APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1979, p. 222-226
0099-2240/79/02-0222/05$02.00/0
Vol. 37, No. 2

Conversion of 2,4,6-Trinitrophenol to a Mutagen by *Pseudomonas aeruginosa*

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Received for publication 7 November 1978

A strain of *Pseudomonas aeruginosa* reduced 2,4,6-trinitrophenol (picric acid) to 2-amino-4,6-dinitrophenol (picramic acid) under anaerobic conditions. Mutagenic assays of picric acid and picramic acid were carried out with histidine-requiring strains of *Salmonella typhimurium*. Picric acid (10 μg per plate) demonstrated mutagenicity (both frame shift and base substitution-type mutations) only after activation with a rat liver homogenate preparation. Picramic acid (1 μg per plate) induced both base pair substitution and frame shift-type mutations without activation by the rat liver preparation.

Disposal of obsolete explosives is a problem within the military because of the pollutant effects these compounds may have in the environment. Past methods of disposing of munition wastes have included dumping at specified landfill areas, dumping in deep water at sea (9) and, when quantities were small enough, incineration. All of these methods carry some potential for harm to the environment. For example, incineration creates a problem of air pollution, and disposal on land risks the possibility that toxic substances will elute or leach into locations where they may threaten aquatic life forms, animals, or humans.

Until recently, ammonium 2,4,6-trinitrophenoxide (ammonium picrate) was used by the military as an explosive. Large quantities of ammonium picrate are still on hand at load-assemble-pack plants or are contained in obsolete munitions. The toxicity, environmental persistence, and biodegradation products of ammonium picrate must be determined before attempting disposal.

In this investigation the biodegradation and mutagenicity of the picrate ion were studied employing 2,4,6-trinitrophenol (picric acid). The capability of a strain of *Pseudomonas aeruginosa*, isolated from a 2,4,6-trinitrotoluene waste pond (16), to reduce picric acid to 2-amino-4,6-dinitrophenol (picramic acid) was studied. The mutagenicity of picric acid and its metabolite, picramic acid, were investigated employing Ames tester strains of *Salmonella typhimurium* (2).

(This work was presented in part at the 77th Annual Meeting at the American Society for Microbiology, New Orleans, La., 8-13 May, 1977.)

MATERIALS AND METHODS

Organism. Preliminary experiments involved attempts to selectively enrich for microorganisms capable of metabolizing picric acid. Mixed cultures were obtained from soil, compost, activated sewage sludge, and estuarine sediment. Incubation for approximately 3 months under anaerobic and aerobic conditions demonstrated no detectable degradation of a 0.1% solution of picric acid. (No decrease in the concentrations of picric acid were detected by thin-layer chromatographic (TLC) analysis; see below). Subsequent experiments utilized a known denitrifier which had been previously identified as *Pseudomonas* y (16). Additional taxonomic studies with this organism demonstrated fluorescent pigment production on pseudomonas agar F (Difco) and abundant growth on nutrient agar at 42°C. Starch hydrolysis was negative, whereas gelatin liquefaction was positive. On pseudomonas agar P (Difco) the organism produced a blue pigment with λ_{max} = 647 nm (at pH 7), and in 2 N HCl λ_{max} = 520 nm. These absorption maximums agree with those reported for pyocyanine (7, 11). Therefore, the organism was identified as *P. aeruginosa*.

Medium. A basal salts medium was employed which contained 0.05% K_2HPO_4, 0.2% $MgSO_4 \cdot 7H_2O$ (8), and a nitrogen-free trace elements solution (5). As a supplemental carbon source, 0.1% yeast extract (Difco) was added to the medium. The concentration of picric acid (Allied Chemical Co., New York, N.Y.) was 0.1%.

The medium was adjusted to pH 6.5 by dropwise addition of HCl and autoclaved for 15 min at 121°C. Analysis of picric acid by TLC (see below) demonstrated no alteration of the concentration of the compound upon autoclaving.

Incubation. A 4-liter Virtis laboratory fermentor, model 40-100AR (Virtis Research Equipment, Gardiner, N.Y.) operated at ambient temperature (25°C) and 200-rpm agitation, was used for anaerobic incubation. Initial medium volume was 3 liters. Oxygen in the medium was depleted by continuous bubbling of oil-free argon through the fermentor jar. Pure cultures

of *P. aeruginosa* were grown aerobically in 500 ml of nutrient broth for 24 h at 25°C, removed from the broth by centrifugation (7,000 × *g*), resuspended in 25 ml of basal salts medium, and added to the fermentor at a concentration of approximately 10^3 cells per ml. The picric acid-containing medium was incubated for 30 days after inoculation. Cell numbers were determined by means of the spread plate technique on nutrient agar (Difco) during the course of the experiment to monitor cell viability.

