by Altom and Strizke (1973). This study also supports the data of Smith (1980), who showed a slightly faster rate of 2,4-D dissipation in clay loam as opposed to that in sandy loam soil. The rapidity of degradation in the clay soil seems consistent with the hypothesis that 2,4-D is rapidly dissociated to its anion in most soils (National Research Council, Canada, 1978) and is repulsed by negative sites on soil colloids, making it more readily available to soil organisms and leaching (Bailey and White, 1970; Weber et al., 1965; Grover and Smith, 1974).

Leaching of 2,4-D to depths lower than the 10-cm sampling horizon may also account for dissipation in our studies. Studies by Smith and Hayden (1976) and Stewart and Gaul (1977) have shown that 2,4-D does not leach to a great extent; however, Wilson and Cheng (1976) reported leaching in soils of low organic matter (<3%). On this basis, the soils from the Englehart location would be most likely to allow leaching. The somewhat greater persistence in these soils and the small amount of rainfall (91-mm total, Figure 3) at the Englehart site suggest that this was not a significant mechanism of dissipation.

In our opinion, the low levels of residue remaining in the soil at the end of the growing season [12–82 μg (kg of dry mass)$^{-1}$] would not pose any environmental hazard. This is especially true if the residues are strongly bound and therefore less available to soil organisms and for transport with runoff water.

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Registry No. 2,4-D, 94-75-7; 2,4-D·dimethylamine, 2008-39-1; 2,4-DP, 120-36-5; 2,4-DP butoxyethanol ester, 53404-31-2.

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Deethylsimazine: Bacterial Dechlorination, Deamination, and Complete Degradation

Alasdair M. Cook* and Ralf Hütter

Enrichment cultures utilizing deethylsimazine (6-chloro-*N*-ethyl-1,3,5-triazine-2,4-diamine) as a sole nitrogen source were obtained from soils that had had long exposure to *s*-triazine herbicides. A bacterium was isolated that utilized deethylsimazine quantitatively as a nitrogen source for growth, but only 1 mol of nitrogen was obtained/mol of deethylsimazine, whereas all six atoms in melamine were utilized. The bacterium, which was identified as a strain of *Rhodococcus corallinus*, converted deethylsimazine to 1 mol of 6-(ethylamino)-1,3,5-triazine-2,4(1*H*,3*H*)-dione, 1 mol of chloride ion, and 1 mol of ammonium ion. 4-Amino-6-(ethylamino)-1,3,5-triazin-2(1*H*)-one was tentatively identified as a transient intermediate in the degradation, which was presumed to be due to a dechlorination followed by a deamination. Growth of *R. corallinus* together with *Pseudomonas* sp. strain NRRL B-12228, which utilized 6-(ethylamino)-1,3,5-triazine-2,4(1*H*,3*H*)-dione, resulted in complete conversion of deethylsimazine nitrogen to cell material.

Deethylsimazine (6-chloro-*N*-ethyl-1,3,5-triazine-2,4-diamine; CEAT, see Table I and Figure 2) is the first compound to be conclusively identified as a microbial (fungal) product from a chloro-*s*-triazine herbicide [Kaufman et al., 1965; Kearney et al., 1965; see also Kaufman and Blake (1970)]. The product is formed in about 70% yield, the other 30% being an unidentified product; traces of ammelide (OOAT) have been tentatively

Mikrobiologisches Institut, Eidgenössische Technische Hochschule, ETH-Zentrum, CH-8092 Zürich, Switzerland.

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Table I. s-Triazines Mentioned in the Text

abbreviation[a]	substituents at the ring carbon atoms	common name
AAAT	amino, amino, amino	melamine
CEAT	chloro, ethylamino, amino	deethylsimazine
CEET	chloro, ethylamino, ethylamino	simazine
CIAT	chloro, isopropylamino, amino	deethylatrazine
CIET	chloro, isopropylamino, ethylamino	atrazine
EEOT	ethylamino, ethylamino, hydroxy	hydroxysimazine
EOAT	ethylamino, hydroxy, amino	*N*-ethylammeline
EOOT	ethylamino, hydroxy, hydroxy	*N*-ethylammelide
IOAT	isopropylamino, hydroxy, amino	*N*-isopropylammeline
IOOT	isopropylamino, hydroxy, hydroxy	*N*-isopropylammelide
OAAT	hydroxy, amino, amino	ammeline
OOAT	hydroxy, hydroxy, amino	ammelide
OOOT	hydroxy, hydroxy, hydroxy	cyanuric acid

