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Tributyl Phosphate Degradation by Immobilized Cells of a *Citrobacter* sp.

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ABSTRACT

Tributyl phosphate (TBP), a plasticizer and solvent, is used in nuclear fuel reprocessing, generating TBP wastes laden with residual uranium. A *Citrobacter* sp. accumulated heavy metals via a phosphohydrolase(s) that precipitated metals with inorganic phosphate liberated from an organic phosphate "donor" molecule (TBP). Mutant analysis suggested that TBP hydrolysis was not attributable to a previously documented acid phosphatase (monoesterase). Purified monoesterase had little activity against phospho di- and triesters, had no requirement for Mg^{2+} or Mn^{2+}, and was EDTA-resistant. Conversely, TBP cleavage by immobilized cells was enhanced by Mg^{2+}, and ininhibited by Mn^{2+} and EDTA. A separate phosphotri/diesterase was implicated.

Index Entries: Phosphoesterase; tributyl phosphate; biodegradation; immobilized cells; *Citrobacter* sp.

INTRODUCTION

Tributyl phosphate (TBP), a solvent and plasticizer (*1*), finds special use in nuclear fuel reprocessing, where it is employed extensively in the extraction of uranium and plutonium from waste fission products (*2*).

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Selective reduction of Pu permits its recovery from the aqueous phase, with retention of U in the TBP solvent (*2*). The latter can be recycled following U removal via a carbonate "scrub" (*3*), but the ultimate disposal of TBP wastes laden with residual U remains an outstanding problem.

A *Citrobacter* sp. accumulates heavy metals via the activity of an atypical acid-type phosphatase that cleaves an organic phosphate "donor" molecule (substrate) to precipitate liberated inorganic phosphate with heavy metals as cell-bound $MHPO_4$ (*4*). A metal-accumulative bioprocess was developed in which cleavage of the phosphate monoester glycerol 2-phosphate supported removal of Cd and U as $CdHPO_4$ and UO_2HPO_4, respectively, from flows presented to immobilized cells held within a flow-through "bioreactor" (*4*). The phosphate donor requirement for Cd uptake was fulfilled also by TBP (*4,5*); cleavage of the latter to yield inorganic phosphate was strongly implicated, but these early studies did not address the underlying enzymology of the mechanism of phosphate release from the phosphate triester.

Very little information is available on the biodegradation of TBP, although organophosphate triester breakdown is relatively well documented in the context of nerve gas (*6*) or pesticide (*7*) biodegradation. Alkyl phosphates (either *per se* or as intermediates in the breakdown of other organophosphates [*6,7*]) are relatively nontoxic (e.g., LD_{50} for TBP for rats is 3 g kg^{-1} [*8*]), which is reflected in the paucity of available literature (reviewed in *9*). Dialkyl phosphates are stable and resistant to chemical hydrolysis (*10,11*), and their environmental accumulation might be anticipated, as degradation products from organophosphate triesters.

The chemical hydrolysis of TBP is generally held to follow the pattern:

$$\underset{\text{tributyl phosphate}}{(C_4H_9O)_3PO} + HOH \rightarrow \underset{\text{dibutyl phosphate}}{(C_4H_9O)_2PO(OH)} + n\text{-butanol} \quad (1)$$

$$\underset{\text{dibutyl phosphate}}{(C_4H_9O)_2PO(OH)} + HOH \rightarrow \underset{\text{monobutyl phosphate}}{(C_4H_9O)PO(OH)_2} + n\text{-butanol} \quad (2)$$

$$\underset{\text{monobutyl phosphate}}{(C_4H_9O)PO(OH)_2} + HOH \rightarrow \underset{\text{phosphoric acid}}{H_3PO_4} + n\text{-butanol} \quad (3)$$

The net yield of 1 mol of phosphate/mol of TBP would require three degradatory steps to support the removal of 1 mol of metal in an integrated process coupling TBP breakdown to U removal. Development of the latter formed one ultimate aim of this investigation.

The present study comprised a preliminary survey into the enzymology of TBP cleavage, with phosphate release as an overall criterion of activity. Phosphotriester cleavage generally occurs via the activity of appropriate triesterases (*6*). The initial step in TBP breakdown (eq 1) may, in fact, proceed spontaneously in solution (*13*); the substrate in vivo would be dibutyl phosphate (DBP), and early data (4) suggested that 2 mol of TBP or 1 mol of glycerol 2-phosphate gave an equivalent removal

of Cd^{2+} in accordance with this hypothesis and with the scheme for TBP breakdown.

