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Ametryne and Prometryne as Sulfur Sources for Bacteria

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Bacteria were isolated that could utilize quantitatively the s-triazine herbicide prometryne [*N*,*N*'-bis(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine] or ametryne [*N*-ethyl-*N*'-(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine], or both, as a sole source of sulfur for growth. The success of enrichments depended on previous exposure of the soil inoculum to s-triazine herbicides. Deaminoethylametryne [4-(1-methylethyl)amino-6-(methylthio)-1,3,5-triazine-2-(1H)-one], methylsulfonic acid, and sodium sulfate could also be used as sulfur sources. Utilization of a compound was quantified as the growth yield per mole of sulfur supplied. Yields were about 6 kg of protein per mol of sulfur. The product of the desulfuration of an s-triazine was identified as the corresponding hydroxy-derivative. This is the first substantiated report of the utilization of these s-triazines as sulfur sources by bacteria.

Studies on the biodegradation of xenobiotics stress utilization of the compounds as carbon sources (e.g., reference 25), whereas other elements have received little attention as nutrient sources. Indeed, attempts to desulfurate coal and oil microbially have used organisms isolated to utilize the sulfur-containing compounds as carbon sources (e.g., reference 19).

Ametryne [*N*-ethyl-*N*'-(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine] and prometryne [*N*,*N*' - bis(1 - methylethyl) - 6 - (methylthio)-1,3,5-triazine-2,4-diamine] (Fig. 1) were investigated because they are widely used herbicides and are available in highly pure form. There is a claim (21) for the fungal utilization of prometryne. The data, however, do not appear to verify the claim, because the molar growth yields with sulfate and prometryne as sulfur sources differed by a factor of about 150, and the amount of prometryne supplied was apparently eightfold lower than the amount measured as disappearing during the incubation.

We now describe (i) enrichment cultures to obtain organisms utilizing ametryne or prometryne as a sole added source of sulfur, (ii) the enhanced occurrence of the organisms in environments exposed to s-triazine herbicides, and (iii) confirmation of the utilization of the s-triazines in culture.

MATERIALS AND METHODS

Materials. Ametryne, prometryne, hydroxyametryne [4-(ethylamino)-6-(1-methylethyl)amino-1,3,5-triazine-2-(lH)-one], hydroxyprometryne [4,6-bis(1-methylethyl)amino - 1,3,5 - triazine - 2 - (lH) - one], deaminoethylametryne [4-(1-methylethyl)amino-6-(methylthio)-1,3,5-triazine-2-(lH)-one], and *N*-isopropylammelide [6-(1-methylethyl)-amino-1,3,5-triazine-2,4-(lH,3H)-dione] (see Fig. 1) were supplied by Ciba-Geigy AG (Basel, Switzerland). The identity of each s-triazine substrate was confirmed by mass spectrometry. The spectrum of prometryne agreed with published data (23), and the spectrum of ametryne is given elsewhere (1). In the following mass spectrum, the two most intense ions are presented for every 14 mass units above *m/z* 34 (relative intensity > 1). Deaminoethylametryne, 43(25), 44(17), 57(5), 58(25), 69(31), 74(21), 83(6), 84(4), 92(4), 95(5), 111(21), 112(31), 125(8), 126(8), 139(3), 143(4), 153(12), 158(17), 167(2), 170(1), 185(100), 186(11), 200(93,M^+), 201(15), 202(6). This spectrum agrees with known disintegration patterns (12, 23).

