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Role of Citrate as a Complexing Ligand Which Permits Enzymically-Mediated Uranyl Ion Bioaccumulation

P. Yong, L. E. Macaskie

School of Biological Sciences, The University of Birmingham, Birmingham B15 2TT, UK

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Microorganisms can be used to remove toxic heavy metals from liquid industrial wastes (Macaskie & Dean 1989). Traditional biotechnological waste treatments have utilized non-living biomass or have accumulated metals into living cells, with the associated problems of metal toxicity and a requirement for cell viability or growth (Macaskie 1990 1991; Volesky 1990; Gadd 1992). One potentially useful system utilizes the enzymically-mediated biomineralization of heavy metals at the surface of bacterial cells (Macaskie 1990). This well-documented system harnesses a metal-resistant phosphatase enzyme overproduced by a *Citrobacter* sp. originally isolated from metal-polluted soil; metal uptake is mediated by the activity of this enzyme, which persists in non-growing cells, to liberate HPO_4^{2-} from glycerol 2-phosphate with stoichiometric deposition of heavy metals (M) as cell-bound $MHPO_4$ (Macaskie 1990; Macaskie et al. 1992).

Recent attention has focused on the treatment of wastes from nuclear power and nuclear fuel reprocessing activities, together with discharges of native uranium in mining wastes. Previous investigations using the *Citrobacter* sp. demonstrated removal of uranium and the transuranic elements, plutonium and americium (Macaskie 1991; Tolley et al. 1993; Macaskie et al. 1994). It was shown that although uranium is inhibitory to the growth of the *Citrobacter* strain (Plummer and Macaskie 1990), and the activity of the cellular phosphatase (Tolley et al. 1992), uranyl phosphate accumulates as polycrystalline HUO_2PO_4 at the cell surface. This precipitated metal is indistinguishable from crystalline $HUO_2PO_4.4H_2O$ prepared by chemical methods (Macaskie et al. 1992). The rate of uranyl removal into the growing crystal is primarily dependent on the rate of phosphate release by the enzyme catalysed reaction. This is inconsistent with the reported toxicity of uranyl ion to the mediating phosphatase; however, in the presence of an excess of substrate, the rapid rate of phosphate release facilitated metal precipitation without toxic effect. Under substrate-limiting conditions uranyl toxicity was seen (Tolley et al. 1991). The present investigation shows that the inhibition of *Citrobacter* sp. phosphatase is related to the concentration of uranyl ion, and that citrate buffer can protect against this toxicity, and permit metal bioaccumulation. The toxicity pattern is dependent upon the substrate used; possible reasons for these effects, and environmental implications are discussed.

MATERIALS AND METHODS

The *Citrobacter* sp. was as described previously (Macaskie 1990). Minimal medium

