Effect of Dietholate (R-33865) on the Degradation of Thiocarbamate Herbicides by an EPTC-Degrading Bacterium[1]

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The persistence of EPTC (*S*-ethyl dipropylthiocarbamate) and related thiocarbamate herbicides in soil is microbiologically controlled. An EPTC degradation proficient bacterial strain, TE1, isolated from soil also degraded butylate (*S*-ethyl diisobutylthiocarbamate) and vernolate (*S*-propyl dipropylthiocarbamate). Addition of the extender, dietholate (*O,O*-diethyl *O*-phenyl phosphorothioate), to cultures of this bacterium inhibited degradation of the three thiocarbamate herbicides. This effect appeared to be related to the inhibitory effect of dietholate on growth of TE1. Dietholate also inhibited growth of two *Rhodococcus* strains but did not affect growth of various other bacterial species. Inhibitory effect of dietholate on bacterial growth and EPTC degradation was dependent upon dietholate concentration in the medium and lasted until dietholate itself was degraded by TE1 cells. Similar inhibition of EPTC, butylate, and vernolate degradation was observed in soils incubated with dietholate.

Soil microorganisms are mainly responsible for the degradation of thiocarbamate herbicides in natural environments (Kaufman, 1967; Fang, 1969; Lee, 1984). The enhanced biodegradation (Harvey and Schuman, 1981; Obrigawitch et al., 1983; Menkveld and Dekker, 1984; Wilson, 1984; Skipper et al., 1986) and the reduced efficacy of thiocarbamates in soils during successive years of application (Rahman and James, 1983; Gray and Joo, 1985; Harvey et al., 1986) have been attributed to the adaptation of soil microorganisms to the herbicides (Kaufman et al., 1970; Graves et al., 1976, Kaufman and Edward, 1983). Kaufman (1986) has cautioned about potential problems that could arise in soils exposed to structurally related herbicides due to the development of enhanced biodegradation. Moorman (1986) found no significant difference in the number of microorganisms in soils adapted or nonadapted to EPTC (*S*-ethyl dipropylthiocarbamate). He hypothesized that the reason for adaptation was not the increase in microbial population but was probably the result of selective stimulation of specific microbial groups in the general population and/or physiological and enzymatic alterations in the adapted microflora.

The use of microbial inhibitors to control biodegradation of herbicides in soils and thereby to extend the duration of their efficacy (persistence) was suggested by Kaufman et al. (1970). For example, several methylcarbamates were shown to act as competitive inhibitors to control the degradation of the herbicide CIPC (isopropyl *m*-chlorocarbanilate). Dietholate (*O,O*-diethyl *O*-phenyl phosphorothioate) has been used as an extender for thiocarbamate herbicides in soils. It retards the degradation of EPTC, butylate (*S*-ethyl diisobutylthiocarbamate), and vernolate (*S*-propyl dipropylthiocarbamate) in soil and improves significantly the duration of their efficacy (Dexter, 1979; Capper, 1982; Obrigawitch et al., 1982, 1983; Gooden et al., 1984). It has been suggested that dietholate exerts this effect through inhibition of soil microbial activity (Skipper et al., 1986). However, the mechanism of inhibition of thiocarbamate degradation by dietholate has not been elucidated.

Dietholate improved the efficacy of EPTC in EPTC-adapted soil but did not enhance the performance of butylate and vernolate in such a soil (Rahman and James, 1983). Obrigawitch et al. (1983) suggested cross-adaptation of EPTC-adapted microorganisms for vernolate with little cross-adaptation for butylate. Skipper et al. (1986) recently reported some cross-adaptation of butylate-adapted microorganisms for EPTC but little cross-adaptation for vernolate in South Carolina soils. Similar results have been reported by Wilson (1984) who found enhanced EPTC degradation in butylate-adapted soil but no effect on butylate degradation in EPTC-adapted soil or in soil previously exposed to vernolate. These studies have suggested some microbial or enzymatic specificity for EPTC and butylate degradation.

We recently described the isolation of an efficient EPTC-degrading bacterial strain (TE1) from EPTC-adapted soil (Tam et al., 1987). Initially this strain was identified as an *Arthrobacter* species but is now believed to be related to the *Rhodococci*, on the basis of morphology and cell wall analysis (Goodfellow and Anderson, 1977). It was shown that this strain can degrade EPTC and is able to grow on EPTC as the sole carbon source. This study was undertaken to determine the degradation of butylate and vernolate by TE1. In vitro degradation of thiocarbamate herbicides by TE1 as affected by dietholate was also investigated.

