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Actinide and Lanthanum Toxicity Towards a *Citrobacter* sp.: Uptake of Lanthanum and a Strategy for the Biological Treatment of Liquid Wastes Containing Plutonium

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Recent attention has focussed on the discharge of actinide species into the environment as wastes from nuclear power and nuclear fuel reprocessing activities, in addition to the more traditional hazards posed by the discharge of native uranium in mining wastes. The chemistry of uranium, plutonium and other actinides is complex, especially in environmental situations (Bulman 1978; Allard and Rydberg 1983) and is complicated by, in some cases, the co-existence of a variety of oxidation states each displaying a different pattern of pH-dependent hydroxylation behaviour (Allard et al. 1980). Actinides form complexes with inorganic anionic species and also with organic species such as citrate; in the presence of the latter hydrolysis is suppressed and soluble complexes are formed (Bulman et al. 1987). Other factors further complicate solution chemistry, e.g. radiolytic oxidation of americium(III) and plutonium(IV) in saline solution has been suggested (Buppelman et al. 1986). Actinides complex strongly with humic and fulvic acids (Allard and Rydberg 1983; Bulman et al. 1987); humic substances can effect Pu(VI) reduction (Choppin and Allard 1985). Photochemical reduction of both PuO_2^{2+} and UO_2^{2+} is well-documented (Toth et al. 1980; Bell et al. 1983). The situation is complicated further if the actinide species are subject to rapid decay, since the nascent daughter elements will assume different chemical and solution behaviour from the parent nuclide. It is difficult to perform and interpret studies on potentially unstable actinides per se and this investigation employs a chemical model system of analogous metal species to study their toxicity towards a microorganism originally isolated from metal-polluted soil (see Macaskie and Dean 1984).

The model system employs uranium (uranyl, UO_2^{2+}) as a representative of the actinide (An)(VI) valency state; the solution behaviour of U and Pu is similar (Allard et al. 1980). The use of commercially available (depleted) $UO_2(NO_3)_2$ employs mainly ^{238}U, of decay pattern: $^{238}U \xrightarrow[\text{half life}]{4.5\times10^{9}\ \text{year}} {}^{234}Th \xrightarrow[\text{half life}]{24.1\ \text{day}} {}^{234}U \xrightarrow[\text{half life}]{2.5\times10^{5}\ \text{year}} {}^{230}Th$; the concentration of generated thorium would be negligible. Solutions of U(IV) are unstable and may oxidise spontaneously; the analogous (Allard et al. 1980) thorium (IV) was chosen as a stable representative of An(IV). Trivalent actinides resemble the stable lanthanides (Bulman 1978; Choppin 1980) with similar ionic radii and chemical behaviour; lanthanum (III) was chosen to represent the An(III) state.

This study formed a preliminary investigation to assess the comparative toxicity of the three valency states with the ultimate aim of predicting which forms of waste discharge would be the most amenable to biological detoxification. The microbiological component of this test system, a Citrobacter sp., has been extensively

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studied (Macaskie and Dean, 1984, 1989). The organism overproduces a phosphatase enzyme which, given a suitable phosphate donor molecule, liberates HPO_4^{2-} to precipitate with heavy metals as cell-bound $MHPO_4$. This may constitute a detoxification response (Macaskie and Dean 1984); one present aim is to assess the toxicity of actinide valency states anticipated to be present in environmental situations and process outflows. Not all microorganisms possess such potential detoxification mechanisms and information on the toxicity of these metal species was sought also by the parallel use of a phosphatase-deficient mutant. These studies indicate that the An(VI) state is by far the most toxic. These findings are discussed in terms of the likely speciation behaviour of the three metals. It is suggested that the An(III) forms are amenable to biological desolubilization; implications in the treatment of plutonium wastes are considered.