Early studies on dialkyl (dimethyl) phosphate degradation by *Aerobacter aerogenes* (*14*) suggested the participation of a phosphohydrolase of phosphodiester and monoester specificity (*15*), but the metal sensitivity of this enzyme (*15*) precluded its use in the present context. A separate monoesterase of this strain was not active against dimethyl phosphate. An acid phosphatase of *Penicillium funiculosum* was reported to cleave both phosphomonoesters and diesters, primarily those with aryl leaving groups (*16*).

Bioprocess application of TBP degradation would be facilitated by an understanding of the kinetics of the degradatory enzyme(s) as operating in the immobilized cells. An important first step requires identification of the component enzyme(s) and the contribution made by each, with identification of the rate-limiting step. The present article discounts a primary role for the well-documented *Citrobacter* acid phosphatase in TBP breakdown, and suggests the participation of separate and distinct phosphotriester and/or diesterases.

METHODS

Microbial Strains

The parent strain, *Citrobacter* sp. N14, was as described previously (*4*). The phosphatase specific activity of batch cells of this strain (units: nmol product min^{-1} mg bacterial $protein^{-1}$) was 235± 19 (*17*). The phosphatase overproducing strain dc5c, selected by Cu-stress (*17*), had a phosphatase activity approx. threefold to excess over the parent strain (*17*). Details of individual batches used in the present investigation are given where appropriate. The phosphatase-deficient mutant M1 was obtained by mutagenesis using ethyl methane sulfonate (EMS). Mid-logarithmic phase cells (Luria broth: 10 g/L^{-1} of tryptone, 5 g/L^{-1} of yeast extract, and 10 g L^{-1} of NaCl; 2–3× 10^8 cells mL^{-1}) were harvested, resuspended in half the original volume, supplemented with Tris buffer (to 200 m*M;* pH 7.5), and treated with EMS at a vol:vol ratio of suspended cells to EMS of 67:1. The mixture was shaken (2 h) at 30°C, diluted into 20 mL of broth (EMS-free), and concentrated 10-fold by centrifugation and resuspension (2 mL). The survivors were plated at approx. 100 colonies $plate^{-1}$ onto Luria agar plates supplemented with the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP; disodium salt, Sigma; 0.6 mg $plate^{-1}$) to visualize white phosphatase-deficient mutants. The phosphatase deficiency of strain M1 was confirmed in liquid culture, with product release (*below*) against the test substrate *p*-nitrophenyl phosphate of 10.4± 2.8 nmol *p*-nitrophenol min^{-1}/mg $protein^{-1}$ (cf above; mean±

standard error; six experiments). This deficiency was confirmed by assay (M. R. Tolley and L. E. Macaskie, unpublished) using cell extracts obtained by mechanical disruption (French Pressure Cell; Sim Aminco: SLM Instruments Inc.).

Media and Growth Conditions

Standard minimal salts medium contained (g L^{-1}): Tris, 12.0; $(NH_4)_2SO_4$, 0.96; KCl, 0.62; $MgSO_4 \cdot 7H_2O$, 0.063; $FeSO_4 \cdot 7H_2O$, 0.00032. The respective carbon and phosphorus sources were glycerol (2 g L^{-1}) and glycerol 2-phosphate (disodium salt hydrate; B.D.H. Ltd., 0.67 g L^{-1}); the pH was adjusted to 7.0–7.1 with 2*M* HCl. Carbon-limiting medium for continuous culture was identical, except that the glycerol concentration was reduced to 0.6 g L^{-1}, and antifoam (polypropylene glycol) was incorporated at 1 mL 10 L^{-1} of medium. Cells were adapted to the minimal medium by several daily subcultures (aerobic, 30°C) prior to experiment. For immobilization, cells (3 L) were grown in batch culture to the late logarithmic-early stationary phase of growth (16 h; 30°C), with aeration provided by vigorous streams of sterile air. Following harvest by centrifugation, the cells were washed twice with isotonic saline (8.5 g L^{-1} of NaCl) and stored pelleted at 4°C until use (generally overnight). Phosphatase was purified from 70-L batches (4×17.5 L) with harvest in a continuous-flow centrifuge (Heraeus 17 RS) and saline wash as above. The pellets were stored at −20°C prior to use.