MATERIALS AND METHODS

Bacterial Strains. The following bacteria were used: EPTC-degrading soil isolate, TE1 (Tam et al., 1987), *Escherichia coli* K-12, *Bacillus thuringiensis* SSP *thuringiensis* HD-2 (Gonzalez et al., 1981), *Bacillus cereus*, *Arthrobacter globiformis* (ATCC 8010), *Arthrobacter oxidans* (ATCC 14358), *Pseudomanas putida* strain PAWI, *Rhizobium meliloti* strain SU47, *Azotobacter chroococcum*, *Rhodococcus erythropolis* (ATCC 15963), and *Rhodococcus rhodochrous* (ATCC 13859). The bacteria grown in basal minimal salts nitrogen (BMN) medium (Behki and Khan, 1986) was supplemented with 1–2 mg/mL glucose or glycerol at 29 °C. The media for growth of *R. rhodochrous* and *R. meliloti* were nutrient broth and tryptone yeast extract, respectively. *E. coli* was grown at 37 °C.

Herbicides. The herbicides were supplied by Stauffer Chemicals, Richmond, Ca, and were 95+% pure. Sterile stock solutions of EPTC (300 μg/mL), vernolate (100 μg/mL), and butylate (40 μg/mL) in BMN medium were kept cold. A stock solution of dietholate (25 mg/mL) was made in ethanol and stored at −20 °C.

Soil. The soil used in this study was clay loam soil from a corn field (Brandon, Manitoba) exposed to four suc-

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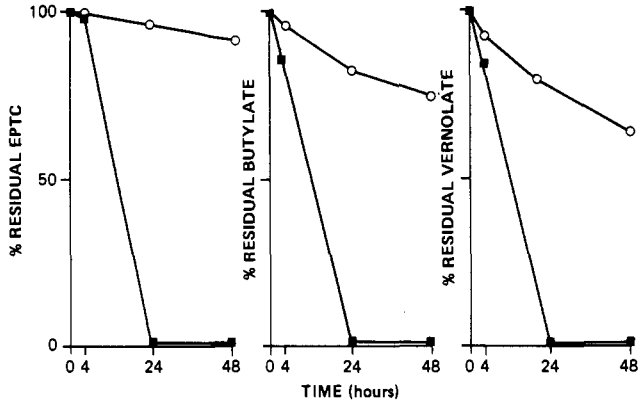

Figure 1. Degradation of EPTC, butylate, and vernolate by TE1 and the effect of diethalate on the degradations: ■, no diethalate added; ○, diethalate added.

cessive annual applications of Eradicane (Tam et al., 1987).

Degradation of EPTC, Butylate, and Vernolate. To determine the degradation of the thiocarbamates by bacterial cultures, cells were centrifuged and the supernatant (1 mL) was extracted by vortexing with 2 mL of hexane. An aliquot (2–5 μL) of the extracted hexane was injected into a gas chromatograph for quantification of the herbicide residue as described later. The values were corrected for the loss of EPTC, butylate, and vernolate determined in uninoculated controls incubated simultaneously under identical conditions. Ethanol equivalent to diethalate was added to the appropriate controls.

Cell Growth with Diethalate. The bacterial cultures were adjusted to an initial OD (600 nm) of 0.07–0.10, and EPTC and diethalate were added. The OD of the cultures was monitored spectorophotometrically (Beckman Model DU6) during incubation at 29 °C in a gyrotory shaker (New Brunswick Scientific) at 120 rpm.

Degradation of Diethalate. The amount of diethalate degraded was determined in the hexane extracts of cell-free supernatant culture medium by a similar procedure as described for the three thiocarbamate herbicides. Preliminary experiments showed the recovery of diethalate in bacterial cultures by this method exceeded 93%.

Gas Chromatography. The gas chromatograph was a Varian Model 3700 equipped with a thermionic specific detector. A 15 m × 0.5 mm silica Megabore column coated with DB-5 (1.5 μm) was operated at 120 °C. Detector and injector port temperatures were maintained at 280 and 170 °C, respectively. The nitrogen carrier flow was 15 mL/min. Under these conditions EPTC, vernolate, butylate, and diethalate gave peaks with retention times of 3.5, 5.1, 5.6, and 11.5 min, respectively. The identity of the compounds was confirmed by comparing the GC retention times with those of authentic samples, cochromatography, and finally gas chromatography–mass spectrometry. A high-resolution mass spectrometer, Model VG ZAB-2F, connected to a varian GC Model 3700 was used. The mass spectra were recorded at 70 eV.