MATERIALS AND METHODS

The Citrobacter sp. was as described previously (Macaskie and Dean 1984). Mutant 1p4a was from laboratory stock with its identity confirmed using the API system of classsification. Cells were grown in inorganic salts medium (Macaskie and Dean 1984) with glycerol 2-phosphate as the non metal-precipitable phosphate source. For cultures not requiring metal addition buffering was by 100mM tris-HCl (pH 7) with glycerol as the routine carbon source at 2 g/l (22mM). For metal-supplemented cultures tris buffer was avoided due to observed metal precipitation. MOPS (3-(N-Morpholino)propanesulphonic acid-NaOH buffer (50mM, pH 7) provided suitable buffering with minimal metal complexation; citrate (10mM) was routinely added to maintain metal solubility (10mM trisodium citrate-citric acid, pH 7). The carbon source was either glycerol (22mM) or citrate; in the latter case the citrate concentration was increased to 20mM. Cells were maintained aerobically (30°C) by daily subculture in the appropriate medium. For toxicity trials the cells were grown (metal-unsupplemented) to the mid-logarithmic phase of growth (A_{600} of 0.3-0.4; Varian series 634 spectrophotometer; approx. $2\text{-}3\times10^8$ cells/ml) prior to metal addition to the required final concentration (30 ml final volume). The metals were of 'Analar' grade (B.D.H.) and were stored as 100mM stock solutions (light-protected in the case of U) and were self-sterile for the duration of the experiments. The salts employed were $UO_2(NO_3)_2.6H_2O$, $Th(NO_3)_4.6H_2O$ and $La(NO_3)_3.6H_2O$; control experiments established that no precipitation occurred in the growth or resuspension media. The latter (10mM citrate buffer, pH 7 or 4 as required, supplemented with 2mM glycerol 2-phosphate and metals at 5mM, with additional buffering provided by MOPS-NaOH or MES (2-Morpholino)ethanesulphonic acid-NaOH; 50mM to pH 7 or 4 respectively), was used for studies using 'resting' cells. Cultures were harvested by centrifugation at $2\text{-}3\times10^8$ cells/ml from metal-unsupplemented growing cultures, washed once in sterile isotonic saline (8.5g/l of NaCl) and shaken in the resuspension medium (20 h, 30°C). Uranium-challenged cells (both resting and growing) were light-protected throughout. Surviving cells were visualised by dilution plating onto metal-unsupplemented nutrient agar (Difco). Counts made immediately after metal introduction established that metal-shock per se did not reduce cell viability. For both parent and mutant strains phosphatase activity was determined in metal unsupplemented cultures as described previously (Macaskie et al. 1988). Phosphatase specific activity is defined as nmoles of product (here p-nitrophenol) liberated/min/mg bacterial protein. Cell disruption employed toluene where required (Macaskie et al. 1988). Cellular uptake of lanthanum was determined as described previously for U (Macaskie et al. 1988). La was estimated in spent resuspension media (with correction for adsorption to the glass of cell-free control flasks) using alizarin red S (Rinehart 1954). La uptake was expressed as percentage of the bacterial dry weight using parallel La-free resuspensions for dry weight determinations; no bacterial growth occurred during the resuspension period (4 h).

RESULTS AND DISCUSSION

Preliminary experiments confirmed the phosphatase deficiency of strain 1p4a (Table 1). This was reflected also in the ability of the two strains to accumulate La; after 4 h resuspension in La (5mM)-supplemented medium the uptake of La was 72% and 9% of the bacterial dry weight for the parent and 1p4a strains respectively. The choice of carbon source did not affect phosphatase activity (Table 1), which was conserved over the pH range 5.5-8 with 68% retention of activity (N14) at pH 4 (Fig 1). Disruption of the cells by toluenization did not visualise additional activity and it was concluded that strain 1p4a was phosphatase-deficient. However the low residual activity (Table 1) supported normal growth in metal-unsupplemented media containing glycerol 2-phosphate as the sole phosphorus source (Figs 2 and 3).