Correspondence to: L. E. Macaskie

(MM) contained (g/L): Tris buffer, 12.0; $(NH_4)_2SO_4$, 0.96; glycerol 2-phosphate (G2P; disodium salt, BDH Ltd), 0.67; KCl, 0.62; $MgSO_4.7H_2O$, 0.063; $FeSO_4.7H_2O$, 0.00032; glycerol, 2.0 (pH to 7.0 with 2M HCl). Cultures for both immobilized cell preparations (see below) and free cell experiments were grown in 3 L batches (30°C) with forced aeration, inoculated using 100ml of overnight culture from cells pre-grown in MM. The cultures were harvested at stationary phase (16-24hr) by centrifugation and washed in isotonic saline (8.5g/L NaCl) twice. For free cell experiments, the cells were resuspended in isotonic saline (1/20 of the original volume). The concentrated cell suspensions were diluted to an OD_{600} of 0.3-0.6 (0.276 mg of bacterial protein/mL/unit of OD_{600}; Jeong 1992) into 20mM 3-(N-Morpholino) propanesulphonic acid (MOPS) (adjusted to pH 7 with NaOH), with or without uranyl nitrate and citrate buffer (pH 7.0) (concentrations as specified in individual experiments) and pre-equilibrated at 30°C for 1.5 hr. For routine assay, the substrate was p-nitrophenyl phosphate (PNPP: BDH Ltd., 0.4 ml of a 12mg/mL solution per 2mL assay) and the reaction was quenched (time as appropriate) by the addition of 0.2M NaOH (5mL). Product (p-nitrophenol: PNP) was estimated at A410nm (Perkin-Elmer spectrophotometer) versus PNP standards similarly prepared. One unit of phosphatase liberates 1 nmol product/min/mg bacterial protein. Cell-free controls established that metal precipitation did not occur in the metal buffer mixture, and discounted spontaneous hydrolysis of PNPP. Samples (taken before onset of uranyl phosphate precipitation) for assay for inorganic phosphate (Pi: 0-5mM) were diluted 30-fold. 0.6mL of H_2SO_4 (1.67M) containing sodium molybdate (2.5%, w/vol; final concentration) was added into each 1mL assay solution and phosphate was visualized by the addition of 0.4mL $SnCl_2$ (fresh solution; 0.25mL stock solution was diluted in 100mL of 1MHCl; stock was made by dissolving 1.5g $SnCl_2$ in 2.5mL concentrated HCl) with estimation of the blue complex at A720nm. For the determination of the rate of product (PNP or Pi) release, the reaction was initiated by the addition of PNPP or G2P (concentrations as specified in individual experiments). Timed samples were quenched in NaOH or H_2SO_4-MoO_4 solution as appropriate, and assayed for liberated PNP or Pi respectively (see above). Recovery of phosphatase activity following removal of uranyl ion was examined as follows. Cells were resuspended in 20mM MOPS buffer (20mL, pH 7, citrate free) with varying concentrations of uranyl nitrate (BDH Ltd.) (1.5 hr), then washed three times (7000 rpm; 15min) with isotonic saline (40mL) in the presence of 1mM citrate buffer (pH 7), prior to assay. In some experiments uranyl ion uptake and phosphate release were determined together. Trials were done using cells harvested as before and resuspended in solutions (20mL) containing 20mM MOPS buffer (pH 7) and 5mM G2P (phosphatase substrate and phosphate donor for metal uptake) with or without uranyl ion and citrate buffer (pH 7, 30°C) as described in individual experiments. Timed samples were chilled and centrifuged (12000 rpm, 3min), with storage of the supernatants in an ice bath before assay for released Pi and residual UO_2^{2+}. Solutions for uranium assay were diluted in 20mM MOPS buffer (pH 7) and assayed as follows. The stock uranium solution (calibrant), and the test solution were diluted 40-fold and acidified by the addition of 0.1mL of 0.75M HCl to 2mL assay solution. Uranium was visualized by the addition of 0.1mL of 0.15% (wt/vol; aq) arsenazo III (BDH Ltd.) with estimation of the pink complex at A652nm. For citrate utilization tests, the cells were incubated in the challenge mixture (1 or 2mM citrate) containing 20mM MOPS buffer (pH 7), 5mM G2P, and either 1mM $UO_2(NO_3)_2$ or 2mM $NaNO_3$ at 30°C. Timed samples were treated with NaH_2PO_4 (5mM final concentration) to precipitate residual UO_2^{2+}, and centrifuged. To each 2mL sample of supernatant was added 0.2mL of 1M $(NH_4)_2SO_4$ buffer (adjusted to pH 2.5 with 1M

H_2SO_4) and 0.2mL of 20mM $Fe_2(SO_4)_3$; the yellow iron citrate complex was estimated at 405nm using a citrate free sample as a reference. Some experiments utilized immobilized cells. Five grams wet weight of harvest (biomass) was immobilized in a polyacrylamide gel (PAG) and shredded as described previously (Macaskie et al. 1987). The shredded gel was divided into five equal parts by weight, washed with isotonic saline three times, packed into 5 replicate columns (constructed in the laboratory from adapted disposable universal bottles: vol = approx. 25mL) and challenged with a flow of composition citrate buffer (pH 7, 1 or 2mM), G2P; (5mM), and MOPS (pH 7, 20mM) supplemented with uranyl nitrate (1mM) at 30°C. Metal removal by the immobilized cells was determined by assay of the residual uranyl in the column outflow, as above, and comparison with the inflow solution. Column challenge solutions were prepared every few days from concentrated stock solutions.