The purity of ametryne, prometryne, or deaminoethylametryne was assayed by elemental analysis. Calculated for ametryne ($C_9H_{17}N_5S$): C, 47.5; H, 7.5; N, 30.8; S, 14.1. Found: C, 47.4; H, 7.6; N, 30.6; S, 14.1. Calculated for prometryne ($C_{10}H_{19}N_5S$): C, 49.8; H, 8.0; N, 29.0; S, 13.3. Found: C, 49.6; H, 7.9; N, 29.0; S, 13.3. Calculated for deaminoethylametryne ($C_7H_{12}N_4OS$): C, 42.0; H, 6.0; N, 28.0; S, 16.0. Found: C, 40.5; H, 6.2; N, 27.0; S, 15.6. There was thus no significant extraneous sulfur in these s-triazines. Methylsulfonic acid (>99%) was bought from Fluka (Buchs, Switzerland). $Na_2{}^{35}SO_4$ was purchased from the Radiochemical Centre (Amersham, Bucks, U.K.). All other chemicals were of the highest purity available commercially.

Media. The growth medium used for sulfur-limited growth was in 10 mM potassium phosphate buffer, pH 7.3, and contained 25 mM NH_4Cl, 0.25 mM $MgCl_2$, carbon sources (see below), a sulfur source (≤20 μM sulfur), trace elements, and, when necessary, vitamins. The buffered solution of NH_4Cl and $MgCl_2$ was autoclaved, and sterile solutions of trace elements, vitamins, carbon source(s), and a sulfur source were added aseptically. Trace elements (22; this solution was supplemented with 100 mg of $CaCl_2$ per liter and was supplied at 1 ml/liter) were sterilized by autoclav-

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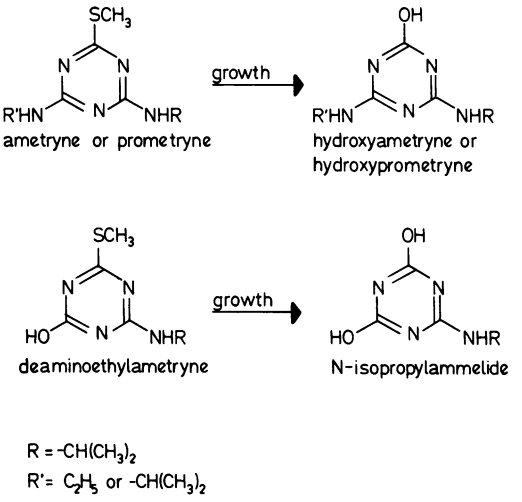

FIG. 1. Methylthio-s-triazines as sulfur sources for *Pseudomonas* sp. strain 26.

ing. Carbon sources, s-triazines, or vitamins (24) in solution were sterilized by passage through membrane filters of 0.2-μm pore diameter and of the appropriate chemical resistance (SM 113 or SM 119; Sartorius, Göttingen, West Germany). The stock solution of ametryne, prometryne, deaminoethylametryne, or methylsulfonic acid was 2 mM in ethanol. The term "buffered salts" refers to 10 mM potassium phosphate buffer, pH 7.3, containing 25 mM NH_4Cl and 0.25 mM $MgCl_2$. Difco (Detroit, Mich.) nutrient agar was used.

Enrichment and isolation of bacteria. Enrichment cultures were used to obtain isolates able to utilize ametryne or prometryne as a sole and limiting source of sulfur for growth. Inocula for enrichments were prepared from sewage or soil. Municipal sewage from a primary settling tank (Werdhölzli, Zürich, Switzerland) was centrifuged (20,000 × *g* for 10 min at 4°C), and the supernatant fluid was discarded to reduce the quantity of extraneous sulfur source(s). The pellet was resuspended in buffered salts solution and washed twice more. The pellet was then suspended in buffered salts solution for use as inoculum. Samples (20 g) from each of four agricultural soils (from Ciba-Geigy Research Stations in Bex, Vaud, Switzerland, and Vufflens, Vaud, Switzerland, and two soils from the Swiss Federal Research Station in Wädenswil, Zürich, Switzerland) that had been exposed to one to seven treatments (Table 1) with s-triazine herbicides were suspended in 100 ml of buffered salts solution, shaken at 30°C for 1 h, and allowed to settle for 30 min. The supernatant fluid was passed through a Whatman no. 1 filter, and the organisms in the filtrate were prepared for inoculation as described for sewage. Enrichment cultures (3 ml in culture tubes closed with plastic caps) were nonsterile and contained 30 μM sulfur source, 5 mM glucose, 5 mM succinate, 10 mM glycerol, and 0.4 ml of inoculum. The inoculated tubes were incubated at 30°C on an orbital shaker (2 rps). The first enrichment culture, including a control without sulfur source, grew in 1 to 3 days. All enrichments were subcultured into homologous medium. Cultures that gave more growth (estimated visually) than the control without a sulfur source were subcultured into homologous medium. After three s-triazine-limited subcultures, positive enrichments were streaked on nutrient agar plates. A representative of each colony type was picked from the agar plates, and isolates that were able to utilize s-triazines were recognized by their growth in sterile selective medium (homologous with enrichment medium). Positive isolates were streaked on nutrient agar now supplemented with a vitamin solution. Isolates were picked from the latter plates and grown in the appropriate selective medium supplemented with vitamins. This procedure of alternated growth in selective liquid medium and nonselective agar plates was used to minimize the risk of loss of degradative ability sometimes experienced on repeated streaking on nonselective media (3) and to reduce the chance of carrying a persistent contaminant through the isolation (8). When the procedure yielded three successive homologous plates, an isolate was considered pure.