[a] We have developed a set of abbreviations for these simple *s*-triazines, whereby the structure leads to the abbreviation and the abbreviation gives the structure (unlike the trivial names and their abbreviations; Cook and Hütter, 1981a). The four-letter code has one letter for each substituent [A = $-NH_2$; O = -OH; C = -Cl; E = $-NHC_2H_5$; I = $-NHCH(CH_3)_2$] and a T for the *s*-triazine ring.

identified. CEAT is observed in waters draining from fields that have been treated with chloro-*s*-triazine herbicides [e.g., Muir and Baker (1976) and Roberts et al. (1979)] and is presumed to be a (microbial) metabolic product from, e.g., simazine, in contrast to the nonbiological dechlorination of, e.g., simazine to hydroxysimazine (EEOT) on clay mineral surfaces (Jordan et al., 1970). The N-dealkylation of appropriate chloro-*s*-triazines to CEAT is also known in plants [e.g., Wichman and Byrnes (1975)] and is widespread in mammalian liver [e.g., Dauterman and Muecke (1974) and Khan et al., (1979)].

Little is know about the (bio)degradation of CEAT. It does not accumulate in soil in field studies [e.g., Ramsteiner et al. (1971) and Muir and Baker (1978)] and disappears from streams leaving areas treated with *s*-triazine herbicides (Roberts et al., 1979), but its fate is unknown. Foster et al. (1980) observed not only dechlorination of CEAT in soluble extracts from liver preparations but also apparent spontaneous hydrolysis of the chloro substituent, though the latter could be an artifact (Dauterman and Muecke, 1974). Giardina et al. (1982) also reported spontaneous decay of CEAT at an unknown low concentration, and they identified a product that is presumed to arise from a microbial reaction at the level of the (alkyl)amino ring substituent.

We have now isolated a bacterium that can utilize CEAT quantitatively as a sole and growth-limiting source of nitrogen for growth. Dechlorination appears to precede deamination, and the stoichiometric product is 6-(ethylamino)-1,3,5-triazine-2,4(1*H*,3*H*)-dione (EOOT), all of whose nitrogen atoms serve as a nitrogen source for a second bacterium (*Pseudomonas* sp. strain D; Cook and Hütter, 1981a).

EXPERIMENTAL SECTION

Materials. The *s*-triazines used (see Table I for abbreviations) were described by Beilstein et al. (1981). CEAT was recrystallized from toluene to 99% purity; it contained negligible *s*-triazine contaminants (<1%) and was essentially free of ammonium ion (mole fraction <0.04). All other chemicals were of reagent grade or better.

Apparatus, Analyses, and Identification of Metabolites. The apparatus used was described by Cook and Hütter (1981a). *s*-Triazines were routinely measured and tentatively identified by HPLC using the improved method (Cook et al., 1983a) of Beilstein et al. (1981). Ammonium ion was routinely determined by using the nitroprusside reaction (Weatherburn, 1967), and ammonium ion was identified by the specific reaction with glutamate dehydrogenase (EC 1.4.1.3) (da Fonseca-Wollheim et al., 1974). Chloride ion was determined by displacement of thiocyanate ion from mercuric thiocyanate (Bergmann and Sanik, 1957), and chloride ion was identified by complementary use of a chloride electrode (Type 96-17; Orion Research, Cambridge, MA) coupled to an ionalyzer (701A, Orion) in the sample addition mode as described in the manufacturer's instructions. Growth was measured as protein (Cook and Hütter, 1981a).

