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Biodegradation of Metal-Nitrilotriacetate Complexes by a *Pseudomonas* Species: Mechanism of Reaction[1]

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A nitrilotriacetate (NTA)-degrading *Pseudomonas* species was shown to degrade Ca, Mn, Mg, Cu, Zn, Cd, Fe, and Na chelates of NTA at nearly equal rates when the appropriate metal concentrations are low enough to avoid toxicity from the freed metal. Ni-NTA, however, was not degraded. When higher concentrations of metal-NTA substrates were used, soil stimulated degradation of Cu, Zn, and Cd complexes, probably as a result of binding toxic freed metals. The metal associated with the NTA substrate does not appear to be transported into the cell, since metals do not accumulate in the cells and the presence of NTA reduces metal toxicity. The data are consistent with the hypothesis that an envelope-associated component, probably a transport protein involved in binding, is responsible for the disassociation of the metal from the NTA. Both soil and this NTA-degrading organism destabilize the metal-NTA complex, which suggests that in the natural environment both would act to limit mobilization of metals as soluble NTA chelates. Crude soluble enzyme preparations degrade Fe-, Mn-, and Na-NTA complexes but not Cu-NTA.

Nitrilotriacetic acid (NTA) is currently marketed as a synthetic organic chelant for a variety of industrial, agricultural, and detergent uses. NTA has now been shown to be biodegraded in a variety of sewage treatment, water, and soil environments. But questions have been raised concerning the extent of degradation of certain heavy metal-NTA chelates in these environments. In activated sludge systems, Gundernatsch (4) found Cu-NTA not to be degraded and Ni-NTA to be poorly degraded. Björndal et al. (1) found no degradation of Cu- and Cd-NTA and reduced degradation of Zn- and Ni-NTA in a mineral salts medium seeded with sewage sludge, but in an activated sludge treatment system they found Cu- and Cd-NTA, but not Ni-NTA, to be degraded. Walker (13) indicated that degradation was reduced or totally inhibited by Ni, Cr, Cu, and Cd in media inoculated with a sewage seed. Fe-NTA was found by several authors (1, 4, 9) to be rapidly and completely degraded. Swisher et al. (10) showed that NTA chelates of Fe, Pb, Cd, Ni, Cu, and Zn are degraded to some extent in river water. Tiedje and Mason (11) showed that NTA added to soil as chelates of Ca, Fe, Mn, Zn, Cu, Pb, or Ni is degraded at equally rapid rates, whereas added Hg and Cd complexes are degraded more slowly. In these soil studies, the more rapid degradation of the more toxic metal chelates is apparently due to substantial displacement of the added metal from NTA. Thus, the rate of NTA metabolism in an environment is a function not only of the biodegradability of a particular metal-NTA complex but also of the metal-NTA species present which are determined by the environment, i.e., the presence of other metals and complexing or precipitating agents and the stabilities of each.

The mechanism of metal displacement from NTA during the course of NTA degradation is not known, though two basic mechanisms have been proposed (1, 10). In one case only free NTA is attacked, with free NTA being continuously replenished by reequilibration at the expense of the remaining chelate. In the second mechanism the metal-NTA complex itself is attacked. Both mechanisms can be evaluated with regard to cell uptake and to dissimilation; however, understanding the former process (uptake) should be critical to assessing whether NTA facilitates transport of heavy metals into cells.

This work utilized a *Pseudomonas* sp. previously isolated from soil which readily metabolizes NTA (12). We report on the facility with which this organism can metabolize various metal-NTA chelates in buffered systems and in soil systems, and we suggest possible mech-

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anisms of attack by this species on the metal-NTA complex.

MATERIALS AND METHODS

Cultures. A *Pseudomonas* species isolated from soil and capable of complete and rapid metabolism of the NTA molecule (12) was used for all studies. The medium and preparative methods used for this organism have been described (12). The final cell preparations were washed and resuspended in either 0.02 M potassium phosphate buffer, pH 7.3, or 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.3.