Continuous cultures (30°C) were maintained in a tower (air-lift) fermenter constructed in the laboratory according to published specifications (*18*). The culture was maintained under carbon limitation at D = 0.13 (0.25 μ_{max}), where μ_{max} corresponded to a doubling time of 1.5 h in carbon-sufficient medium at 30°C. Harvest of the lumen contents (*below*) was performed after 6 d of growth.

Preparation of Immobilized Cells

For biofilm growth, biomass support (polyurethane reticulated foam type "Firend" 20; Recticel Ltd. [*19*]) was incorporated as 1-cm cubes tied within the lumen of the tower fermenter (*19*). After 6 d of continuous growth (with discard of the outflow culture), the retained supports were withdrawn visibly coated with biofilm. The coated supports were washed gently in saline and stored at 4°C until use (up to several weeks). Whole cells from batch cultures were immobilized in polyacrylamide gel as described previously (*5*). The gel was shredded by passing through a stainless-steel sieve (*5*) to give extruded particles of cross-section c.1.5 mm. Each bulk shredded immobilization (5 g wet wt of cells) was divided into 1-g aliquots; each aliquot of shredded material contained approx 10–12 mg dry wt of cells (dry wt:wet wt ratio of 1:10; L. E. Macaskie, unpublished). It should be noted that this did not represent the total biomass available, since the substrate diffusional depth was not established. With

reticulated foam, the biomass loading per unit of foam area was similarly not determined. This is not relevant, since with thick biofilms the available biomass is related to the diffusional threshold of substrate into the film; this was similarly not determined. All experiments were therefore performed under standard conditions of biomass loading, gel preparation, and experimental protocol vs appropriate controls.

Purification of Phosphatase

All preparatory steps were performed at 4°C with routine incorporation of 10 m*M* 2-mercaptoethanol into purification buffers. The harvested cells were suspended in 20 m*M* MOPS (*N*-morpholino propane sulfonic acid)-NaOH buffer, pH 7.0, at a final concentration of 0.2 g wet wt of cells mL^{-1}. DNAase II (Sigma, type V) was added (1 mg to 10 g wet wt of cells), and the cells were disrupted by two passages through the French Pressure Cell (*above*). Cellular debris and unbroken cells were removed by centrifugation (18,000 rpm^{-1}; 1 h; Sorvall). The supernatant was then ultracentrifuged (40,000 rpm^{-1}; 2 h; Beckman ultracentrifuge). The high-speed supernatant was brought to 30% ammonium sulfate saturation and separated by centrifugation (15,000 rpm^{-1}; 20 min). The supernatant was raised to 60% $(NH_4)_2SO_4$ saturation; phosphatase activity was associated with the precipitate and retained. The sample was resuspended and desalted by dialysis overnight against 20 m*M* MES (*N*-morpholino-ethane sulfonic acid)-NaOH buffer, pH 6.0, and loaded onto a SP-Sephadex C-50 cation exchange column (2.8×28.5 cm) in the same buffer. The phosphatase, which appeared in the void volume, was concentrated via ultrafiltration (Amicon type CF 25 membrane), and dialyzed against 20 m*M* diethanolamine buffer, pH 9.4. The preparation was chromatographed on a QAE-Sephadex A-50 anion exchange column (2.8 cm×24.5 cm), equilibrated, and run in the diethanolamine buffer containing a 0–0.25*M* linear sodium chloride gradient. Fractions containing phosphatase activity were pooled and desalted by dialysis, and the buffer was changed to 20 m*M* potassium phosphate, pH 6.8, by ultrafiltration, as before. The sample was chromatographed on a hydroxyapatite column (2×9.5 cm), pre-equilibrated with 20 m*M* potassium phosphate buffer, pH 6.8, and run in the phosphate buffer in a 20–300 m*M* linear potassium phosphate gradient. Pooled phosphatase fractions were ultrafiltered, diluted in 20 m*M* MOPS-NaOH buffer, pH 7.0, supplemented to 25% ammonium sulfate, applied to a phenyl sepharose column (2×8 cm) run in the MOPS buffer, and eluted with a decreasing (25–0%) linear gradient of ammonium sulfate.

The purity of the purified enzyme was confirmed by the appearance of only one band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (*20*) using a stacking gel concentration of 2.5% and a resolving gel of 12.5%. Gels were stained with Coomassie Brilliant Blue R 250 (0.1%) dissolved in water:methanol: glacial acetic acid (5:5:2 by vol).