TABLE 1. Source and history of materials used for inoculation, and the success of enrichments and isolations

Source of inoculum	Pretreatment with s-triazine herbicides (yr)	Total amt of s-triazine herbicide (g/m^2)	No. of enrichments (maximum = 6)	No. of pure isolates obtained
Municipal sewage	0	0	0	0
Orchard soil	1	0.7	0	0
Orchard soil	3	1.8	1	0
Cornfield soil	3	1.2	6	3
Orchard soil	7	10.5	5	6

Stock cultures of each isolate were maintained in liquid culture in screw-cap tubes containing minimal medium with vitamin supplement and the appropriate s-triazine as the sole sulfur source. Agar of all grades gave growth without added sulfur, so agar slants were not used for storage as they did not select for the desired degradative capacity.

Limited taxonomy was done, following the principles of *Bergey's Manual* (8), to ascertain that the strains were unlikely to be serious pathogens. Morphology was studied by phase-contrast microscopy, the Gram reaction was deduced from growth on MacConkey agar plates (Hoffmann-La Roche, Basel, Switzerland), and Oxiferm and Enterotubes (Hoffmann-La Roche) were used in combination with the methods of Stanier et al. (26).

Quantification of growth and substrate utilization. Experiments to measure bacterial growth yields with limiting sulfur sources were done at 30°C with 60-ml cultures in 500-ml fluted Erlenmeyer flasks mounted on an orbital shaker (2 rps). Media containing the sulfur source at five concentrations between 0.0 and 20 μM sulfur were inoculated (0.3%, vol/vol) with a culture grown in a limiting amount of s-triazine. The carbon source was 10 mM glucose. Before incubation, a sample of the culture was taken aseptically and centrifuged (15,000 × *g* for 20 min at 4°C), and the supernatant fluid was stored frozen. When the culture

TABLE 2. Properties of three strains isolated to utilize ametryne or prometryne as sulfur source

Strain designation	s-Triazine in enrichment	Sulfur sources utilized[a]	Vitamin requirement	Taxonomic characteristics
15	Ametryne	Sulfate, ametryne	+	Gram-negative, nonmotile, oxidase-negative rod
22	Ametryne	Sulfate, ametryne	+	Gram-negative, nonmotile, oxidase-negative rod
26	Prometryne	Sulfate, ametryne, prometryne, deaminoethylametryne, methylsulfonate	0	Gram-negative, motile rod; obligate aerobe, oxidase positive, arginine hydrolase activity, fluorescent pigment[b]

[a] Sulfate, ametryne, prometryne, deaminoethylametryne, and methylsulfonate were tested.
[b] The organism grew at neutral pH and utilized acetate and glucose as sole carbon sources.

was fully grown (1 to 3 days), another sample of the culture supernatant fluid was taken and stored frozen, and growth was quantified as protein. The samples of culture supernatant fluid were thawed and analyzed for s-triazines. In separate experiments, the utilization of $Na_2{}^{35}SO_4$ was measured radiochemically in samples of the culture supernatant fluid.