Growth Media and the Isolation of Organisms. The growth medium, a salt solution containing carbon source(s) and a nitrogen source, was described by Cook and Hütter (1981a). Enrichment cultures were inoculated with organisms from soils in 10 different experiments from Swiss research stations belonging to Ciba-Geigy AG, Basel. The soils had total exposures of 1.2–40 g/m^2 over 3–20 years, and an average exposure of 12 g/m^2. Microorganisms from two groups of five soils were prepared for inoculation as previously described (Cook and Hütter, 1981a), and in a separate experiment, 5 g each of the 10 soils was shaken in 200 mL (final volume) of growth medium in 1-L screw-cap bottles containing an atmosphere free of dinitrogen; until the soil had been diluted out by three transfers, no estimate of growth could be made in the latter experiment. Incubation times of 1–2 weeks were necessary, but otherwise the procedure of Cook and Hütter (1981a) was followed to obtain pure cultures.

In some experiments, *Pseudomonas* sp. strain NRRL B-12228 (strain D; Cook and Hütter, 1981a) was used.

Quantification of Growth and Substrate Utilization. Bacterial growth yields with limiting nitrogen sources and 10 mM glycerol as the carbon source were obtained as in Cook and Hütter (1981a), except for experiments with CEAT, which was slowly hydrolyzed to EOAT in the acid used to store samples. Samples containing CEAT (and appropriate controls) were centrifuged to remove bacteria (23000*g* for 20 min at 4 °C), and the supernatant fluid passed through a membrane filter (0.22-μm pore diameter) and stored frozen.

Growth kinetics were examined in 300-mL aerated cultures at 30 °C in the apparatus of Harvey et al. (1968), essentially as described by Cook and Hütter (1981a). The carbon source was 10 mM glycerol. Portions of outgrown CEAT culture media were evaporated to give about 10 mM EOOT, which was purified and identified (Cook and Hütter, 1981a). Cells for use in cell suspension studies were grown in 0.9-L batches in the apparatus of Harvey et al. (1968), essentially as in Cook and Hütter (1981a), except that longer incubations were required.

RESULTS

Enrichment and Isolation of Organisms. Enrichment cultures to obtain organisms that could utilize CEAT or CIAT as the sole nitrogen source were prepared. The inocula were either (a) cells washed out of 10 soils (in two groups of five) or (b) the 10 whole soils. CIAT did not support significant growth. Three enrichements were obtained with CEAT as the nitrogen source, one from each enrichment culture, but only one enrichment (no. 11, from inoculum a) gave complete utilization of CEAT. Culture

Table II. Growth Yields, Substrate Utilization and Product Formation of Strain 11 and/or Strain D When Utilizing Different Nitrogen Sources

nitrogen source	organism	protein growth yield, g/mol of substrate	N presumed utilized, mol/mol of substrate	protein growth yield, g/mol of N	N source remaining after growth	product, mol/mol of substrate
NH_4^+	11	40	1	40	ND[a]	ND
CEAT	11	39	1	39	EOOT	0.98
EOAT	11	46	1	46	EOOT	0.99
IOAT	11	49	1	49	IOOT	1.03
EOOT	11	1	0	0	EOOT	0
IOOT	11	1	0	0	IOOT	0
AAAT	11	264	6	44	ND	ND
OAAT	11	246	5	49	ND	ND
OOAT	11	184	4	46	ND	ND
OOOT	11	150	3	50	ND	ND
NH_4^+	D	43	1	43	ND	ND
CEAT	D	1	0	0	CEAT	0
EOAT	D	1	0	0	EOAT	0
CEAT	11 + D	215	5	43	ND	ND

[a] Not detectable.

Table III. Data from the UV Spectra of Authentic *s*-Triazines and of Materials Formed by Strain 11

substance[a,b]	wavelength, nm max	min	max
authentic CEAT	215[c]	249	260
authentic EOOT	202	210	226[c]
product from CEAT	202	210	226[c]
product from EOAT	202	210	226[c]
authentic EOAT	209[c]	227	231
transient intermediate from CEAT[d,e]	209[c]	227	231
authentic IOAT	210[c]	226	231
authentic IOOT	202	210	226[c]
product from IOAT	202	210	226[c]

[a] Abbreviations as in Table I. [b] Fractions of the eluate from an analytical HPLC column were examined in a double-beam spectrophotometer. The reference cuvette contained a liquid phase that had been passed through the column. CEAT was analyzed in a mixture of methanol (50% v/v) and 100 mM potassium phosphate buffer, pH 6.7. The other compounds were analyzed in a mixture of methanol (25% v/v) and the phosphate buffer. The spectra of many *s*-triazines are very sensitive to changes in solvent [cf. Cook and Hütter (1981a)]. [c] Peak with the higher molar absorption coefficient. [d] This intermediate was observed in growing cultures and in nongrowing suspensions. [e] This compound was found also after storage of CEAT in acid: we now believe that isolated data in the UV spectra given in Beilstein et al. (1981) are values not for the chlorinated compound but for the acid-hydrolyzed product (i.e., the hydroxy analogue of CIAT).