Protein Determination

Protein was assayed in whole cells by the Lowry method (*21*) and in enzyme preparations by the method of Bradford (22) with bovine serum albumin as standard.

Assay of Phosphatase Activity

Phosphomonoesterase activity was measured in whole cells by the liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate as described previously (*17*). Triesterase activity was measured by substitution of tris *p*-nitrophenyl phosphate (Sigma) to a final substrate concentration of 5 m*M*. Tris *p*-nitrophenyl phosphate is only sparingly water soluble. It was dissolved in dimethyl sulfoxide (DMSO) and diluted to the required final concentration in the assay. The resulting precipitate was removed by centrifugation prior to estimation of the liberated *p*-nitrophenol at A 410 m*M* vs *p*-nitrophenol standards similarly assayed. Routinely, phosphatase assays were stopped by the addition of 0.2*M* NaOH, which also intensified the yellow color of the *p*-nitrophenol. Tris *p*-nitrophenyl phosphate was hydrolyzed in alkali; for these assays, the reaction was stopped by reduction of the temperature to 4°C, and immediate removal of the cells and residual insoluble substrate by centrifugation. For cell extracts or purified enzyme, the sample (0.1 mL) was added to 0.9 mL of 0.2*M* MOPS-NaOH buffer, pH 7 (30°C). The reaction was initiated by the addition of 0.2 mL of *p*-nitro-phenyl phosphate (12 mg mL^{-1}, or as otherwise specified), and terminated by the addition of 2.5 mL of 0.2*M* NaOH. The liberated *p*-nitrophenol was estimated vs a *p*-nitrophenol standard with a molar extinction coefficient of 17,874 mol^{-1} cm^{-1} determined under assay conditions. It was confirmed that *p*-nitrophenol and inorganic phosphate were liberated stoichiometrically, by assay of inorganic phosphate (*23*). Enzyme activity towards glycerol 2-phosphate and alkyl phosphates was determined by assay of phosphate release. The triester TBP was only sparingly water soluble; the aqueous dispersion was shaken vigorously prior to use and during assay. Phosphate release assays were stopped using 2.5*M* H_2SO_4. It was confirmed that all substrates gave no reaction in the appropriate assay. One unit of enzyme activity is defined as that liberating 1 nmol of product min^{-1} mg of $protein^{-1}$.

Determination of Phosphate Release by Immobilized Cells

As with enzyme preparations, preliminary tests established stoichiometric liberation of *p*-nitrophenol and inorganic phosphate from *p*-nitrophenyl phosphate. Immobilized cells (1 g of gelled material or 3 biofilm-loaded reticulated foam cubes) were preequilibrated with gentle shaking in 27 mL of citrate buffer, pH 6.9. The reaction was initiated by the addition

of substrate (50 m*M*) to final concentrations of 2 m*M* and 5 m*M* of buffer and substrate, respectively; the final reaction vol was 30 mL. The incubations were shaken at 30°C. Samples (0.3 mL) of the perfusing mixture were withdrawn at intervals into 0.2*M* NaOH (for *p*-nitrophenyl phosphate) or 2.5*M* H_2SO_4 (for glycerol 2-phosphate or alkyl phosphate); product (*p*-nitrophenol or inorganic phosphate) was estimated as described above. Phosphoesterase activity was expressed as μmol of product liberated/g of gelled material or per 3 biofilm-loaded reticulated foam cubes (30-mL reaction vol).

Treatment of Data

The determination of very low concentrations of phosphate was performed in dedicated glassware that had been washed in "Analar" H_2SO_4 (2.5*M*) and rinsed extensively in deionized, glass-distilled water. All determinations were in triplicate. Any sample with a variation > 10% between triplicates was discarded. The background (negligible) level of phosphate was determined upon substrate addition. This was subtracted from the phosphate concentration in the timed samples. Generally all experiments were performed at least twice, with data expressed as means ± SEM, for experiments performed three or more times.