Analytical methods. Growth rates were obtained by following cell density turbidimetrically at 500 nm, using round (0.5-inch [ca. 1.3-cm] diameter) cuvettes in a Bausch & Lomb Spectronic 20 colorimeter. Cell material was quantified as protein (rather than turbidity or dry weight) to eliminate trivial complications arising from the accumulation of lipid or carbohydrate storage polymers. Bacteria were precipitated with 0.5 M trichloroacetic acid (final concentration) and collected by centrifugation (20,000 $\times g$ for 20 min at 4°C), after which the supernatant fluid was discarded, and the walls of the tube were dried with a cotton swab. The pellet was suspended in 0.66 M NaOH and assayed for protein (16). In calculations from published data, the protein in microorganisms was considered to be 0.5 × dry weight (17).

s-Triazines in solution were determined by high-pressure liquid chromatography with a reversed-phase column (1). The mobile phases used were mixtures of methanol and potassium phosphate buffer, pH 6.7. Ametryne and prometryne were eluted with methanol plus 10 mM buffer (7:3, vol/vol). Hydroxyametryne, hydroxyprometryne, and deaminoethylametryne were eluted with methanol plus 100 mM buffer (1:1, vol/vol). *N*-isopropylammelide was eluted with methanol plus 100 mM buffer (1:4, vol/vol). Hydroxyametryne, hydroxyprometryne, and *N*-isopropylammelide were tentatively identified by cochromatography with authentic material. A portion (e.g., 250 ml) of solution containing the tentatively identified hydroxyametryne or hydroxyprometryne was pumped on to a Lobar RP-8 column (31 by 2.5 cm; E. Merck AG, Darmstadt, West Germany) that was equilibrated with 60% (vol/vol) methanol in water, and the s-triazine was eluted in the same solvent. The fractions containing the s-triazine were dried on a rotary evaporator, and portions were examined by UV spectroscopy and mass spectrometry.

Spectrophotometric analyses were done with a Bausch & Lomb Spectronic 88 spectrophotometer equipped with a flow-through cell of 1-cm path length or with a Kontron Uvikon 800 spectrophotometer. Radioactivity was measured in a Beckman LS7000 liquid scintillation analyzer with Aquassure counting fluid (New England Nuclear Corp., Boston, Mass.). Mass spectrometry was done by direct probe insertion in a Hitachi-Perkin Elmer RMU 6 mass spectrometer, using electron impact ionization at 70 eV. Methane was assayed with a gas chromatograph as described by Daughton et al. (9).

RESULTS

Isolation of strains. The enrichment medium was chosen to allow growth only of those organisms which could utilize ametryne or prometryne as a sole source of sulfur. The sulfur concentration was chosen to allow the synthesis of about 120 μg of protein per ml (5), which gave a simple visual test for growth (i.e., turbidity), whereas any contamination of sulfur in the enrichment medium did not support significant growth. The sulfur source was pure and growth limiting (6).

A suitable choice of inoculum was also essential for successful enrichments (Table 1). Inocula with little or no exposure to s-triazine herbicides were unsuccessful, whereas repeated exposure to the xenobiotics usually gave positive enrichments.

A successful enrichment did not necessarily lead to a pure culture (Table 1). However, the addition of vitamins to the solid and liquid cultures allowed us to isolate nine pure cultures, all bacteria, three of which grew fast (about 10 generations in 2 days compared with 10 generations in 7 to 10 days for the slow-growing isolates).