RESULTS AND DISCUSSION

Effect of Diethalate on the Degradation of EPTC, Butylate, and Vernolate by TE1. The EPTC-degrading bacterial strain, TE1, isolated from the enrichment culture of EPTC-adapted soil, also degraded butylate and vernolate (Figure 1). The addition of 15 μg/mL diethalate to TE1 cultures (OD 0.08) in BMN + glycerol medium containing 35 μg/mL of the thiocarbamate inhibited the degradation of all the three herbicides. The results show that this bacterial isolate from EPTC-adapted soil showed little specificity for the in vitro degradation of the three thiocarbamates. This is contrary to the observations of Obrigawitch et al. (1982) who reported little or no cross-adaptation for butylate degradation in EPTC-adapted soil. It is, however, possible that soil may contain microbial strains specifically involved in the degradation of certain thiocarbamate herbicides as suggested by the results of Wilson (1984) on studies with butylate-adapted soil.

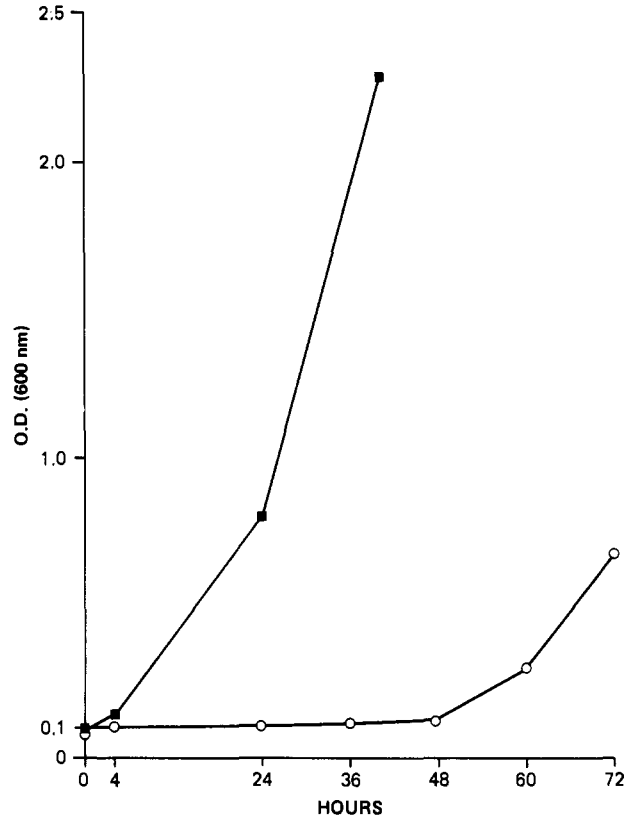

Figure 2. Effect of diethalate on the growth of TE1: ■, no diethalate added; ○, diethalate added.

Table I. Degradation of EPTC by TE1 Cell Suspension in Phosphate Buffer and in Unwashed Cells

addition, μg/mL	EPTC degraded, % 2 h	4 h	7 h
resuspended cells[a]			
EPTC (30)	34	54	71
EPTC (30) + diethalate (15)	0	0	0
unwashed cell[a]			
EPTC (30)	65	91	100
EPTC (30) + diethalate (15)	9	17	25

[a] The cell density at 0 time adjusted to 0.6 at 600 nm.

Effect of Diethalate on the Growth of TE1 Cells. It was observed during the incubation experiments that cultures containing diethalate showed no visible increase in turbidity at 48 h. This indicated that diethalate exerted an inhibitory effect on growth of TE1 cells. The effect of diethalate on the growth of TE1 culture in BMN + glycerol + EPTC medium was monitored and is shown in Figure 2. There was no growth of TE1 in the presence of diethalate over a 48-h incubation period. Thereafter, the culture resumed slow growth. This suggests that the inhibitory effect of diethalate on thiocarbamate degradation described above may be due to inhibition of growth of TE1 and cessation of metabolic activities. The latter was examined by incubating EPTC with washed (2X) TE1 cells resuspended in phosphate buffer with and without diethalate addition. The results in Table I show that EPTC was degraded by the resuspended cells at a lower rate than in unwashed cells, reflecting the reduced metabolic rate