RESULTS AND DISCUSSION

Previous work had demonstrated that uranyl ion is growth inhibitory while thorium and lanthanum are not detectably so, even at concentration as high as 10mM (Plummer and Macaskie 1990). The present study confirmed uranyl toxicity; this was concentration-dependent (Fig.1a) irrespective of the substrate used. No uranium was lost from the supernatant during the incubation. With 0.2 mM uranyl ion the retention of activity was 80% and 60% against PNPP and G2P respectively in the presence of 2mM citrate (Fig.1a). Uranyl ion toxicity is dependent on the concentration of citrate; reduction of the latter to zero resulted in an 80% loss of activity at 0.2 mM uranyl ion with almost complete loss of activity at 1mM UO_2^{2+} (Fig.1b). Uranyl ion toxicity is partially reversible; release of product was partially restored by washing with citrate. Approximately 20%-30% of the activity was not recovered; this was largely independent of the uranyl concentration (Fig.1b). The pattern of inhibition by uranyl ion was examined using Lineweaver-Burk plots. Fig. 2a shows that the apparent K_m is increased by uranyl ion (K_m=2.50mM and 3.33mM with 0.2mM and 0.4mM UO_2^{2+}, respectively, as compared to a K_m of 1.25 mM without uranyl ion using PNPP as the substrate). V_{max} is restored by increasing the concentration of PNPP. This suggests that the inhibitory effect is a competitive-type inhibition; possibly uranyl ion forms a complex with PNPP in the solution. Using G2P as a substrate (Fig.2b), the K_m is uranyl-independent while the Vmax is decreased by UO_2^{2+} and cannot be restored by addition of excess substrate. Possibly the uranyl ion here binds to the enzyme itself. This presupposes that the UO_2^{2+} binding to the G2P is less tight than to PNPP, and that the affinity of the enzyme for UO_2^{2+} is greater than that of the metal for G2P; with G2P there would also be more free UO_2^{2+} to bind to the enzyme. This would explain why activity was restored if PNPP was used; in contrast previous studies had shown that uranyl ion toxicity to the enzyme was irreversible if G2P was the substrate (Tolley 1993).

In contrast to the metal binding behavior of PNPP and G2P, the role of citrate as a complexing ligand is well documented (LgK_1=8.5; LgK_2=10.8, Dean 1985). The concentration of free UO_2^{2+}in citrate solution is low because of formation of the metal-citrate complex. Since free uranyl ions are toxic to the phosphatase, citrate buffer could confer protection by binding UO_2^{2+}; a protective effect was observed which was citrate concentration and substrate dependent (Fig.3). Citrate buffer alone did not affect product release in uranyl-unsupplemented solution (not shown). The toxicity of uranyl ion is dependent on the concentration of citrate. When PNPP was used as a substrate a

