infused zinc in their urine.

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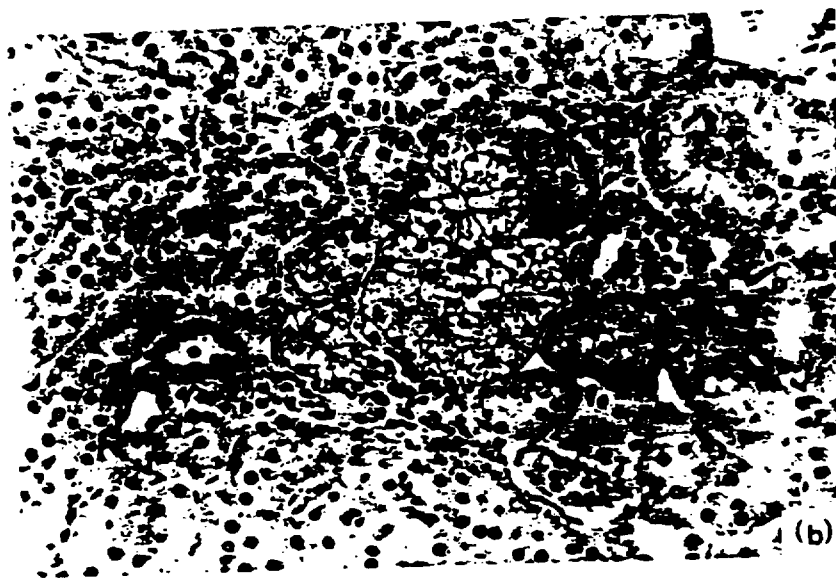


Plate 1. Photomicrographs of kidneys from rats 6 hr after a gavage of 7.3 mmol NTA/kg body weight: (a) kidney of a rat given iv 0.3 mmol ZnSO₄/kg body weight 30 min after the NTA gavage; (b) kidney of a rat given iv saline after oral NTA. Haematoxylin and eosin $\times 130$.

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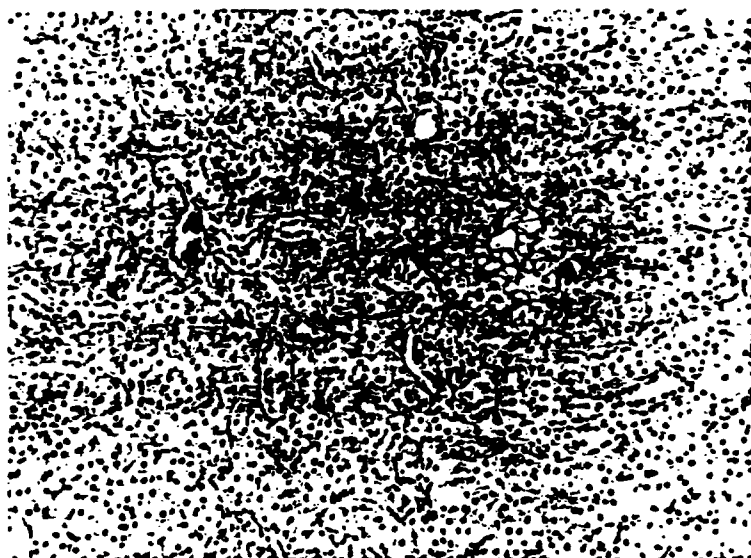


Plate II. Photomicrograph of the kidney of a rat infused for 72 hr with 6 mmol NTA/day. The photomicrograph is from an animal infused with MgNaNTA but similar effects were induced in the kidneys of animals infused with $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$, $\text{K}_3\text{NTA} \cdot \text{H}_2\text{O}$ or CaNaNTA at the same exposure rate. Haematoxylin and eosin $\times 70$.



Plate III. Kidney section from a rat fed a diet containing 2% $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ for 3 days. Note the focus of tubules with vacuoles and hyperplasia. Haematoxylin and eosin $\times 350$.

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Plate IV. Photomicrograph of the kidney from a rat infused with $2.25 \mu\text{mol ZnNaNTA/day}$ for 2 days. Note proximal convoluted tubule with vacuoles. Haematoxylin and eosin $\times 130$.



Plate V. Photomicrograph of a kidney section of a rat infused iv with $125 \mu\text{mol HgCl}_2/\text{kg}$ 24 hr for 4 hr. Haematoxylin and eosin $\times 70$.

Table 4. Kidney Zn and NTA concentrations after ZnNaNTA infusions

Parameter	Results following ZnNaNTA infusion at ($\mu\text{mol/kg/day}$)	
	400	1200
Kidney excess Zn*(mmol/g)	240 ± 21	1020 ± 65
Kidney NTA (mmol/g)	174 ± 22	588 ± 110
Kidney Zn:NTA	1.43 ± 0.26	1.81 ± 0.22
Urine Zn:NTA	0.33 ± 0.04	0.62 ± 0.04

*Kidney Zn in rats infused with ZnNaNTA minus kidney Zn in rats infused with saline.

Each value is the mean $\pm$ SEM for samples from three rats infused with the indicated level of ZnNaNTA for 3 days.