Table II. Effect of Dietholate (15 μg/mL) on the Growth of Various Bacterial Species

bacterial cultures	OD (600 nm) at time				
		5 h		24 h	
	0	-DE	+DE	-DE	+DE
E. coli K12	0.12	0.29	0.28	2.30	2.20
Pseudomanas putida PAWI	0.09	0.22	0.18	1.95	1.88
B. thuringiensis HD-2	0.12	0.19	0.21	1.70	1.65
B. cereus	0.15	0.23	0.20	1.85	1.80
A. globiformis	0.07	0.30	0.33	1.42	1.10
A. oxidans	0.07	0.25	0.19	1.31	1.04
EPTC$^-$ mutant of TE1	0.07	0.14	0.07	1.10	0.08
Rhizobium meliloti	0.07	0.16	0.08	1.24	1.14
Azotobacter chroococcum (NRC 18002)	0.07	0.285	0.318	nd	nd
Rhodococcus erythropolis (ATCC 15963)	0.07	nd	nd	2.1	0.083
Rhodococcus rhodochrous (ATCC 13859)	0.07	nd	nd	1.59	0.119

(residual) in resuspended cells. It was observed that in spite of the initial high cell density dietholate inhibited EPTC degradation completely. There was a small degradation in the presence of dietholate with unwashed cells.

Effect of Dietholate on the Growth of Other Bacteria. Dietholate has been referred to as microbial inhibitor that exerts its effect on thiocarbamate degradation via inhibition of microbial growth or enzymatic activity (Skipper et al., 1986). The results in Table II show that dietholate is not a general inhibitor of microbial growth. It did not inhibit the growth of representatives of *Bacillus*, *Pseudomonas*, *Arthrobacter*, *E. coli*, *Rhizobium*, or *Azotobacter* species. In addition, dietholate showed no inhibitory effect on growth of *Fusarium oxysporum* and *Fusarium solani* (results not shown). However, dietholate inhibited severely the growth of TE1 (Figure 2) and derivatives of TE1 (results shown for only one EPTC degradation deficient mutant; Table II). Growth of two strains of *Rhodococcus* was as severely inhibited as that of TE1. Some bacterial strains isolated from soil showed enhanced growth in the presence of dietholate during 24-h incubation (results not shown). Addition of EPTC (up to 100 μg/mL) had no effect on growth of any of the bacterial strains tested. These results rule out the possibility of dietholate as a general microbial inhibitor. Instead, the inhibition appears to be species specific but not related to the ability of the cell to degrade the thiocarbamate. Lee (1984) isolated from EPTC-adapted soil members of *Bacillus* and *Pseudomonas* and strains of fungi capable of degrading EPTC. Our data show that the growth of these species was not inhibited by dietholate. Thus, it is difficult to explain how dietholate inhibited the degradation of EPTC in the New Zealand soils harboring the strains isolated by Lee (1984) if in fact they were actively involved in EPTC degradation.

Effect of Dietholate Concentration on EPTC Degradation by TE1. Degradation of EPTC (40 μg/mL) was monitored in the presence of 5, 10, 15, and 20 μg/mL of dietholate for 4 days in TE1 cultures growing in BMN + glycerol medium. The results in Table III show that the degradation of EPTC was dependent on the amounts of dietholate added. The extent of this effect was found similar to the degree of inhibition of growth of TE1 cells with the dietholate concentration (results not shown). It is evident from these results that dietholate concentration influenced the amount of EPTC degradation by TE1. The rapid breakdown of EPTC after about 72 h of incubation coincided with the release of growth inhibition of the cultures by dietholate (Figure 2). These observations suggest the possibility of dietholate degradation by TE1

Table III. Effect of Dietholate Concentration on EPTC Degration by TE1[a]

dietholate added, μg/mL	EPTC degraded, %		
	36 h	72 h	96 h
5	32	80	100
10	9	41	61
15	2	23	44
20	0	13	27

[a] OD of culture 0.2 at 600 nm.