Chemical analysis. The loss of picric acid from the culture medium was measured by TLC. Samples taken periodically from the fermentor were filtered through a 0.45-μm filter (Millipore Corp.) to remove bacterial growth. To determine picric acid concentration, a 1-ml sample of the filtered medium was diluted 10-fold with distilled water and extracted with 2 ml of methyl ethyl ketone after the addition of approximately 3 g of NaCl. The efficiency of this method of extraction was 98.6%. The extracts (4 μl) were spotted on Quanta/gram LQD-F plates (Quantum Industries, Fairfield, N. J.). The plates were developed to 10 cm with 50/50/1 (vol/vol/vol) benzene–acetone–acetic acid. The R_f value of picric acid under these conditions was 0.17. The picric acid spots were scanned with a photodensitometer (Photovolt Corp., Edison, N.J.) equipped with an ultraviolet source. Peak areas were converted to picric acid concentrations by using a calibration curve obtained from the analysis of known picric acid solutions. The calibration curve was linear up to a 100-μg/ml concentration in original solution.

For preliminary identification of the degradation products, 10 ml of sample medium was extracted with 5 ml of methyl ethyl ketone after the addition of approximately 3 g of NaCl (extraction efficiency = 100%). The extract (5 μl) was analyzed on Silica Gel G TLC plates (Analtech, Canoga Park, Calif.) with the solvent system 95/5 (vol/vol) benzene–acetone. The TLC plates were developed three times and visually examined, scanned, and quantitated as described above. The calibration curve for picramic acid was linear up to 1.5 μg/ml.

For confirmation of picramic acid, a 300-ml sample of fermentor liquid was adjusted to pH 3.2 (with HCl), resulting in the formation of an unidentified brown precipitate. The solution was centrifuged, and the dark yellow supernatant was extracted three times with 25 ml of benzene. The benzene solution was back extracted with dilute HCl, pH 3.2, until TLC analysis indicated all the picric acid had been removed. Evaporation of the benzene and vacuum drying of the residue yielded 12.8 mg of a red crystalline material. Nuclear magnetic resonance spectra were obtained in trifluoroacetic acid on a Varian model T-60 (Varian, Palo Alto, Calif.). Ultraviolet-visible spectra were recorded on a Varian model 219 ultraviolet-visible spectrophotometer (Varian, Palo Alto, Calif.).

Mutagenesis assay. The mutagenic assay used was that of Ames, which employs histidine-requiring auxotrophs of *S. typhimurium* (2). The procedure for carrying out this assay has been previously described (2, 15). *S. typhimurium* strains TA 1535 and TA 100 were used to detect base substitution mutations; frame shift mutations were determined with strains TA 1538 and TA 98.

For metabolic activation studies, reduced nicotinamide adenine dinucleotide phosphate-dependent microsomal enzymes in a rat liver homogenate were added to the system. The procedure for this assay has also been previously described (1, 15).

RESULTS

Incubation of *P. aeruginosa* with picric acid resulted in a 2-log increase in cell number during the first 24 h. Bacterial counts remained between 10^5 and 10^7 cells per ml for the remaining 30 days, during which time the pH of the medium dropped from 6.5 to 6.3. The increase in cell numbers was probably due to consumption of supplemental yeast extract. Reduction of picric acid by *P. aeruginosa* was first shown by a color change in the medium from yellow to reddish brown.

LC analysis of partially reduced picric acid solutions with a 95/5 (vol/vol) benzene–acetone solvent system indicated three substances were present with R_f values of 0.47, 0.32, and 0.0. No other colored components were observed. Two potential metabolic products, 2,4-dinitrophenol and 2,6-dinitrophenol, were chromatographed separately under the described conditions and appeared as yellow spots with R_f values of 0.42 and 0.58, respectively. These dinitrophenols were not detected in the test medium. Two of the three spots observed with the solvent system had R_f values identical to authentic picric and picramic acid. The third spot, which remains at the origin, has not yet been identified.

The nuclear magnetic resonance and ultraviolet-visible spectra of the recovered red crystals and picramic acid were in excellent agreement. Nuclear magnetic resonance spectra consist of two doublets δ 8.5 and 8.8 (external tetramethylsilane) with coupling constants $J_H = 3$ cycles/s in trifluoroacetic acid. The ultraviolet-visible spectra of both the acidic and basic forms of the red material in water matched authentic picramic acid with $\lambda_{max} = 404$, 313, and 230 nm for the basic forms and $\lambda_{max} = 356$, 283, and 224 nm for the acidic form, giving isosbestic points at 366, 343, 301, 258, and 238 nm. Therefore, the red crystalline compound was identified as picramic acid.