For the enzyme activity study, the harvested cells from 8 liters of culture medium were resuspended in 15 ml of Tris buffer and broken by sonic treatment. The cellular debris was removed by centrifuging at 20,000 × *g* for 20 min. Since reduced nicotinamide adenine dinucleotide is required for cell-free metabolism of NTA (J. M. Tiedje, M. K. Firestone, B. B. Mason, and C. B. Warren, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, P183, p. 171), it was added to the enzyme assay vessels in molar quantities twice that of the NTA used.

Oxygen uptake. A portion of the oxygen uptake studies was done by using a refrigerated Warburg manometer (Aminco) held at 30 C. The flasks contained 0.2 ml of 20% KOH in the center well, 2.0 ml of the washed cell suspension (approximately 3 mg [dry weight]/ml) in the main reservoir, and 1.0 ml of metal, substrate, or metal-substrate mixture in the side arm. One gram of Brookston sandy loam soil was added where indicated.

For the enzyme activity study, the reduced nicotinamide adenine dinucleotide was placed in the side arm, whereas the metal-NTA chelate and 1 ml of the crude cell preparation were in the main reservoir. Hence, metabolism of NTA began with the addition of reduced nicotinamide adenine dinucleotide. This arrangement was required because of the presence of reduced nicotinamide adenine dinucleotide-oxidizing enzymes in the crude preparation other than the NTA-dependent reduced nicotinamide adenine dinucleotide oxidase. All oxygen uptake data from the Warburg have thermobarometer and endogenous values subtracted unless otherwise indicated.

An oxygen electrode (YSI model 53; Yellow Springs Instrument Co.) and a modified Beckman oxygen analyzer (model 777, Beckman Instruments, Inc.) connected to a recorder were used for studies with lower concentrations of metals and NTA. The system was calibrated by the method of Robinson and Cooper (7). The cell suspension (2.9 ml) at one-half the concentration of that used for Warburg studies was maintained at 30 C. An endogenous oxygen uptake rate was established for each sample prior to addition of 0.1 ml of the metal-NTA mixture. All rates given for oxygen uptake have endogenous values subtracted.

Substrate preparation. For all studies the sodium salt of the substrate (acetate, malate, or NTA) was used. The NTA was the trisodium salt monohydrate obtained from Aldrich Chemical Co. The metals used were as follows: $CuCl_2 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$, $FeCl_3 \cdot 6H_2O$, $ZnCl_2$, $CaCl_2 \cdot 2H_2O$, $CdCl_2$, $NiCl_2 \cdot 6H_2O$, and $HgCl_2$. The following preparation procedures were used for making the metal-NTA chelated substrates to avoid precipitation of the metal. For phosphate buffer experiments, the metal chloride and NTA were first dissolved in water to allow chelation, and then phosphate buffer was added to give the desired buffer molarity. The pH was adjusted to 7.3 with NaOH as necessary.

For Tris buffer experiments both the metal and NTA were dissolved in the buffer and then combined to give the desired metal-NTA ratio. The pH was adjusted to 7.3 with NaOH. For the oxygen electrode studies in Tris buffer, the metals were dissolved in 3 mM HCl and then combined with NTA in water. The chelate was diluted to the proper concentration in Tris buffer. Unless otherwise indicated, the metal and NTA were each 1 mM in the Warburg and atomic absorption studies and 0.02 mM in oxygen electrode studies. In all cases the NTA and metal were combined before addition to cells to insure chelation.