The results of the initial metal toxicity trials are shown in Figs 2 and 3. It is possible that apparent metal toxicity was interpretable as a physiological starvation for growth source phosphate, with HPO_4^{2-} liberated by enzymic cleavage being desolubilized as metal phosphate. However identical results (U-challenge) were obtained with the glycerol 2-phosphate concentration increased by 10mM (not shown). Growing cells expressed no toxicity towards lanthanum or thorium (concentrations as shown) in either medium (Fig 2). The high tendency of Th to form $Th(OH)_4$ at neutral pH was not evident as precipitation; the extremely strong actinide(IV) citrate complex formation (see below) probably suppressed hydroxylation. Uncomplexed Th may be present as anionic species at pH 7 (Table 2), having minimal affinity for cellular cation-interactive sites. In contrast to Th and La, uranium elicited a concentration-dependent growth inhibition of each strain essentially independently of the challenge medium, i.e. forcing the cells to use citrate as the growth carbon source did not enhance toxicity. This could be accounted for by greater complexing of the uranyl ion by the increased concentration of citrate in the latter; indeed U-toxicity was ameliorated completely by increasing the concentration of citrate to 50mM (not shown). The data of Fig 3 establishes that phosphatase activity does not confer resistance to uranium (although phosphatase-mediated uptake of uranium by strain N14 has been established previously; Macaskie et al. 1988). Indeed, strain 1p4a was the more resistant at 5mM U (Fig 3). The question of differential toxicity was addressed further, under conditions in which metal speciation changes might occur, with respect to both hydroxylation behaviour (Table 2) and metal-citrate complex formation (see below). For example in the absence of citrate effects the predominant form of An(VI) (here U(VI)) at neutral pH would be $UO_2(OH)^+$ and $(UO_2)_3(OH)_5^+$, whereas for An(III) the predominant species would be probably the trivalent cation and $La(OH)^{2+}$ (Table 2). Adjustment of the pH to 4 would tend to shift the uranyl ion towards the UO_2^{2+} form, while La would be predominantly La^{3+}. Cell growth did not occur at pH 4, although the cells retained substantial phosphatase activity (Fig 1). The effect of pH on metal toxicity was investigated using

Table 1 Phosphatase specific activities of strains N14 (parent) and 1p4a as employed in this study

Strain	Growth medium carbon source	Phosphatase specific activity (nmoles p-nitrophenol/min/mg protein) mean	standard error	no. expts.	Reference
N14	glycerol	326.9	+/- 3.4	3	Hambling et al 1987
N14	glycerol	235.7	+/- 19.0	20	Macaskie et al 1988
N14	citrate	292.5	+/- 18.6	3	This study
1p4a	glycerol	2.0	+/- 0.6	7	This study
1p4a	citrate	negligible		2	This study

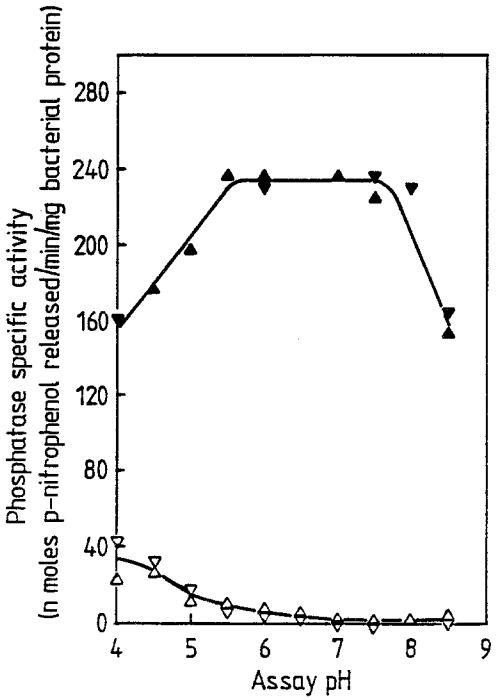

Figure 1. Phosphatase-pH activity profile for Citrobacter sp. strains N14 and 1p4a. Cells were grown as described, harvested and assayed for phosphatase activity in MES or MOPS buffers (pH 4-7 and 7-8.5 respectively). Closed symbols: N14. Open symbols: 1p4a. Data from two experiments.