RESULTS

Phosphate Release from Glycerol 2-Phosphate and Tributyl Phosphate (TBP) by Polyacrylamide Gel- and Biofilm-Immobilized Cells

Although polyacrylamide gel (PAG)-immobilized cells are often employed as a useful model system, large-scale biotreatment processes often employ biofilms self-immobilized on inert supports (*24*). Biofilms of the *Citrobacter* sp. N14 were prepared on reticulated foam cubes (Methods). Preliminary experiments validated the use of PAG-immobilized cells as a model system, and established identical patterns of phosphate release from glycerol 2-phosphate and TBP in the biofilm and PAG-immobilization systems (Fig. 1). TBP was an inferior substrate. The respective rates of phosphate release from glycerol 2-phosphate and TBP were 24.3 ± 3.3 and 1.6 ± 0.5 μmol of phosphate h^{-1} g^{-1} of gelled material (means ± SEM; eight determinations using several batches of PAG-immobilized cells). Three hydrolytic cleavages would be required to liberate 1 mol of phosphate/mol of TBP (Introduction). In comparative terms, the rate of reaction against TBP was therefore increased from the above value to c. 20% of that against glycerol 2-phosphate. Accurate determination of the relative rates would require estimation of liberated butanol and intermediate reac-

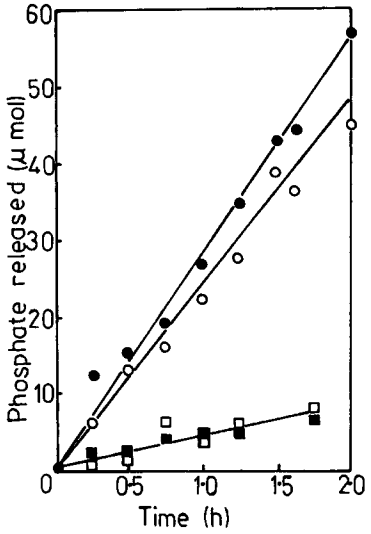

Fig. 1. Phosphate release from glycerol 2-phosphate (●, ○) and tributyl phosphate (■, □) by polyacrylamide gel-immobilized (filled symbols) and biofilm-immobilized (open symbols) cells of *Citrobacter* sp. N14. Data from gelled cells were from three experiments. The biofilm data were from a representative experiment.

tion products (dibutyl phosphate and monobutyl phosphate), which was outside the scope of this preliminary investigation. Furthermore, in terms of application to a metal bioaccumulative process, the efficiency of phosphate liberation would govern the ultimate efficiency of the process. The underlying enzymology of TBP biodegradation was investigated further.

Phosphomonoesterase and Phosphotriesterase Activities of Phosphatase-Overproducing Mutants

A technique has been described for the enrichment of phosphatase-overproducing mutants (*17*). A survey of the phosphoesterase activities of c. 200 survivors of such enrichments gave no correlation in the rate of product (*p*-nitrophenol) release from *p*-nitrophenyl phosphate (monoester) and tris *p*-nitrophenyl phosphate (triester) using free cells harvested from mid-logarithmic phase batch culture. This provided preliminary evidence for separate phosphomono- and triesterase activities, which was confirmed using PAG-immobilized cells of the phosphatase-overproducing (*17*) strain dc5c. Although this strain gave two- to threefold enhancement of phosphomonoesterase activity (*17*), degradation of TBP occurred at an identical rate to the parent strain, N14. These data were not conclusive, since uptake of TBP may have been the rate-limiting step for each strain; further confirmation was sought using a phosphomonoesterase-deficient mutant.