Metal determination. To determine the fate of the metals, the following procedures were used. Samples were incubated in Warburg flasks or in centrifuge tubes (17 by 100 mm) on a rotary shaker. Immediately after oxygen uptake was complete, the cells and/or soil were removed by centrifugation at 3,600 × *g* for 15 min. Samples with Fe additions were acidified with 2 drops of 1 N HCl and mixed well before centrifuging. Experiments to determine the fate of the metal were run in 0.05 M Tris buffer. In this buffer, Cu, Zn, and Mn remain soluble at a pH of 7.3, but Fe remains in solution only as the chelate of NTA. As the NTA was metabolized a $Fe(OH)_3$ precipitate formed. Acidifying the samples was sufficient to resolubilize the Fe before centrifuging. Controls were included for each metal to determine if precipitate was interfering with the metal determination; controls were metal alone in buffer, metal with live cells, metal with azide-treated cells, metal-NTA alone, and metal with a nonchelating substrate (malate or acetate). After centrifuging, the supernatant fluid was removed, and the cells were suspended in 2.5 or 3.0 ml of buffer (volume equal to supernatant fluid removed). In studies to determine possible iron excretion from the cell, the cells were removed by centrifuging when oxygen uptake was half completed. The cells were resuspended in 2.5 ml of buffer, incubated for 1 h, and then centrifuged again. The first and second supernatant fluids and the cells were analyzed for iron content.

Labeled NTA was used to determine the amount of NTA remaining in solution; 0.5 ml of supernatant fluid was added to 15 ml of Bray solution and assayed by liquid scintillating counting (12). The [^{14}C]COOH-labeled NTA was a gift from Procter and Gamble Co. The metal concentrations in the supernatant fluids and cells were determined by atomic absorption by using a Perkin-Elmer 303 atomic absorption spectrophotometer. Stability constants are reported as log K_1 for constants of the first metal under the conditions given and as log $K_{M'}$, as used by Warren (14), for the actual stability constant at biological pH.

RESULTS

Oxidation of metal-NTA chelates. Oxygen uptake, measured by Warburg manometry, was used to follow NTA metabolism in three systems: in phosphate buffer, in phosphate buffer plus 1 g of soil (Fig. 1), and in Tris buffer (Fig. 2). The capacity of soil to enhance microbial metabolism of the NTA chelates is illustrated in Fig. 1. In addition, no oxygen uptake was observed with Ni, Cd, Hg alone, or Hg plus soil (not shown in Fig. 1). Soil with cells was used as an endogenous control, and soil alone or with substrate produced no measurable oxygen uptake. It is apparent that the presence of soil enhanced oxygen uptake with Zn-, Cu-, Cd-, and Ni-NTA chelates, whereas the NTA added with Hg remained untouched. In addition to those metals shown in Fig. 1, Ca-, Fe-, and Mn-NTA chelates added with and without soil gave oxygen uptake curves almost identical to that shown for Na-NTA.

The data shown in Table 1 illustrate the effect of soil on the fate of the metal. Unchelated metal ions, particularly Cu, Fe, and Zn, were readily removed from solution by soil. When the metals were added as the NTA complex, a characteristic equilibrium was established for each metal under these experimental conditions in which the majority of the metal remained with NTA, except for Zn. When

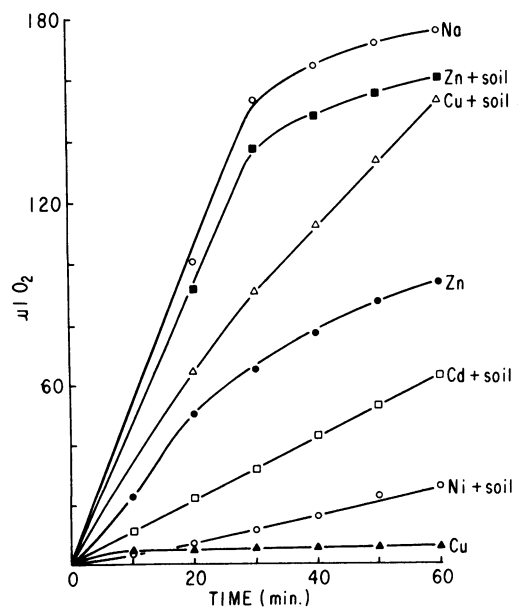

FIG. 1. *Oxygen uptake of cells incubated with metal-NTA chelates in phosphate buffer with and without soil.*

TABLE 1. *Metals in solution after incubation of metal or metal-NTA with soil or soil plus cells*

Metal	Additions to soil[a] (components in solution after 60 min)			
	Metal (µmol)	Metal + ^{14}C-labeled NTA		Metal + NTA + cells[b] (µmol of metal)
		Metal (µmol)	NTA (µmol)	
Cu	0.06	1.95	2.97	0.30
Fe	0.00	1.14	1.92	0.12
Mn	0.81	2.40	2.82	0.72
Zn	0.00	1.23	3.00	0.03

[a] Inoculation vessel contained 1 g of soil and 3 µmol of metal and NTA (where used) in 3 ml of 0.05 M Tris buffer.