The isolated organisms fell into three groups: (i) the slow-growing organisms, which all had similar morphology on nutrient agar plates, (ii) strains 15 and 22, and (iii) strain 26. Only the three fast-growing strains (15, 22, and 26) were examined further (Table 2). The strains which had been isolated with ametryne as a sulfur source could utilize only ametryne, whereas

TABLE 3. Cell growth yields, substrate utilization, and product formation during sulfur-limited growth

Organism	Sulfur source (20 μM)	Growth yield[a] (kg of protein/mol of S)	Substrate remaining after growth[b]	Product (about 20 μM)
15	SO_4^{2-}	5.9		
	Ametryne	6.7	ND	Hydroxyametryne
22	SO_4^{2-}	6.4		
	Ametryne	6.1	ND	Hydroxyametryne
26	SO_4^{2-}	5.4[c]		
	Ametryne	6.1	ND	Hydroxyametryne
	Prometryne	5.5[d]	ND	Hydroxyprometryne
	Deaminoethylametryne	6.3	ND	*N*-Isopropylammelid[e]
	Methylsulfonic acid	5.1	NA	NA

[a] Defined as slope of the line "final protein concentration" versus "initial concentration of sulfur source." There was negligible growth in the absence of added sulfur source.
[b] ND, not detectable; NA, not assayed.
[c] Specific growth rate (μ), 0.58 h^{-1}. The specific degradation rate was thus 30 μkat/kg of protein.
[d] Specific growth rate (μ), 0.40 h^{-1}. The specific degradation rate was thus 20 μkat/kg of protein.
[e] Identified tentatively by cochromatography with authentic material in a high-pressure liquid chromatograph and by UV spectrophotometry.

strain 26, isolated on prometryne, displayed a wider substrate spectrum (Table 2).

Strain 26 was the only isolate that did not require vitamins, and this enabled us to use commercially available systems to help in identification: strains 15 and 22 were not identified. Strain 26 was identified as a fluorescent pseudomonad (Table 2).

Quantitative utilization of the sulfur sources. Each strain grew with sulfate as a limiting sulfur source with a yield of about 6 kg of protein per mol of S supplied (Table 3). The utilization of $Na_2{}^{35}SO_4$ was confirmed radiochemically; less than 6% of the radioactivity from the supplied $Na_2{}^{35}SO_4$ remained in the supernatant fluid after growth. The growth yield compared well with that calculated from the sulfur content of growing cells (2.4 kg of protein per mol of S; 18) and with previous data (about 4 kg of protein per mol of S; 5) for a strain of *Pseudomonas acidovorans*. The data thus confirm quantitative utilization of Na_2SO_4 as a limiting sulfur source for growth.

The growth yields of each strain with ametryne or prometryne as the limiting sulfur source closely resembled those with sulfate as a sulfur source (Table 3). Ametryne and prometryne disappeared from the medium during growth, and compounds which cochromatographed with authentic hydroxyametryne and hydroxyprometryne, respectively, were formed stoichiometrically (Table 3). Each product had a UV spectrum that differed from that of the substrate, and the product from ametryne (or prometryne) had an absorption maximum at 216 nm (or 217 nm) compared with 216 nm (or 217 nm) for authentic hydroxyametryne (or hydroxyprometryne). The identifications were confirmed by mass spectrometry of hydroxyametryne (Fig. 2) and hydroxyprometryne (not shown). Ametryne and prometryne were stable in sterile control experiments and contained no hydroxyderivatives, and cells growing with sulfate as a sulfur source produced no material that interfered with these

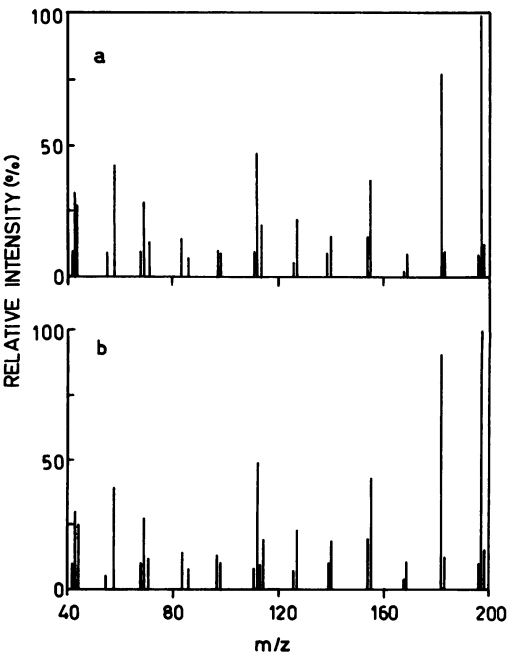

FIG. 2. Mass spectra of (a) the product produced by strain 26 from ametryne and (b) authentic hydroxyametryne, molecular weight 197.