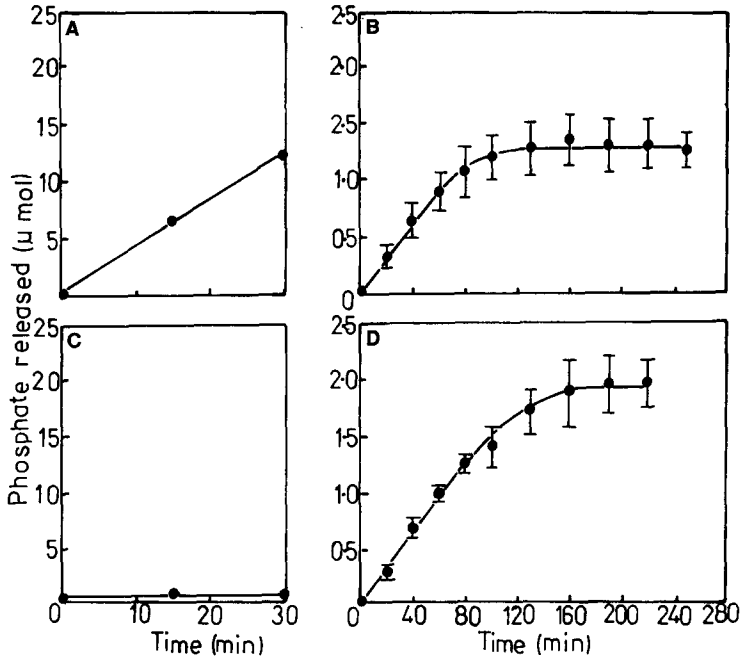

Fig. 2. Phosphate release from glycerol 2-phosphate (left) and tributyl phosphate (right) by *Citrobacter* sp. 2a,b: Phosphate release by the parent strain, N14. 2c,d: Phosphate release by the phosphomonoesterase-deficient mutant, M1. Phosphate release from glycerol 2-phosphate was a representative experiment; that from TBP was mean ± SEM from 13 and 5 experiments for strains N14 and M1, respectively.

Phosphomonoesterase and Phosphotriesterase Activities of a Phosphatase-Deficient Mutant

The phosphatase-deficient mutant M1 was obtained by mutagenesis using EMS (Methods). Little product release was observed from *p*-nitrophenyl phosphate (Methods) or from glycerol 2-phosphate (Fig. 2c), but the rate of phosphate release from TBP was identical for the parent and mutant strains (Fig. 2b,d). Phosphate release was not sustained, but ceased after 1.5–2 h, as with strain N14. The duration of activity and the extent of phosphate release were enhanced in the monoesterase-deficient strain (Fig. 2b,d). The cessation of activity was probably not attributable to substrate depletion. The total phosphate liberated (1–2 μmol/incubation) represented only about 5% of the available TBP, assuming a concentration of TBP in the aqueous phase of approx 1–1.5 m*M* (*12*). It was assumed that replenishment of TBP from the organic into the aqueous phase was not a rate-limiting step. Accordingly, sonicated substrate dispersion gave no enhancement of phosphate release, although no attempt was made to quantify the concentration of TBP in aqueous solution in the emulsions.

Stability of Phosphomonoesterase and Phosphotriesterase Activities under TBP Challenge

Inactivated PAG-immobilized cells were removed from the reaction vessel after 1.5 h, washed in isotonic saline, and rechallenged with TBP. No phosphate release was observed, confirming that substrate depletion was not responsible for the loss in activity. In addition, these experiments established that no spontaneous degradation of TBP occurred in the test system. Controls had previously shown that cell-free gels or TBP-unsupplemented PAG-immobilized cells yielded negligible inorganic phosphate (not shown).

Previous work using Cd accumulation as a measure of phosphate release from TBP by PAG-immobilized cells suggested a lability of the TBP-degrading enzyme(s) (*5*). This lability was confirmed in the present work (*above*) and provided further evidence for the participation of a separate enzyme in phosphotriester degradation. Tributyl phosphate-challenged PAG-immobilized cells (260-min samples; Fig. 2b) were withdrawn, washed in isotonic saline, stored for 1 wk at 4°C, and rechallenged with glycerol 2-phosphate or TBP. No phosphotriesterase activity was observed, although the monoesterase activity was very similar to freshly harvested cells.

Substrate Specificity of Citrobacter Phosphatase

The above studies would attribute the phosphomono- and triesterase activities to separate and distinct enzymes. This was tested by examination of the substrate specificity of the purified phosphomonoesterase in vitro (Table 1). Phosphate monoesters were cleaved extensively. It is noteworthy that the rate of phosphate release from glycerol 2-phosphate was 30% of the rate of appearance of the chromophore product *p*-nitrophenol from *p*-nitrophenyl phosphate. In the latter, phosphate and chromophore moieties were liberated stoichiometrically. A similar relationship was observed using biofilm-immobilized whole cells (not shown), and tends to validate comparisons between the immobilized cell and in vitro assay systems. The purified enzyme had negligible activity against phosphodiester or triester substrates (Table 1). Quantitative data were questionable using the triester substrates tris *p*-nitrophenyl phosphate and TBP owing to their low water solubility, but cleavage of the soluble analog triethyl phosphate was similarly negligible (Table 1).