[b] No NTA remaining after 60 min of incubation with cells.

cells were added to metabolize the NTA, the metals were removed from solution in quantities similar to that found for metal-only additions.

The capacity of the *Pseudomonas* cells to attack metal-NTA complexes is further illustrated in Fig. 2, which shows oxygen uptake in Tris buffer. In addition no oxygen uptake was observed for Cd and Hg. The inhibition shown by Cu, Zn, Ni, Cd, and Hg apparently results from metal toxicity. But it should be noted that in Tris buffer less inhibition is observed for Cu and Ni than in phosphate buffer (Fig. 1). Since Tris is a weak chelant, it could be lowering the toxicity of these metals. The stability constants (log K_1) for Cu-Tris and Ni-Tris chelates are 3.98 and 2.86, respectively (8). Under the same conditions (0.1 M KNO_3, 20 C), the log K_1 for Cu-NTA is 11.5, and for Ni-NTA, 11.5 (8). Despite the fact that the Tris is 50 mM compared to 1 mM NTA, the Tris should not effectively compete with NTA for these metals; thus, the reduced toxicity of Ni and Cu in Tris buffer over phosphate buffer could be explained by Tris chelation of metal that is freed as a result of NTA uptake.

If the inhibition observed for certain metal-NTA chelates in the Warburg study results from the metal toxicity rather than an inability to break the metal-NTA chelate, then lowering the concentration of the metal below the toxicity level should result in an increased ability to metabolize the metal-NTA chelate of these metals. By using an oxygen electrode to measure oxygen uptake, it was possible to lower the concentration of metal-NTA from 1.0 mM (Warburg studies) to 0.02 mM, a concentration at which the rate was substrate limited. A K_m of

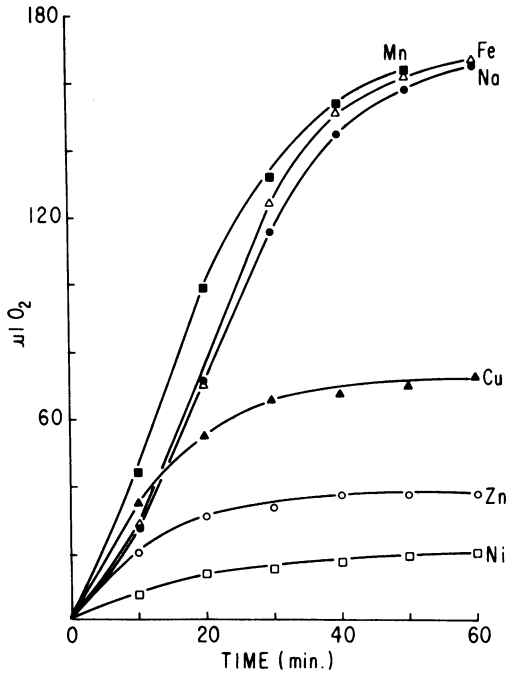

FIG. 2. *Oxygen uptake of cells incubated with metal-NTA chelates in Tris buffer.*

18.0 µM and a V_{max} of 0.21 µmol of O_2 per min (4.3 mg [dry weight] of cells) were determined for the rate-limiting step in NTA dissimilation. The inhibition associated with Cu, Cd, and Zn was essentially eliminated, whereas Ni remained inhibitory at this level (Table 2). The level of Cu, Zn, and Cd used was evidently very close to the toxic level since a second injection of 0.06 µmol of the same substrate showed much lower oxygen uptake rates than for the first addition. Further evidence for metal toxicity causing the inhibition of oxygen uptake was found from studies which showed that low levels of Cu (0.13 mM) totally inhibited malate and acetate oxidation.