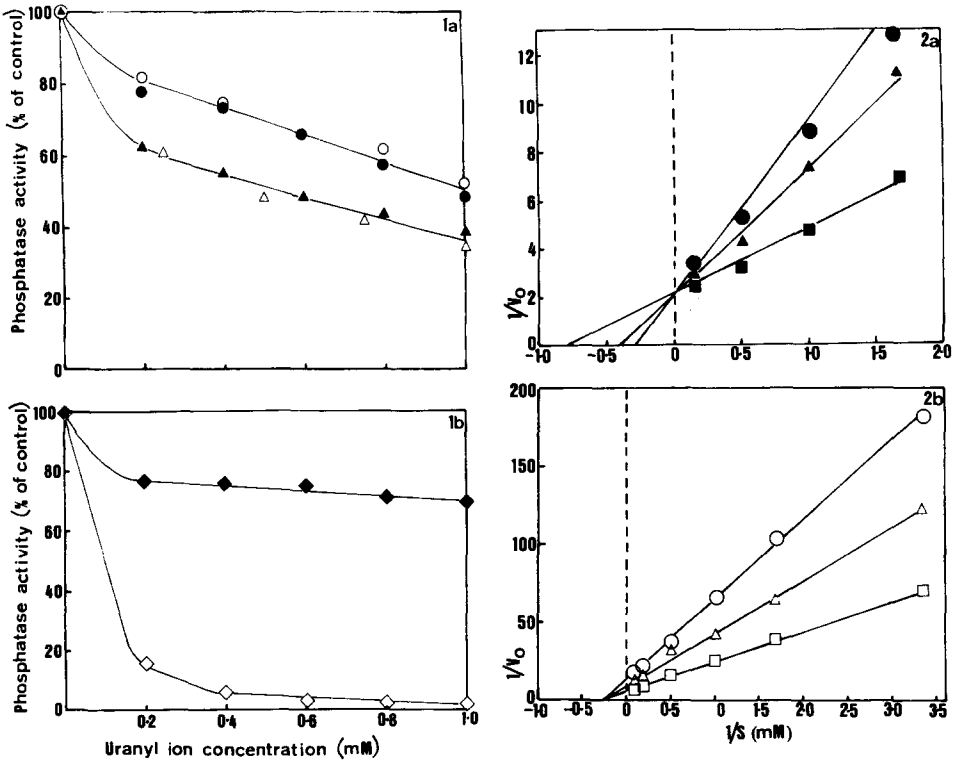

Figure 1a. The effect of uranyl ion on phosphatase activity. The cells were resuspended in 20mM MOPS/2mM citrate buffer, pH 7.0, and varying concentrations of uranyl ion (1.5hr). Cells were assayed for p-nitrophenol release from 6mM PNPP (●, ○) or phosphate release from 5mM G2P (▲,△) for two batches (filled and open symbols; the specific activity was 504 and 524 units for batches I and II, respectively.) Control: product release by uranyl--unsupplemented cells (expressed as 100%).

Figure 1b. Reversibility of uranyl ion inhibition. Cells were suspended in 20mL of 20mM MOPS buffer (pH 7; citrate free) with varying concentrations of uranyl ion, at 30°C for 1.5 hr, then washed with 40mL isotonic saline in the presence of 1mM citrate buffer (pH 7) three times. The concentration of uranyl ion in the final supernatant was negligible by assay. Cells were assayed for PNP release in the presence or absence of uranyl ion before and after washing (open and filled symbols, respectively). Control: PNP release by uranyl-unsupplemented cells (expressed as 100%).

Figure 2. Double reciprocal plots relating the initial rate of product release (V_O: μmol product/min/mg bacterial protein) to the substrate concentration (S) in the presence of uranyl ion. 2a. Inhibition of PNP release from PNPP; 2b. Inhibition of Pi release from G2P. The cells were resuspended in 20mM MOPS/1mM citrate buffer, pH 7.0, supplemented with varying concentrations of substrate without uranyl ion (■, □), or in the presence of uranyl ion at: ▲,△, 0.2mM; and ●, ○, 0.4mM.

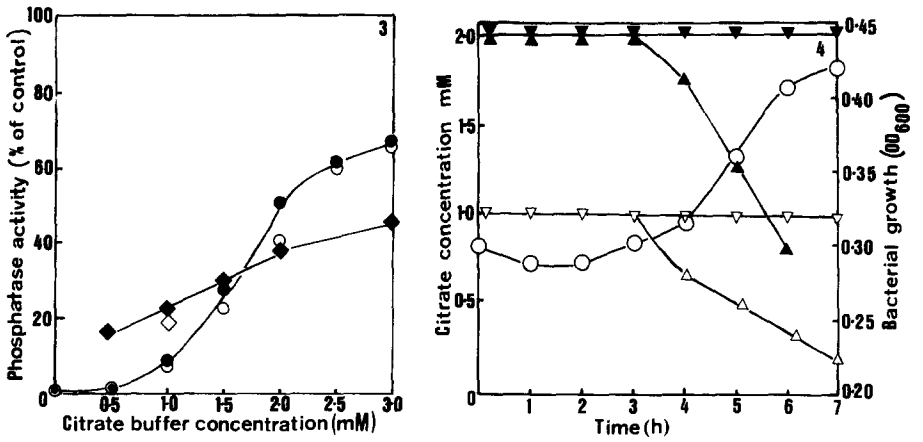

Figure 3. The effect of citrate on the phosphatase activity of cells challenged with 1mM uranyl ion. Cells were resuspended in 20mM MOPS buffer supplemented with 6mM PNPP or 5mM G2P, 1mM uranyl ion and varying concentrations of citrate (as shown). PNP and Pi release were also determined using uranyl-unchallenged cells as a control (expressed as 100%): ● , ○ , PNP release; ◆, ◇ , Pi release. Data are from two experiments; batches I (filled symbols) and II (open symbols) were as previously.

Figure 4. Cell growth and citrate utilization in the challenge solution containing either 1mM $UO_2(NO_3)_2$ or 2mM $NaNO_3$. Data are shown from representative batches of cells. ○, Cell growth in $NaNO_3$-supplemented solution (1 or 2mM citrate). Citrate removal from 2mM citrate solution supplemented with: ▲ , $NaNO_3$: ▼ , $UO_2(NO_3)_2$. Citrate removal from 1mM citrate solution supplemented with: △ , $NaNO_3$; ▽, $UO_2(NO_3)_2$.