The Role of Phosphodiesterase in TBP Degradation

Biodegradation of TBP was proposed to proceed through sequential hydrolyses via the intermediate dibutyl phosphate (DBP; *see* Introduc-

Table 1
Substrate Specificity of Purified *Citrobacter* Phosphatase[a]

	Substrate	Activity, %
Phosphate monoesters	*p*-Nitrophenyl phosphate	100
	BCIP	64.64±3.12
	Glycerol 1-phosphate	88.81±3.47
	Glycerol 2-phosphate	29.95±2.92
Phosphate diesters	bis *P*-Nitrophenyl phosphate	0.87±0.17
	Dibutyl phosphate	0.81±0.33
	Diethyl phosphate	0.94±0.28
Phosphate triesters	tris *p*-Nitrophenyl phosphate	0.37±0.23
	Tributyl phosphate	0.48±0.21
	Triethyl phosphate	0.28±0.24

[a]Phosphatase was purified to give a single band on SDS-PAGE. The enzyme was assayed by estimation of liberated inorganic phosphate. All substrates were tested at 1 m*M* concentration. Enzyme activity was expressed as % of the activity against *p*-nitrophenyl phosphate; data are the means ± SEM from four experiments.

tion). Preliminary experiments using PAG-immobilized cells gave no phosphate release from DBP, but in view of earlier success in identification of a phosphodiesterase active against a dialkyl phosphate (dimethyl phosphate) in the related *Aerobacter aerogenes* (*14*), the participation of a phosphodiesterase in the present system was investigated further. A *Citrobacter* phosphodiesterase was identified in disrupted cells that catalyzed the release of *p*-nitrophenol from bis *p*-nitrophenyl phosphate at a rate of c. 260 nmol of product (*p*-nitrophenol) min^{-1} mg $protein^{-1}$. This enzyme was highly labile, with a half-life of only approx 2 h in the native extract; mercaptoethanol conferred diesterase stability. Incorporation of Mn^{2+} ions stimulated diesterase activity, but conversely, Mn^{2+} inhibited TBP degradation (*below*). It was inferred that the diesterase was not involved in alkyl phosphate breakdown. This was confirmed by competition experiments. Incorporation of DBP into the Mn^{2+}-supplemented reaction mixture with bis *p*-nitrophenyl phosphate at a ratio of DBP to diester substrate of 7:1 did not affect the rate of product release from the latter, suggesting little affinity of the enzyme for DBP.

Further Studies on TBP Biodegradation

Previous studies using whole cells suggested that monoesterase activity was unaffected by added Mg^{2+}, Mn^{2+}, or EDTA (*25*), which was confirmed in the present study using purified enzyme (Table 2). In contrast, incorporation of 5 m*M* Mg^{2+} into the reaction mixture, although not increasing the initial rate of phosphate release from TBP, enhanced the duration and extent of activity (Fig. 3). Conversely, incorporation of EDTA (5 m*M*) reduced phosphate liberation by 50–60%; a similar effect was seen in the presence of 5 m*M* Mn^{2+} (Fig. 3). It could be argued that addition of

Table 2
The Effect of Mg^{2+}, Mn^{2+}, and EDTA on Phosphomonoesterase Activity[a]

Supplements	Phosphatase activity (%)
Unsupplemented	100
Mg^{2+}	100.24±2.85
Mn^{2+}	98.83±6.30
EDTA	100.19±6.08

[a] Supplements were added to give a final concentration of 6 m*M*. Phosphatase activity was expressed as a percentage of the activity of unsupplemented controls.

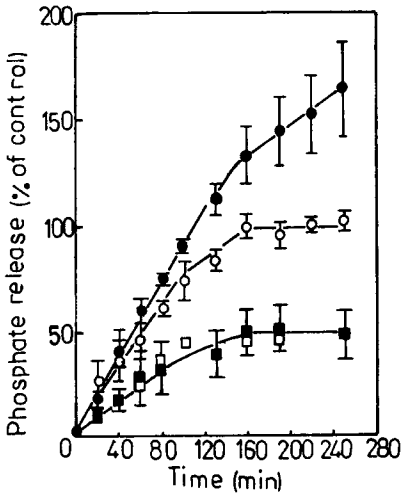

Fig. 3. The effect of Mg^{2+}, Mn^{2+}, and EDTA on phosphate release from TBP. For each control (unsupplemented) experiment (○), the data were calculated as a percentage of the final value. Where supplements were employed (5 m*M* throughout of: ● Mg^{2+}; □ Mn^{2+}, and ■ EDTA), the data were expressed as percentage of the final value for the corresponding unsupplemented control, performed in parallel. Data for Mn^{2+} were the mean of two experiments. Data for Mg^{2+}-supplemented incubations were mean±SEM from four experiments. Mg-supplemented incubations for the mutant M1 gave phosphate release identical to unsupplemented incubations and are not shown. Both strains were inhibited identically by EDTA; the data were pooled to give the mean±SE from three experiments using the pooled data.