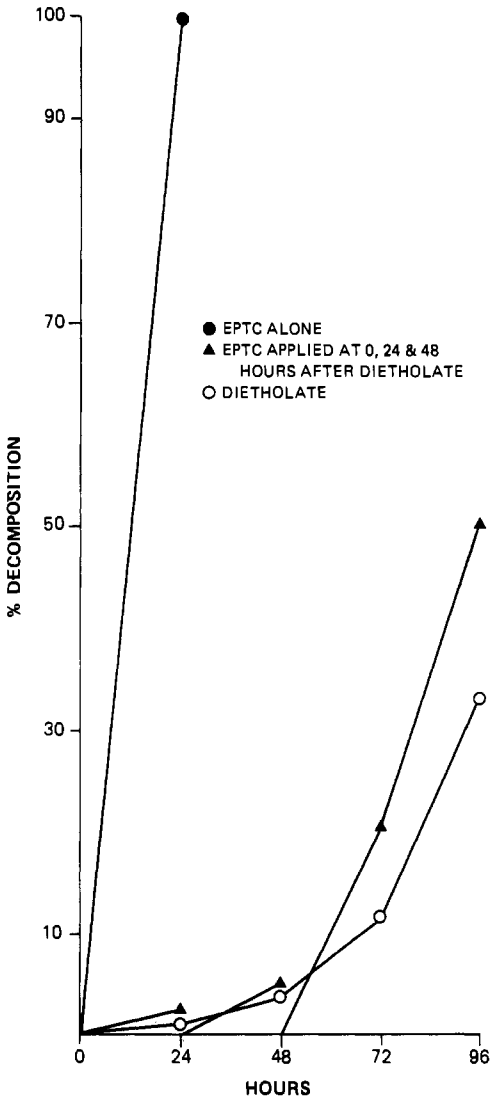

Figure 3. Effect of TE1 exposure to dietholate on the degradation of EPTC and dietholate.

cells. This was confirmed by the results shown in Figure 3. In this experiment TE1 was incubated with dietholate (15 μg/mL). Aliquots of the culture were withdrawn at 0, 24, and 48 h, and 30 μg/mL EPTC was added. The degradation of EPTC and the amount of dietholate in the cultures were determined over the next 24 h. It is quite obvious that EPTC degradation increased with a decrease in dietholate concentration. The rate of EPTC degradation was faster after 72 h and coincided with progressively decreasing dietholate concentration.

A breakdown product of dietholate appeared as a new peak on the gas chromatogram with a retention time of 8.5 min. A GC–MS of this peak showed a molecular ion at m/e 230 and other ions at m/e 215 ($M^+ - CH_3$) and m/e 201 ($M^+ - CH_2CH_3$). The spectrum was consistent with the mass spectrum of dietholate oxon. The oxon was formed in progressively increasing amounts with a corre-

Table IV. Degradation of EPTC, Butylate, and Vernolate by EPTC-Adapted Soil with and without the Addition of Diethola[a]

chemicals added	recovered, μg/mL	% degraded
EPTC	0	100
EPTC + diethola	11.8	59
butylate	0	100
butylate + diethola	9.5	65
vernolate	0	100
vernolate + diethola	10.8	62
EPTC control (no soil)	33.2	
butylate control (no soil)	34.6	
vernolate control (no soil)	33.3	

[a] 2 g of soil suspended in 10 mL of BMN medium was supplemented with 35 μg/mL of EPTC, butylate, or vernolate ± 15 μg/mL diethola.

sponding decrease in the amounts of diethola. It is worth noting that the degradation of diethola was dependent upon the cell density of the culture.

Effect of Diethola on EPTC, Butylate, and Vernolate Degradation with EPTC-Adapted Soil. Diethola inhibited the degradation of the three herbicides almost equally in the EPTC-adapted soil in 64 h (Table IV). The results suggest cross-adaptability of this soil to the three thiocarbamates, similar to the results obtained with the isolate TE1 from the EPTC enrichment culture of this soil.

Results of this study provide insight into the probable mode by which diethola extends the efficacy of thiocarbamate herbicides in soils. Diethola inhibits their degradation, thereby prolonging their persistence. The effect lasts until diethola itself is degraded. This is consistent with the observation that diethola increased the half-life of EPTC by 2.5-fold (Obrigawitch et al., 1983).

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Registry No. EPTC, 759-94-4; butylate, 2008-41-5; vernolate, 1929-77-7; diethola, 32345-29-2.