The surprisingly low rate of metabolism of Fe-NTA is evidently not a result of toxicity. When 0.06 µmol of Na-NTA was injected into the Fe-NTA incubation 10 min after addition of Fe-NTA, the rate of oxygen uptake was identical to that previously observed for Na-NTA. This reduced rate of oxygen uptake for Fe-NTA with the oxygen electrode is not consistent with the rapid oxygen uptake shown for higher levels of Fe-NTA in the Warburg studies.

Incubation of ^{14}C-labeled NTA with cells under the same conditions as described for the oxygen electrode studies showed that $^{14}CO_2$ evolution for Cu-, Fe-, and Ni-NTA was 84, 55, and 8% of that for Na-NTA. Since previous work has shown that $^{14}CO_2$ evolution directly correlates with NTA disappearance and oxygen uptake (12), these data confirm the oxygen electrode finding that Ni-NTA, though not Fe-NTA, is resistant to degradation.

Fate of metal after metabolism of NTA. The data in Table 3 show the fate of Cu, Fe, and Mn after incubation of cells with 1.0 mM NTA or malate in Tris buffer for 60 min. Oxygen uptake data showed that NTA metabolism was complete for Fe and Mn, whereas about 22% of the NTA was metabolized when chelated with Cu. Malate metabolism was complete in 60 min in the presence of Fe and Mn, and no oxygen uptake was evident in the presence of Cu. Table 3 shows higher levels of Cu and Fe with malate-incubated cells than NTA-incubated cells, whereas similar Mn was found with NTA and

TABLE 2. *Rate of oxidation of metal-NTA chelates as measured by oxygen electrode*

Metal-NTA substrate	Oxygen uptake (µmol of O_2/min)		
	First injection	Second injection	Subsequent Na-NTA injection
Mg	0.14	0.14	
Mn	0.14	0.13	
Ca	0.12	0.12	
Na	0.13	0.11	
Cu	0.11	0.06	0.08
Cd	0.10	0.02	
Zn	0.08	0.03	
Fe	0.03		0.13
Ni	<0.01	<0.01	0.11

TABLE 3. *Position of metals after metabolism of substrates in the presence of equimolar quantities of metals*

Substrate	Cu (µmol)		Fe (µmol)		Mn (µmol)	
	With cells	In solution	With cells	In solution	With cells	In solution
NTA	0.2	2.6	0.8[a]	2.3	0.4	2.7
Malate	0.8[b]	1.8	2.0[a]	0.5	0.3	2.5
Malate + azide[c]	0.8[b]	2.0	1.5[a]	0.8	0.3	2.7

[a] Large quantities of Fe associated with cells appear to result from Fe insolubility or sorptivity to cells.

[b] Values are from 1.5 µmol of Cu data normalized to 3.0 µmol. Reduced levels of Cu were necessary in the malate incubation to reduce toxicity.

[c] Azide (500 µg/ml) was added to inhibit uptake.

malate-oxidizing cells. Thus, there seems to be no significant increase in metal found with cells metabolizing NTA as compared to cells metabolizing a nonchelating substrate. The quantities of metal found with the cells probably result from sorption to cells rather than from uptake, since cells incubated with the same substrates but inhibited with 500 μg of azide per ml showed similar quantities of metals with the cells after centrifugation.

It is possible that the metal is being taken into the cell during assimilation of NTA and subsequently excreted; hence, there would be no significant increase in the quantity of metal found with the NTA-metabolizing cell at the end of the incubation. However, cells that were interrupted midway during Fe-NTA metabolism showed no excretion of Fe when immediately resuspended and incubated in fresh buffer for 1 h.