sigmoidal effect was observed. This was not seen using G2P, where the response was linear (Fig.3). These data are consistent with the scheme proposed above. With G2P (proposed to play a minimal role in uranyl complexing) citrate and enzyme would compete directly for available UO_2^{2+}, and uranyl toxicity would be proportional to the citrate concentration, as seen. With PNPP the rate of reaction is uranyl concentration-dependent (Fig.2a) and strong complexing of PNPP to the uranyl ion can be postulated to account for both the recovery of the enzyme following substrate and uranyl removal (Fig.1b) and restoration of V_{max} with increased PNPP concentration. If it is assumed that citrate and PNPP compete directly for available UO_2^{2+} (enzyme inhibition per se is not a major factor here because the free UO_2^{2+} is very small) then the data of Fig.3 may confirm this hypothesis. At a low citrate concentration PNPP complexation to UO_2^{2+} is maximal and V_0 is low. At intermediate citrate concentrations the carboxylic acid takes the burden of the uranyl ion to liberate PNPP and increase V_0. At high citrate concentrations according to:

$$\text{Citrate} + UO_2^{2+} \rightleftharpoons \text{Citrate-}UO_2^{2+} \qquad LgK_1=8.5;$$

and

$$\text{Citrate-}UO_2^{2+} + UO_2^{2+} \rightleftharpoons \text{Citrate-}(UO_2^{2+})_2 \qquad LgK_2=10.8,$$

the available uranyl ion will be citrate-bound, restoring the saturating concentration of free PNPP and with this the V_{max}. This possible explanation is an oversimplification, however. Even at high concentrations of citrate only 70% protection was achieved (Fig.3; c.f. extensive washing with citrate recovered only 80% of the activity; Fig. 1b).

Furthermore the challenge concentration of UO_2^{2+} was only 1mM, representing a PNPP; uranyl ratio of 6:1. It is unlikely that all of the PNPP would be uranyl-bound. Interpretation of the data may be made difficult if microbial utilization of citrate occurred during the experiment; indeed, both growth and citrate utilization were seen during uranyl-unsupplemented challenge (Fig.4). However, the toxicity of uranyl ion prevented citrate utilization within the time scale of the experiment (Fig.4)

The formation of a metal citrate complex in solution reduces the free concentration of uranyl ion (see above) which, in turn, would retard the formation of uranyl phosphate precipitate at a given rate of phosphate release (solubility product for HUO_2PO_4 = $2.1x10^{11}$). Accordingly, uranyl ion removal by resuspended cells is citrate-dependent. Using G2P as the substrate, the most rapid onset was seen in the solution without citrate buffer; a delay was seen in citrate-supplemented solutions (Fig.5). Uranyl uptake by citrate-free suspensions was short-lived, probably attributable to the high concentration of free uranyl ion in the solution and resulting enzyme inhibition. However, here the precipitation of uranyl phosphate can occur efficiently in the presence of low concentrations of phosphate. Hence free uranyl can be removed rapidly; this, in turn, retards the onset of uranyl toxicity, permitting short-lived removal. The low-level of uranyl uptake from citrate-free solution was not attributable to depletion of the metal; 90% of the initial uranyl ion remained in solution after 7hr. In the solution with 1mM citrate buffer, the concentration of free uranyl ion would be reduced by citrate binding; metal precipitation was retarded but the concentration of remaining free uranyl ion was sufficient to inhibit phosphate release by the cells. With 2mM citrate buffer, the concentration of free uranyl ion was reduced efficiently by citrate binding and the toxicity of uranyl ion was reduced accordingly, such that the rate of phosphate release was increased, which facilitated and accelerated initiation of formation of uranyl phosphate. From Fig.5 it is apparent that the best situation for rapid uranyl uptake by resuspended cells is from citrate-free solution. However, a precipitate was seen in cell-free solution on standing. It is possible that the deposited uranyl was in the form of hydroxide on the cells; this was not tested. The data suggest that optimal uranyl uptake by free cells is from the solution with 2mM citrate buffer; here the precipitate is HUO_2PO_4 (Macaskie et al., 1992). The identity of the accumulated uranyl phosphate was confirmed by X-ray powder diffraction.