analyses. The data confirmed that strains 15, 22, and 26 could utilize ametryne and that strain 26 could utilize prometryne as a sole source of sulfur for growth with excretion of the corresponding hydroxyderivative (Fig. 1). Strain 26 (but not strains 15 and 22) could also utilize deaminoethylametryne quantitatively, yielding *N*-isopropylammelide (Fig. 1) which was identified by cochromatography and UV spectroscopy.

We suspected that methylsulfonic acid could be an intermediate in the degradation of the thiomethyl-s-triazines and found that strain 26 (but not 15 or 22) could utilize methylsulfonic acid as a sulfur source (Table 3). We then tested whether methane was a product of growth with methylthio groups as sulfur sources. No methane was released with sulfate, ametryne (strains 15, 22, and 26), prometryne, deaminoethylametryne, or methylsulfonic acid as a sulfur source. The organisms did not utilize methane that had been added to culture headspaces.

DISCUSSION

The enrichment, at low and growth-limiting concentrations of the sulfur source, was designed to utilize the sulfur-containing compound quantitatively, and this aim was achieved (Table 3). Substrate of high purity was essential to prevent spurious enrichments (3). Equally important in enrichments to utilize these xenobiotic compounds was the choice of inoculum (Table 1), which had to have experienced extensive exposure to s-triazine herbicides.

The enrichment was designed to utilize a portion of a substituent on the s-triazine ring. Only this moiety of the molecule was removed, and no ring cleavage was obtained. This result is similar to that of Kearney et al. (14, 15), who used simazine as a carbon source for microorganisms; only a carbon-containing substituent was removed. A direct approach to ring cleavage, enriching to utilize the ring nitrogen, has been successful (7).

The evidence for the quantitative utilization of ametryne, prometryne, or deaminoethylametryne is a series of controlled, independent, quantitative determinations of growth, substrate utilization, and formation of an identified product (Table 3 and Fig. 1). This is the first report of the bacterial degradation of methylthio-s-triazines as sulfur sources, though qualitatively similar reactions have been reported in soil, plants, and animals (e.g., reference 11). A report on the fungal degradation of prometryne (21) may be invalid, because eight times as much prometryne seemed to disappear as was added, and we calculate the growth yield with sulfate to be higher (295 kg of protein per mol of S) than expected (2.4 to 6.7 kg of protein per mol of S; Table 3; 5, 18) and to be 148-fold higher than the yield with prometryne as a sulfur source.

Whereas the fate of the s-triazine ring in the sulfur source is now known (Fig. 1), the fate of the methyl group has not been established. Desulfuration of prometryne in soil is reported to proceed through the sulfone (13), and we hypothesized that methylsulfonate could be a subsequent intermediate, but only strain 26 could utilize methylsulfonate. In no case was the methyl group released as methane. The system of C-S bond cleavage in methylsulfonic acid thus differs from that of C-P bond cleavage in methylphosphonic acid, where methane is released (9), just as the cleavage of phenylsulfonic acid and phenylphosphonic acid differs (4). Cleavage of the C-S bond was reviewed by Cain (2).

The aim of this work was to obtain organisms to detoxify unwanted thiomethyl-s-triazine herbicides at point sources. Ametryne was not detected in the wastewater from ametryne production, so there appears to be no need for ametryne degraders in the treatment of production wastewaters. However, there may be an application for the organisms in the treatment of spills (10) or in the detoxification of pesticide on apparatus (20).

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