Table IV. Mass Spectral Identification of EOOT Produced from CEAT by Strain 11

m/z[a]	relative intensity: authentic EOOT	product from CEAT
157	13	9
156	100 (M)	100
142	4	4
141	53	55
129	12	6
128	82	76
113	4	5
112	7	7
98	6	6
91	2	1
85	22	30
84	6	9
70	13	14
69	44	55
56	3	6
55	22	34
44	63	93
43	40	71

[a] In the mass spectra, the two most intense ions are presented for every 14 mass units above m/z 34 (relative intensity > 1). The fine detail in the spectrum of the product is not significantly different from that of the authentic material.

11 had a growth yield of 285 g of protein/mol of CEAT (i.e., 57 g of protein/mol of nitrogen) compared with 58 g of protein/mol of ammonium ion, so all five nitrogen atoms/mol of CEAT were apparently being utilized for growth by the mixed culture. The culture did not fix dinitrogen.

From enrichment culture 11, a pure culture, strain 11, was isolated that utilized CEAT as a sole source of nitrogen. The organism was a bacterium. It was a nonmotile, oxidase-negative, uniform coccobacillus that divided, yielding V and Y forms. Strain 11 was obligately aerobic, and electron micrographs showed the smooth surface characteristic of Gram-positive organisms. This coryneform organism [cf. Buchanan and Gibbons (1974)] produced a red pigment that did not diffuse into the growth medium; it was identified by Seiler's (1983) key. There was an 8 (of 8) character fit in assigning the organism to the *Rhodococcus* group (one of six groups in Seiler's classification) and a 10 (of 10) character fit in assigning the isolate as a strain of *Rhodococcus corallinus* (one of seven subclusters in the *Rhodococcus* group).

Growth and Product Formation of Strain 11. Strain 11 had a growth yield of about 40 g of protein/mol of ammonium ion (Table II), a normal value (Luria, 1960; Cook and Hütter, 1981a,b). The organism could utilize quantitatively a wide range of *s*-triazines for growth (Table II) but although capable of using all nitrogen atoms in nonalkylated *s*-triazines (AAAT, OAAT, OOAT and OOOT) it could not degrade (alkylamino)triazinediones, which accumulated stoichiometrically in the growth medium during growth in medium containing CEAT, IOAT, or EOAT.

The product, which remained in the growth medium after growth with CEAT as the sole nitrogen source, cochromatographed (HPLC) with authentic EOOT and had a UV spectrum identical with that of EOOT (Table III). The identification of the product from CEAT as EOOT was confirmed by mass spectrometry (Table IV). CEAT contained negligible *s*-triazine impurities and was stable

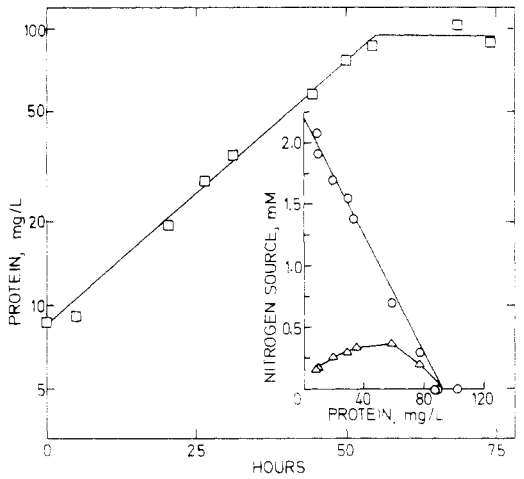

Figure 1. Growth (□) of strain 11 with CEAT as the nitrogen source. The carbon source was 10 mM glycerol. The insert is a differential plot of *s*-triazine concentration vs. the corresponding protein concentration: sum of the concentrations of EOAT plus CEAT (○) and intermediate EOAT (△). The inoculum was an untreated culture growing with CEAT as the sole added nitrogen source; thus EOAT was present at the start of the experiment.