resuspended 'resting' cells. Controls established no loss in viability throughout the 20 h experimental period for metal-unchallenged cells at either pH. The responses given by strains N14 and 1p4a were identical and the data were pooled to give the results shown in Table 3. Th and La were again non-toxic at pH 7; survival at 20 h was not significantly different from unchallenged cells. Uranium toxicity was pronounced, with a bacterial survival of four orders of magnitude less than with the other metals. Survival in U-medium at pH 4 was similarly low; presumably in both cases the toxicity effects are those of the stable uranyl-citrate complex. These studies suggest that actinide(VI) bio-treatment processes relying on growing cells may be subject to severe toxicity limitations and justify the use of non-growing (resting) biomass for U-accumulation (Macaskie et al. 1988). The plutonyl ion is much less stable than the analogous uranyl ion, readily undergoing photochemical (Bell et al. 1983) or chemical reduction to, in natural waters, predominantly Pu(IV) (Allard and Rydberg 1983). Despite the high insolubility of $An(OH)_4$ the tetravalent actinides may achieve solubilities exceeding the solubility product due to the formation of polymerized species (Allard and Rydberg 1983). At low pH and under reducing conditions trivalent Pu(III) forms (Allard and Rydberg 1983); this may also occur photochemically (as may the breakdown of polymeric Pu(IV)). In the test system employed here it has been shown previously that the Citrobacter sp. does not display U(VI) reductive capacity (L.E. Macaskie, unpublished). It was not possible to test the toxicity of Th at pH 4 due to observed Th precipitation (possibly due to a shift from anionic to $Th(OH)_4$ forms: ratio of metal to citrate was here 1:2: Tables 2 and 3). However at pH 4 the toxicity of La increased to a level comparable to that of U (Table 3). Glycerol 2-phosphate was assumed to play no significant part in La speciation since removal of this gave identical results

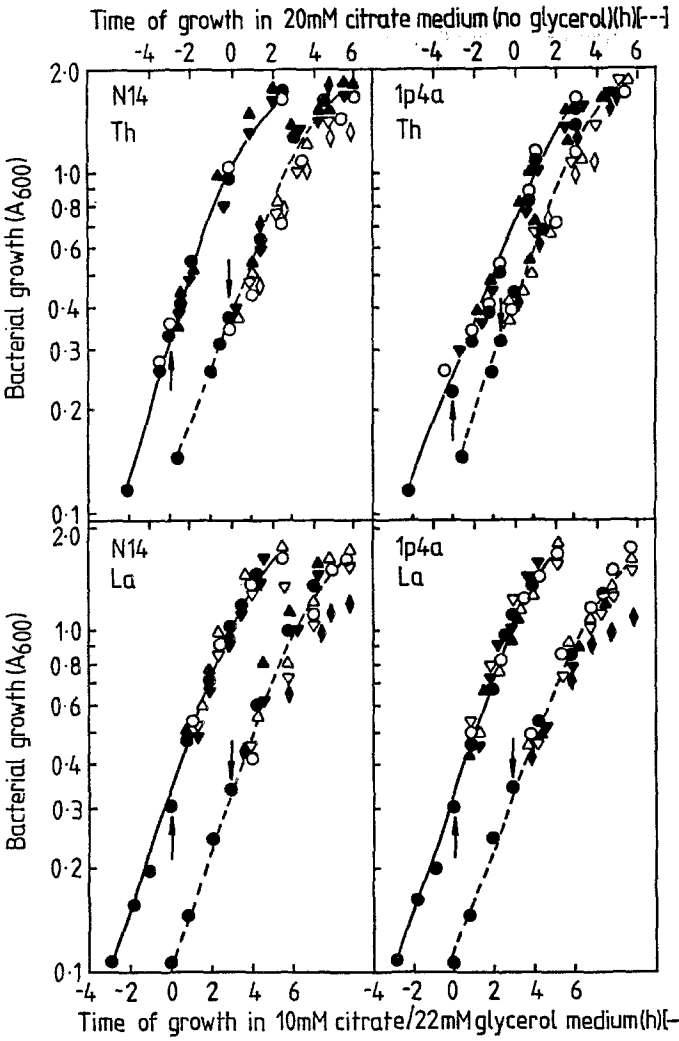

Figure 2. The effect of thorium and lanthanum on growth of the Citrobacter strains in two media (buffered with 50mM MOPS throughout). Solid lines: 10mM citrate/22mM glycerol/2mM glycerol 2-phosphate. Broken lines: 20mM citrate/glycerol-unsupplemented/2mM glycerol 2-phosphate. Metal concentrations 0-10mM. Details as in legend to Fig 3. Maximum concentrations tested in 10mM citrate medium were 5mM due to precipitation during growth.