Uranyl removal by immobilized cells is shown in Fig.6. The efficiency of metal removal is dependent on both the cellular phosphatase activity and concentration of citrate buffer. However, the concentration-dependent effect of citrate was the converse to that obtained using resuspended cells. A flow rate of 1.16 and 1.54 ml/min for batch II, and 0.35 and 0.43 ml/min for batch III, supported the removal of 50% of the uranium from the flow ($FA_{1/2}$) in the solutions of 1 and 2 mM citrate respectively. The column efficiency for uranyl removal from the solution with 1mM citrate buffer was increased by 20% as compared to the solution with 2mM citrate buffer. It is possible that uranyl phosphate deposition protected the enzyme from damage in the heavily loaded cells of the columns more efficiently than in the suspensions of free cells. Other studies (Tolley et al. 1992; Tolley 1993; Macaskie et al. 1994) have suggested that metal phosphate deposition is accelerated in the presence of a previously-accumulated crystal of metal phosphate. This situation would occur in the immobilized cells where yellow deposits of uranyl phosphate were seen. In contrast, the free cells in suspension would lack 'pre-deposited' crystals as the incubation times were shorter.

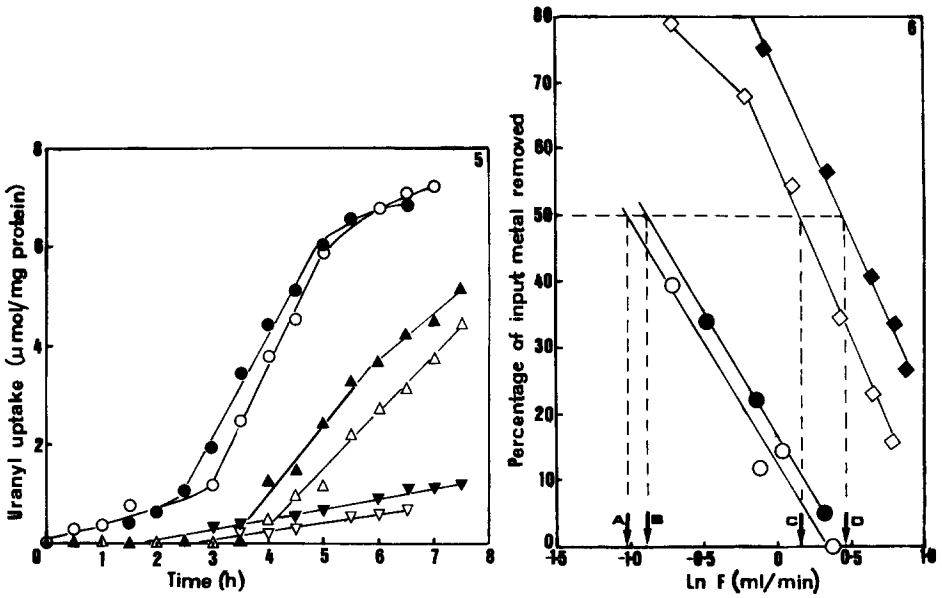

Figure 5. Uptake of uranyl ion by *Citrobacter* sp. Cells were resuspended in 5mM G2P, 20mM MOPS (pH 7), and 1mM uranyl ion in the absence of citrate buffer (●,○) or with citrate buffer at 1mM (▼,▽) or 2mM (▲,△). Data are from two independent experiments using two batches of cells; the specific activity of batches I (open symbols) and II (filled symbols) was as described in legend to Fig. 1.

Figure 6. Removal of uranyl ion by PAG-immobilized cells of *Citrobacter* sp. The cells were harvested and immobilized in PAG as described. The specific activity of batch II (◆,◇) at harvest was 524 units; that of batch III (●,○) was 380 units. The flow-through solution comprised 20mM MOPS buffer (pH 7), 5mM G2P and 1mM uranyl ion with citrate buffer (pH 7) at 1mM (filled symbols) or 2mM (open symbols). Uranyl ion removal was determined at varying flow rates; the $FA_{1/2}$value (arrowed) is that flow rate at which 50% of the input metal is removed. The data are from two experiments. The $FA_{1/2}$values were (mL/min): 0.35 (A); 0.43 (B) (batch III); 1.16 (C); 1.54 (D) (batch II). The variation in the $FA_{1/2}$ value with the phosphatase specific activity of the culture was as described previously (Tolley, 1993).

These studies, done in a chemically-defined system, have environmental implications. Complexing agents are well-known to modulate heavy metal toxicity, but it is shown here that the nature of the substrate utilized may also play a primary role in determining metal speciation, and the outcome of the challenge. Furthermore, in natural environments the majority of microorganisms exist as immobilized biofilms where an "open" (flow-past) and not a "closed" (batch) regime is the norm. Experiments described here, in addition to optimizing a bioprocess with application to waste decontamination, illustrate also the importance of "open" and "closed" regimes in the interpretation of toxicological and bioaccumulation data as applied to heavy metals.

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