for at least 2 weeks in sterile growth medium. In the control cultures with ammonium ion as the nitrogen source, no compounds interfered with the determinations of *s*-triazines. EOOT and IOOT were identified as the products from EOAT and IOAT, respectively, by cochromatography with authentic material and by UV spectrometry (Table III).

Strain 11 grew exponentially with CEAT as sole and limiting nitrogen source (Figure 1). During growth, an intermediate was excreted into the medium and subsequently utilized (Figure 1, insert). The intermediate was tentatively identified as EOAT by cochromatography with authentic EOAT and by UV spectrometry (Table III). When the sum of the concentrations of CEAT plus EOAT was plotted vs. growth (Figure 1, insert), growth was seen to be concomitant with utilization of the nitrogen supply. Product (EOOT) formation was also concomitant with growth (not shown). The specific rate of CEAT degradation during growth could be estimated by dividing the specific growth rate (μ; 0.043 h^{-1}) by the growth yield (Table II) and was 0.3 mkat/kg of protein. In a control experiment, the specific utilization rate of ammonium ion was 0.35 mkat/kg of protein: slow growth of strain 11 was thus characteristic and not due to especially slow utilization of the *s*-triazine. The organism produced slime and/or a storage polymer because the turbidity of the culture rose by 50% after growth (measured as protein) stopped.

Growth of strain 11 in pure culture with CEAT as the nitrogen source yielded EOOT quantitatively (Table II), whereas the original enrichment utilized all five nitrogen atoms in CEAT (see above). We did not isolate further organisms from the enrichment, but we could construct, with strain 11 and *Pseudomonas* NRRL B-12228, a defined mixed culture that could utilize all five nitrogen atoms in CEAT (Table II). The mixed culture eliminated all *s*-triazines from the growth medium and incorporated them into cell material: the pseudomonad could not utilize CEAT or EOAT, and strain 11 could not utilize EOOT.

The chloro substituent of CEAT was released, as chloride ion, concomitant with substrate disappearance (Table V). Release of chloride ion from 0.5 mM CEAT in nongrowing suspensions of strain 11 was found to be quantitative (102–108%) by two independent methods; there was negligible chloride in the CEAT, in the bacteria, or

Table V. Initial Rates of Reactions in the Degradation of CEAT by Suspensions of Nongrowing Cells of Strain 11

reaction	specific rate, mkat/kg of protein
CEAT disappearance	0.44
Cl^- formation	0.46
EOAT (intermediate) release	0.22
EOOT (product) formation	0.21

in the solutions used. The specific rate of CEAT degradation in these experiments was about 0.4 mkat/kg of protein for cells grown with CEAT as the nitrogen source and about 0.2 mkat/kg of protein for cells grown with ammonium ion as the nitrogen source. Just as in the growth experiment (cf. Figure 1), EOAT was detected as a transient intermediate in CEAT degradation in nongrowing suspensions (Table V), and EOOT was the quantitative product.

Ammonium ion was detected by two independent methods during the degradation of CEAT by nongrowing cells of strain 11. The yield of ammonium ion from 0.5 mM CEAT was a maximum of about 0.35 mM, but separate experiments showed that the ammonium ion was consumed by cells under these conditions. The amino substituent of CEAT, which was utilized quantitatively for growth (Table II), was presumably released quantitatively as ammonium ion and consumed in part by the cells.

Neither simazine (CEET) nor atrazine (CIET) was dechlorinated by strain 11. Nongrowing suspensions of strain 11, however, could dechlorinate CIAT (0.04 mkat/kg of protein) to IOAT, which was deaminated to IOOT. We do not know why the organism did not grow detectably with CIAT as a nitrogen source.