(Table 3). Actinide(III) ions are generally difficult to study due to their instability in solution (although americium(III) is the most stable form: Allard 1981). Available data indicate that the citrate complexing capacity of U(III) is low (Udal'tsova 1970); indeed successful complexation of La(III) requires the use of stronger complexing agents (Ryabchikov and Ryabukhin 1970). Data are available for Pu(IV) and Pu(III) citrate complexes. The ratio of the stability constants for Pu(IV) and Pu(III) citrates is stated as 2×10^{16} (pH 5) and 5×10^{17} (pH 6) (see Milyukova et al. 1969). Clearly the tendency of An(III) to complex with citrate is low and in the present case it is suggested that the more antibacterial species is the La^{3+} cation since this non-hydroxylated form would predominate at pH 4 (Table 2) in the absence of substantial citrate complexation. A bacterial

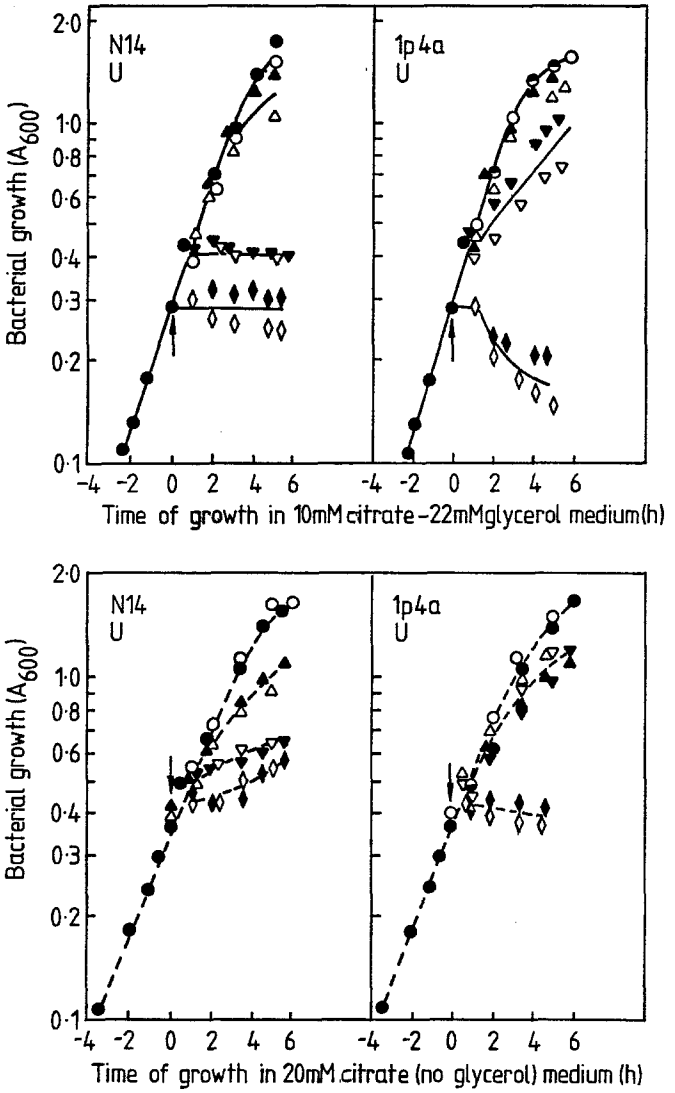

Figure 3. The effect of uranium on growth of the Citrobacter strains. Media details as in legend to Fig 2. The cells were grown (metal-unsupplemented) for 3 h to mid-logarithmic phase and metal was added at the points arrowed to final concentrations of: ● :0; ▲ : 1mM; ▼ :5mM and ◆ 10mM. Open and closed symbols denote independent experiments.

Table 3. Viable cell counts following metal exposure (5mM) for 20 h

Exposure conditions	Control	Uranium	Lanthanum	Thorium
pH 7 + glycerol 2-P	175+/-100(4)	0.017+/-0.003(3)	122+/-51(4)	217+/-110(4)
pH 4 + glycerol 2-P	80+/- 16(4)	0.014+/-0.012(4)	0.019+/-0.014(4)	-
pH 4, no glycerol 2-P	89+/- 11(4)	0.015+/-0.008(5)	0.030+/-0.014(5)	-

The data represent viable cells x 10^6/ml +/- standard error of the mean for the number of experiments in parentheses. Citrate concentration was 10mM. Additional buffering employed MOPS or MES buffers at 50mM.