LITERATURE CITED

Behki, R. M.; Khan S. U. *J. Agric. Food Chem.* **1986**, *34*, 746–749.
Capper, B. E. *Proc. 35th N.Z. Weed and Pest Control Conf.* **1982**, 222–225.
Dexter, A. G. *North Central Weed Control Conference* **1979**, *36*, 156.
Fang, S. C. In *Degradation of Herbicides*; Kearney, P. C., Kaufman, D. D., Eds.; Dekker: New York, 1969; pp 147–164.
Gonzalez, J. M., Jr.; Dulmage H. T.; Carlton B. C. *Plasmid* **1981**, *5*, 351–365.
Gooden, D. T.; Murdock, E. C.; Skipper H. D.; Zublena J. P. *Proc. South Weed Sci. Soc.* **1984**, *37*, 327.
Goodfellow, M.; Anderson, G. *J. Gen. Microbiol.* **1977**, *100*, 99–122.
Gray, R. A.; Joo, G. K. *Weed Sci.* **1985**, *33*, 698–702.
Greaves, M. P.; Davies, H. A.; Marsh, J. A. P.; Wingfield, G. I. *CRC Crit. Rev. Microbiol.* **1976**, *5*, 1–35.
Harvey, R. G.; Schuman, D. B. *Abstr. Weed Sci. Soc. Am.* **1981**, 124
Harvey, R. G.; Dekker, J. H.; Fawcett, R. S. *Abstr. Weed Sci. Soc. Am.* **1986**, *No. 215.*
Kaufman, D. D. *J. Agric. Food Chem.* **1967**, *15*, 582–591.
Kaufman, D. D. *Abstr. Weed Sci. Soc. Am.* **1986**, *No. 218.*
Kaufman, D. D.; Edwards, D. F. *Proc. 5th Int. Congr. Pestic. Chem.* **1983**, *4*, 177–182.
Kaufman, D. D.; Kearney, P. C.; Vont Endt, D. W.; Miller D. E. *J. Agric. Food Chem.* **1970**, *18*, 513–519.
Lee, A. *Soil Biol. Biochem.* **1984**, *16*, 529–531.
Menkveld B.; Dekker, J. H. *Abstr Weed Sci. Soc. Am.* **1984**, *No. 250.*
Moorman, T. B. *Abstr. Weed Sci. Soc. Am.* **1986**, *No. 252.*
Obrigawitch, T.; Roeth, F. W.; Martin, A. R.; Wilson, R. G., Jr. *Weed Sci.* **1982**, *30*, 417–422.
Obrigawitch, T.; Martin, A. R.; Roeth, F. W. *Weed Sci.* **1983**, *31*, 187–192.
Rahman, A.; James, T. K. *Weed Sci.* **1983**, *31*, 783–789.
Skipper, H. D.; Murdock, E. C.; Gooden, D. T.; Zublena, J. P.; Amakiri, M. A. *Weed Sci.* **1986**, *34*, 558–563.
Tam, A. C.; Behki, R. M.; Khan, S. U. *Appl. Environ. Mirobiol.* **1987**, *53*, 1088–1093.
Wilson, R. G. *Weed Sci.* **1984**, *32*, 264–268.

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Absorption, Distribution, and Fate of Neptunium in Plants

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Soil–plant concentration ratios (CR) for neptunium (Np) in bushbean, measured over the range of 5.2×10^{-7} to 4.1 mg of Np/g of soil, are approximately 2 at soil concentrations below 4×10^{-4} mg/g and increase to 12 at higher soil levels. The CR values determined for soybean, bushbean, barley, and alfalfa range from 0.5 to 4 at a soil concentration of 2.6×10^{-6} mg/g. Root absorption by soybean seedlings of Np from solutions containing 7×10^{-7} to 473 mg of Np(V)/mL is generally proportional to concentration but exhibits some saturation in root absorption at higher concentrations. Seed concentrations in bushbean and wheat are a factor of 10 lower than vegetative tissues. Neptunium is transported within the plant in organic complexes containing one or more organic acid residues. Fractionation of plant tissues indicates that Np is substantially more soluble than plutonium, especially in seeds, with approximately 50% of the soluble Np in roots and leaves associated with plant ligands of less than 5000 molecular weight.

The continuing interest in the environmental and biological behavior of the transuranic elements is based on their long half-lives and their potential human toxicity. Studies addressing the transfer of transuranic elements from soils to plants have dealt primarily with plutonium (Pu) and americium (Am), and only to a limited extent with the behavior of curium (Cm) and neptunium (Np), which are much more biologically available. Investigations have generally documented the transfer of the transuranic elements from soils to plants for specific field environs and assessed uptake over a range of elemental concentrations in soils, with a variety of plant species and growth conditions (Watters et al., 1980). The results of these efforts show that the transfer factors from soil to plant vary widely

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