NTA-dependent oxygen uptake by cell-free extract. The first observed step in the enzymatic breakdown of NTA results in the formation of iminodiacetic acid and glyoxylate; this reaction requires oxygen (J. M. Tiedje et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, P183, p. 171). The effect of Na-, Mn-, Fe-, and Cu-NTA substrates on the rate of oxygen uptake by a crude enzyme preparation is illustrated in Fig. 3. The enzyme responsible for the first step of degradation can attack the NTA in the Fe and Mn chelates, but appears to be inhibited by Cu. Destabilization could be caused by the NTA-degrading enzyme or by other components of the crude extract.

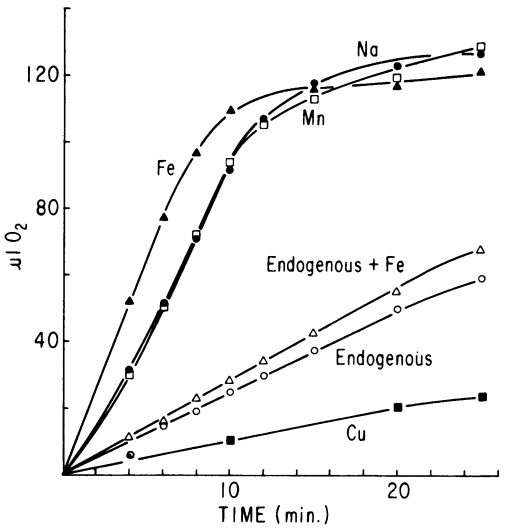

FIG. 3. *Oxygen uptake of crude enzyme preparation incubated with metal-NTA chelates.*

DISCUSSION

Atomic absorption studies showed that soil is an effective competitor for the metals from Cu-, Fe-, Mn-, and Zn-NTA chelates. Soil binding of metals is well known and has been reported to be due to a cation exchange mechanism, a specific adsorption process (covalent bonding), or a soil-mediated precipitation, depending on the metal (3). Hence, it would be expected that in soil metal binding would reduce toxicity of heavy metals as well as create an equilibrium condition in which the dominant NTA chelate would be either Ca or Fe. This interpretation is consistent with our previous soil and metal-NTA studies (11). In the present study, however, NTA-degrading cells were found capable of degrading most heavy metal chelates in the absence of soil, but in the presence of soil higher concentrations could be metabolized probably because the soil served as a "sink" to remove the freed toxic ions.

It is evident that the *Pseudomonas* species used in this study can metabolize most metal-NTA chelates and liberate the metal. For the nontoxic metals, Ca, Mn, and Fe, the rate of metabolism is essentially the same as for Na-NTA in Warburg studies. In oxygen electrode studies with the concentration of metal per cell mass reduced by a factor of 25, Mg, Mn, Ca, Cu, Cd, and Zn chelates are readily metabolized, but the Ni-NTA complex is not attacked. Hence, the metal-NTA chelate is readily degraded if the concentration of metal is low enough to avoid toxicity of the freed metal. Toxicity apparently was not the mechanism preventing Ni-NTA degradation, since the oxidation of a subsequent Na-NTA addition was not inhibited by the presence of the Ni (Table 2).

In Warburg studies no metabolism of NTA was observed in the presence of Hg. The hydroxyl complex of Hg is stronger than the chelate (1); hence the Hg toxicity appears to be independent of any NTA effect.

Cu, Ni, Zn, and Cd can form strong tetradentate complexes with NTA at pH 7.0 with stability constants (log K_{M*}) between 7 and 11, whereas $Fe(OH)_2$, Ca, and Mn form weaker tridentate complexes with stability constants in the range of 2 to 6 (5, 8; M. G. Miles, Ph.D. thesis, Univ. of Leeds, Leeds, England, 1965). When the effects of toxicity were eliminated, the *Pseudomonas* sp. used in this study could readily metabolize the NTA involved in the stronger tetradentate chelates of Cu, Zn, and Cd as well as all the weaker tridentate complexes.