Mg^{2+} removed a possible source of end-product inhibition by removal of liberated phosphate as $MgHPO_4$. Control experiments confirmed a lack of precipitation from solution under assay conditions. Furthermore, if this were the case, Mn^{2+} would have been expected to have had a similar effect, which was not seen. It is also noteworthy that La^{3+}, which had no effect of whole-cell monoesterase activity (*26*), apparently inhibited phos-

phate release from TBP (not shown), although in the present studies, the precipitation of $LaPO_4$ was not quantified.

The stimulatory effect of Mg^{2+} was confined to the wild-type strain N14. Addition of Mg^{2+} did not enhance the TBP-degradative activity of the phosphomonoesterase-deficient mutant M1 (not shown), although this strain was similarly sensitive to EDTA (Fig. 3). The activities of the Mg-stimulated parent strain (N14) and Mg-independent mutant M1 (Mg-unsupplemented) were very similar.

DISCUSSION

Although the *Citrobacter* sp. was reported previously to utilize TBP as a phosphate donor in the uptake of Cd^{2+}, the underlying mechanism of TBP hydrolysis was not addressed (*4*). The present article discounts a primary role for the well-documented (*4*) acid-type phosphatase (monoesterase) previously assumed responsible for metal uptake. No correlation was observed between phosphomonoesterase production and TBP biodegradation using either monoesterase overproducing or deficient strains. These data were not conclusive if the (undetermined) rate of TBP uptake comprised the rate-limiting step. For example, the phosphatase-deficient mutant M1 retained c. 4% of the monoesterase activity of the parent strain (Methods). If it is assumed that the rate of phosphate release from TBP (c. 6–7% of the rate from glycerol 2-phosphate) reflected the rate of uptake of the triester, then within the experimental errors given, the low phosphoesterase activity of the mutant might be sufficient to account for the observed hydrolysis of TBP. However, the purified enzyme had little activity against phosphodi- and triesters.

Confirmation of a secondary enzyme was obtained by the responses to metal ions and EDTA of the phosphoesterase-mediated reactions. The previously documented acid-type phosphatase was insensitive to Mg^{2+}, Mn^{2+}, and EDTA, both in whole cells (*25*) and when purified to homogeneity (this study), whereas TBP degradation was enhanced by Mg^{2+}, and inhibited by Mn^{2+} and EDTA.

The lability of TBP degradation noted previously (*5*) was confirmed in the present work and was partially overcome by Mg^{2+}. This was limited to the parent strain N14; strain M1 was apparently Mg^{2+} insensitive. This strain will find use as a tool to improve further the stability of the system. Such studies are a prerequisite to further bioprocess development.

An interesting observation was made in previous work (*5*). In the presence of glycerol 2-phosphate, Cd accumulation supported by TBP continued unabated for extended periods, although no satisfactory explanation was given. In this lightly buffered flow-through system, the pH rose steadily (*5*); accordingly, in the present work, the pH of the incubations

was routinely checked to ensure sufficient buffering. However, the pH of the bulk solution does not reflect the pH of the periplasmic microenvironment. Spontaneous alkaline hydrolysis of TBP periplasmically may have liberated DBP as the substrate in vivo. However, no evidence for DBP cleavage by immobilized cells was obtained in the present work, and DBP did not appear to compete with bis *p*-nitrophenyl phosphate as a diester substrate. Furthermore, the latter reaction was Mn^{2+} dependent.

The present article does not warrant more speculative discussions, although it should be noted that other authors have observed similar problems in organophosphorus compound biodegradation, particularly the instability of broken cells or cell extracts. It has been proposed (*27*) in the light of similar findings in *Pseudomonas* sp. that uncharged, nonpolar triesters are readily assimilated into the cells, whereas permeability constraints limit the uptake of the charged diester. A more detailed discussion is given in reference (*9*).

It has been recently appreciated that phosphate ester and inorganic phosphate uptake are often linked by a membrane-associated anion-exchange antiport (*28*). If this were occurring in the present system, the detergent properties of the dialkyl phosphate might exert membrane-disruptive effects, resulting in inhibition of uptake of the phosphate ester. If so, then TBP would indeed constitute the preferred and possibly the only feasible substrate if cell integrity is required for alkyl phosphate cleavage.

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