To further differentiate between the renal effects of ZnNaNTA, Zn gluconate and ZnEDTA, the renal Zn levels were determined after 3 days of iv infusion of each of these forms of Zn at c. 550 $\mu\text{mol/kg/day}$. To minimize tissue contamination with entrapped urine Zn, the animals were infused with saline for 4 hr before they were killed. Figure 6 shows that the Zn level in the kidneys from the rats infused with Zn gluconate and ZnEDTA were comparable even though the former excreted <1% of the infused Zn in their urine and the latter excreted >70%. In contrast, the kidney Zn level after infusion with ZnNaNTA was about 100% greater than that after infusion with Zn gluconate or ZnEDTA. This increased kidney Zn level could not be attributed to a higher urinary Zn concentration at the time of death compared with that of rats infused with ZnEDTA. Thus NTA results in a specific renal tissue Zn accumulation that is resistant to washout and that is not simply the result of either increased systemic Zn (as brought about by Zn gluconate) or increased urinary Zn excretion (as caused by ZnEDTA). To determine if the NTA-associated renal accumulation of Zn was due to ZnNaNTA accumulation, the excess kidney Zn (i.e. levels in ZnNaNTA-infused kidneys - levels in saline-infused kidneys) and [^{14}C]NTA concentrations were ascertained after 48 hr of iv infusion of ZnNa[^{14}C]NTA at 400 or 1200 $\mu\text{mol/kg/day}$. At both ZnNaNTA infusion rates there was a greater excess kidney Zn than [^{14}C]NTA (Table 4). Further, since the urine contained more NTA than Zn the increased tissue Zn cannot be attributed entirely to urine contamination. To place the tissue NTA levels in perspective the entrapment of $59 \pm 6 \mu\text{l}$ urine/g tissue could account for all of the kidney NTA at both doses but for only 70 and 400 nmol Zn/g tissue at the low and high doses, respectively. Thus not all of the renal tissue Zn that accumulates during NTA clearance is derived from ZnNaNTA. This becomes even more significant when it is recalled that in feeding studies it was shown that renal clearance of NTA was accompanied by a net Zn resorption.

Discussion

The results of the experiments reported show that the extent of damage induced in the renal PCT during clearance of constant amounts of NTA is directly proportional to the availability of Zn in the circulation. Both the induction of vacuoles in PCT cells and the

subsequent development of hyperplasia in these cells, at a constant level of NTA excretion, are proportional to the availability of Zn in the circulation. In addition, the results show that Zn infused iv is a renal toxin in the absence of NTA when it is retained in the carcass (Zn salts) but its toxicity is markedly reduced when it is excreted in the urine as a complex (ZnEDTA). In this sense Zn nephrotoxicity is similar to mercury nephrotoxicity—mercury salts are potent nephrotoxins but mercurial diuretics are not (for discussion see Zbinden, 1971).

Examination of the effects of NTA on Zn disposition shows that when the UF_{NTA} attains sufficient concentration (>20 μM) it increases the concentration of UF_{Zn} . During renal processing of the UF fraction a large portion of the Zn, but little, if any, of the NTA is resorbed. The increase in Zn resorption is a necessary condition for the initiation of PCT toxicity, and since the initial PCT toxicity is clearly a necessary but not sufficient condition for eventual renal tumorigenesis (Alden *et al.* 1980), doses of NTA that do not initiate the response will not increase the renal tumour incidence relative to that of controls. The demonstration of a distinct threshold for an NTA effect on PCT toxicity certainly negates the validity of any mathematical model for extrapolation of possible toxicity including tumorigenicity at a dose below this threshold from results noted at doses that clearly exceed this threshold. The results noted in chronic ingestion studies are in agreement with this model in that only doses of NTA that increase urinary Zn have resulted in any renal tubular cell lesions and only extreme doses have been associated with renal tubular cell neoplasia (National Cancer Institute, 1977; Nixon *et al.* 1972). In contrast, chronic exposure to NTA doses that do not alter dietary Zn disposition ($\leq 0.03\%$ $\text{Na}_2\text{NTA} \cdot \text{H}_2\text{O}$) do not induce any renal toxicity even after 24 months of ingestion (National Cancer Institute 1977; Nixon *et al.* 1972).

Finally, a calculation of the average exposure of man to NTA from drinking-water, based on measured values in Canada, where NTA has been used in detergents since 1970 (Malaiyandi, Williams & O'Grady, 1979), shows an average exposure of 0.1 $\mu\text{g/kg/day}$ ($35 \text{ ml } \text{H}_2\text{O/kg/day} \times 2.82 \mu\text{g NTA}/10^3 \text{ ml } \text{H}_2\text{O}$). If all of this daily ingested dose of NTA were in the plasma UF pool at one time it would amount to only 0.36 nequiv of NTA/kg or 0.009 μM (assuming 40 ml UF/kg), more than three orders of magnitude less than the plasma UF_{NTA} threshold of >20 μM which is

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necessary to increase the UF_{Zn} level and initiate PCT toxicity. In reality, man shows low absorption of ingested NTA (c. 12%) and rapid urinary clearance (Budny & Arnold, 1973) so that the ingestion of 0.1 $\mu\text{g}\cdot\text{kg}\cdot\text{day}$ would result in UF_{NTA} several orders of magnitude less than the UF_{NTA} threshold established in rats.

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