NTA could be taken into living cells either as a metal-bound species or as a non-metal-associated species (free NTA). If the former were the case, NTA would be facilitating transport of metals across membranes. For the *Pseudomonas* used in these studies, the results suggest that this mechanism is not used, since there was no increase in concentration of metal associated with the cells after metabolism of the NTA chelated with Cu, Fe, and Mn as compared to cells incubated with a nonchelating substrate. If the mechanism involved the transport of the metal across the membrane, one would also expect to find metal toxicity increased by NTA metabolism. This was clearly not the case, since lower levels of Cu were more inhibitory to malate and acetate oxidation than to NTA oxidation. It is impossible, however, to rule out experimentally rapid excretion or equilibration of the metal concentration in the cell with the surrounding medium. Even if this were the case, however, the cell would not be endangered.

If the substrate entering the cell is free NTA, then the following mechanisms of metal removal are feasible. First, the cell could be attacking only the free NTA existing in equilibrium with the metal-NTA. Second, when the NTA enters the cell, a part of the transport system could bind NTA, destabilizing the complex and releasing the metal outside of the cell. Third, the cell could secrete a compound of high chelating capacity which would destroy the metal-NTA complex, thus freeing the NTA for uptake. Bacterial secretion of strongly chelating hydroxamates (log K_1 = 25 to 32 for Fe) to facilitate iron uptake has been reported (6). But the mechanism proposed here would be the reverse, that is, the secretion of a strong chelant to facilitate NTA uptake and exclusion of the metal. Our results indicate that a secretion of a strong chelating agent by the cells was not occurring, since a slight decrease in Cu and Ni toxicity was observed as a result of the weak chelating capacity of Tris buffer. If the cells were secreting a chelant stronger than NTA, then Tris would be unable to compete and hence should have no effect on metal toxicity.

The first mechanism requires uptake or attack on only free NTA existing in equilibrium with the metal-NTA. The quantity of free NTA available for uptake then would be less than the total quantity of NTA present by a factor of between 10^2 and 10^{11}, depending on the metal with which it was complexed. For example, in the oxygen electrode experiment the concentration of NTA added was 0.02 mM. When only the sodium salt of NTA was present, the effective concentration of free NTA was close to 0.02 mM, whereas the effective concentration of free NTA in the Cu-NTA system was reduced by a factor of at least 10^5 (including competing Tris chelation). Yet the rates of metabolism under substrate-limited conditions were nearly identical, 0.13 and 0.11 μmol of O_2 per min, respectively, for Na- and Cu-NTA substrates. The very large reduction in the concentration of the free NTA in the case of Cu did not significantly change the rate of metabolism. This finding implies that the species involved in uptake is not the free NTA alone but the total quantity of NTA present, most of which exists as Cu-NTA chelate. However, it is not possible to conclusively rule out transport of only free NTA, since the transport mechanism may be saturated even at the very low concentrations of free NTA present in the Cu example.

Regardless of the form of the NTA substrate for uptake, it is likely that an NTA-binding protein is part of the transport system, since such proteins have been commonly demonstrated to be associated with uptake of polar substrates in *Pseudomonas* and because of the relatively high affinity of this organism for NTA, $K_m < 10^{-4}$ M. If such a binding protein is present, it is more feasible to assume that the specificity would dictate transport of only one or a limited number of forms of NTA and not the wide variety of tri- and tetradentate substrate complexes. It also follows that during NTA binding the previously associated metal could be destabilized and lost from the complex. It does not appear that the NTA-degrading enzyme could be responsible for the destabilization since both Cripps and Noble (2), working with a similar pseudomonad, and ourselves (J. M. Tiedje and M. K. Firestone, unpublished data), working with this organism, have found that the enzyme responsible for the first degradative step is in the soluble fraction and is not associated with the membrane fraction. Though a specific NTA-binding protein has not been directly demonstrated, it appears to be the most reasonable explanation for the mechanism of metal elimination from the chelate.

Our results show that both soil and microorganisms can destabilize metal-NTA chelates and suggest that in the natural environment both would act to limit mobilization of metals as soluble NTA chelates.

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