DISCUSSION

CEAT is seen to be biodegradable by organisms enriched from native environments. The choice of inoculum was based on our experience (Cook and Hütter, 1982) that long exposure of soils to *s*-triazine herbicides was critical to the success of the enrichments. In this case we chose as the inoculum many soils that had been subject to extensive exposure to *s*-triazine herbicides, and we have had more success than in our previous work with fewer soils and lower exposure (Cook and Hütter, 1981a). Other important aspects of enrichments are discussed elsewhere (Cook and Hütter, 1981b; Cook et al., 1983b).

A bacterium has been isolated that is capable of quantitative utilization of CEAT and other *s*-triazines (Table II). CEAT is degraded stoichiometrically to 1 mol of chloride ion, 1 mol of ammonium ion, and 1 mol of EOOT (Tables II–V). This degradation appears to occur in two steps, first a dechlorination to EOAT, which was detected during growth (Figure 1, insert), followed by a deamination to EOOT (Figure 2). Experiments with cell-free enzymes will be needed to test whether this is the only pathway present [cf. Jutzi et al. (1982)]. This is the first conclusive report of the bacterial dechlorination of an *s*-triazine ring and the first report of the quantitative degradation of a chloro-*s*-triazine to natural products (biomass of the defined mixed culture and chloride ion).

It is difficult to compare our data with those of Foster et al. (1980), who reported dechlorination (albeit partial) of CEAT in preparations from goose liver. Foster et al. (1980) gave no indication of the concentration of biological material used and found CEAT to be unstable (6% dechlorination in 5 h), whereas we find the compound to be stable (2-week incubation) unless exposed to acid. We suspect that their clean up, which involves an acidic alumina column, may be the source of the discrepancy, and

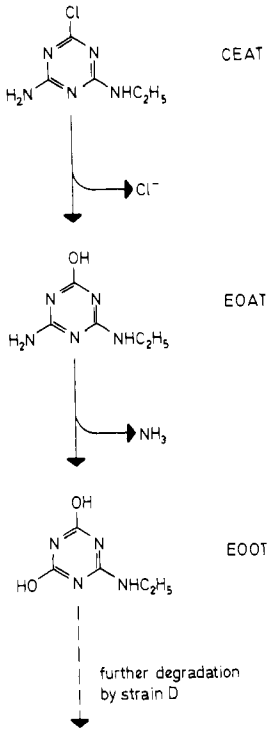

Figure 2. Degradative pathway of CEAT by strain 11.

Dauterman and Muecke (1974) warn against similar artifacts. Giardina et al. (1982) also believe CEAT to be unstable, but they describe an unquantified, chlorinated product, which is presumably formed from CEAT: many unidentified products are also claimed. Fungal dechlorination of atrazine (CIET) has been claimed in an unverified report in which the behavior of the controls is not described (Couch et al., 1965).

The mechanism of dechlorination may be of interest, because Knackmuss (1981) holds it "unlikely that bacteria have evolved enzymes for the direct hydrolysis of the aromatic carbon–halogen bond", whereas Klages et al. (1981) report such an activity in whole cells. In rat liver, the aromatic carbon–chlorine bond of *s*-triazines is displaced, yielding the aromatic carbon–sulfur bond of a glutathion conjugate [e.g., Dauterman and Muecke (1974)], and we have observed the carbon–oxygen bond as the quantitative product from an aromatic carbon–sulfur bond of an *s*-triazine (Cook and Hütter, 1982); the sum of both reaction types would represent an hydrolysis. However, the atypical aromatic character of the *s*-triazine ring (Harris et al., 1968) may allow reactions that are impossible in the benzene ring.

Strain 11 grows slowly (μ = 0.04 h^{-1}), but the specific rate of degradation of CEAT (0.3 mkat/kg of protein) is similar to specific activities of *s*-triazine degradation in our other isolates (about 0.4 mkat/kg of protein; Cook and Hütter, 1981a). It is possible to couple the degradative capabilities of two organisms, strain 11 and strain D, to completely convert CEAT nitrogen into cell material (Table II). A similar combination may be responsible for the disappearance of CEAT observed in fields exposed to chloro-*s*-triazine herbicides [e.g., Muir and Baker (1978)].

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Registry No. CEAT, 1007-28-9; EOOT, 2630-10-6; EOAT, 7313-54-4.

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