Table 2. Expected species of uranium in aqueous solutions (Allard et al. 1980)

Valency	Aqueous species*						
	U^{3+}	$U(OH)^{2+}$	$U(OH)_2^{+}$	$[U(OH)_3]$	$U(OH)_4^{-}$		
U(III), pH 4	10^{-5}	10^{-8}	0	0			
U(III), pH 7	$10^{-5.5}$	$10^{-5.5}$	10^{-8}	$10^{-9.5}$	0		
	U^{4+}	$U(OH)_2^{2+}$	$U(OH)_3^{+}$	$[U(OH)_4]$	$U(OH)_5^{-}$		
U(IV), pH 4	0	10^{-9}	10^{-8}	10^{-8}	10^{-10}		
U(IV), pH 7	0	0	$10^{-10.5}$	10^{-8}	10^{-7}		
	UO_2^{2+}	$UO_2(OH)^{+}$	$(UO_2)_2(OH)_2^{2+}$	$(UO_2)_3(OH)_5^{+}$	$(UO_2)_3(OH)_4^{2+}$	$[UO_2(OH)_2]$	
U(VI), pH 4	10^{-5}	$10^{-6.5}$	10^{-8}	10^{-8}	10^{-11}	10^{-8}	
U(VI), pH 7	$10^{-8.5}$	10^{-7}	10^{-9}	10^{-6}	10^{-10}	$10^{-5.5}$	

* The values are the calculated concentrations (log., M) of dissolved uranium species in water. Insoluble species are shown in parentheses.

predisposition towards enhanced metal toxicity at acid pH is possible but this is not supported by the U-challenge experiments (Table 3).

In conclusion it is suggested that the optimal speciation of actinides to maximize their amenability to biological treatment with minimal toxicity may be as the An(III) form presented at neutral pH. Indeed, using La^{3+} as a stable model the uptake by strain N14 (pH 7: 4 h exposure) was 72 +/- 16% of the bacterial dry weight (mean +/- standard error; three experiments) which, calculated on a molar basis (moles metal accumulated/mg dry weight) is approximately twice that reported earlier for uranium (Macaskie et al. 1988). U(VI) toxicity towards the phosphatase enzyme responsible for metal uptake may be contributory; preliminary data have suggested that the uranyl ion, but not La^{3+} is to some extent inhibitory (L.E. Macaskie, unpublished). On the basis of this study a strategy for the future treatment of Pu wastes may be proposed, exploiting the instability and ease of photoreduction of the plutonyl ion as compared to the corresponding uranyl species, with Pu(IV) arising by disproportionation. It is suggested (Bell et al. 1983) that photochemical reduction of Pu^{4+} may be possible without the introduction of chemical reductants or the generation of chemical products. The disproportionation reaction of Pu(IV) is generally written as $3Pu^{4+} + 2H_2O \longrightarrow 2Pu^{3+} + PuO_2^{2+} + 4H^{+}$ (Bell et al. 1983); under UV illumination light is suggested to be converted into an e.m.f. with excitation of Pu(IV) and a shift towards its disproportionation. However the quantum efficiencies for Pu reductions using UV light are low (Toth et al. 1980); the reaction $U(VI) \longrightarrow U(IV)$ occurs more efficiently, using broad wavelength UV-visible light (Toth et al. 1980). U(IV) can be used as a Pu(IV) reductant according to: (Depoorter & Rofer-Depoorter 1980) $2Pu^{4+} + U^{4+} + 2H_2O \longrightarrow 2Pu^{3+} + UO_2^{2+} + 4H^{+}$. Clearly it would then be feasible to effect a chemical shift of Pu(IV) towards Pu(III) and the results of this study indicate the potential of this approach in Pu-waste treatment. However anions and other complexing agents can change the potentials sufficiently that U(IV) and Pu(IV) can coexist in solution; these studies are ongoing with trials against Pu anticipated in the near future.

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