Quantitative assessment of the depletion of picric acid and the production of picramic acid is shown in Fig. 1. Adsorption of picric acid to fermentor flask side walls and bacterial surfaces caused an initial decrease in picric acid concentration, and therefore chemical analyses were not performed until after 24 h of incubation. The concentration of picric acid in samples extracted after 1 day of incubation, as compared with samples extracted after 30 days of incubation, showed a decrease of approximately 22% (240 to

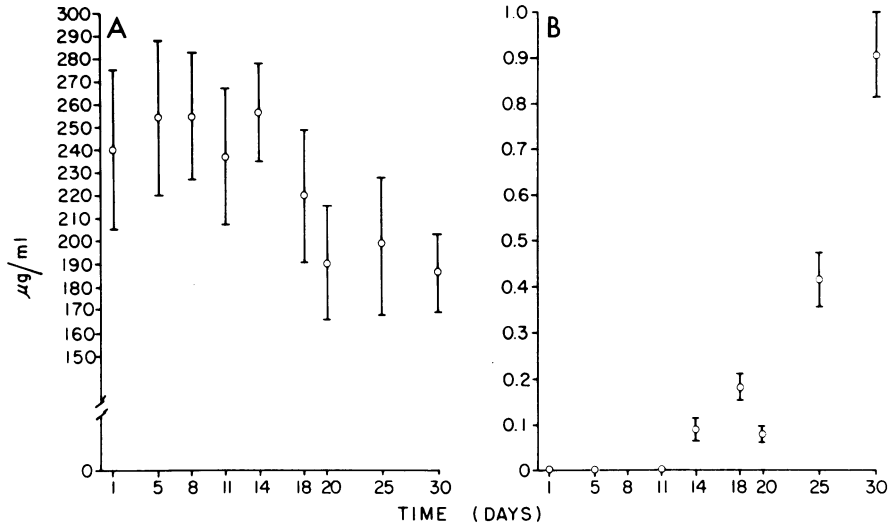

FIG. 1. *Depletion of picric acid (A) and production of picramic acid (B) during 30 days of anaerobic incubation with P. aeruginosa. Each point represents the mean of triplicate analyses; vertical lines represent the standard deviation about the means.*

187 μg/ml). A correction for extraction efficiency has been made with these values. The amount of picramic acid detectable in samples extracted after 30 days was 0.904 μg/ml. Therefore, approximately 1.7% of the picric acid which was lost was converted to picramic acid.

The remaining loss of picric acid may be attributable to adsorption onto bacterial surfaces and to bacterial accumulation, as well as formation of unidentified degradation products. The uptake of picric acid by the *Pseudomonas* organism was demonstrated by thoroughly washing the cells in physiological saline and sonically disrupting a suspension of the organism with a Bronwill sonic oscillator (Bronwill Scientific Inc., Rochester, N.Y.) to cause lysis. After extraction of the supernatant, analysis by TLC demonstrated the presence of picric acid. The amount of picric acid incorporated by the cells was not quantitated.

Screening of picric acid for mutagenicity with Ames tester strains of *S. typhimurium* showed no reversion activity. Strains TA 98 and TA 100 (test organisms for frame shift and base substitution mutations, respectively) manifested enhanced reversion when picric acid (10 μg per plate) was activated by a rat liver microsomal fraction.

The bacterial conversion product, picramic acid, caused reversion of both the base pair substitution and frame shift tester strains without activation. As shown in Fig. 2, the reversion frequency for these tester strains was found to increase linearly over the concentration range tested (1 to 200 μg per plate) for strain TA 100 (1 to 100 μg per plate). The lines shown in Fig. 2 were determined by simple linear regression analysis. Picramic acid appeared more potent as a base substitution mutagen than as a frame shift mutagen in that reversion frequency for TA 100 was 10-fold higher than that of the frame shift tester TA 98. At a concentration of 200 μg per plate, the response of TA 100 was dampened by toxicity of the compound.

DISCUSSION

Investigation of the biodegradation of picric acid demonstrated the compound to be resistant to microbial attack. The mixed microbial cultures used as inocula in preliminary studies were selected from sources in the environment which could conceivably be sites of final deposition of ammonium picrate waste. Under enriched conditions with extended incubation periods, no biodegradation of the picrate ion was observed. The resistance of the picrate ion to microbial attack has been reported by other investigators. Ammonium picrate in wastewater was found to exhibit no biological oxygen demand and at a concentration greater than 300 μg/ml inhibited the normal biological oxygen demand of wastewater (12). Chambers et al. (4) studied the biological oxidations of 48 different phenolic compounds, including picric acid, by phenol-adapted bacteria. They found phenols to be increasingly resistant to bacterial attack as the number of nitro groups on the compound increased. Oxygen uptake in the presence of nitrated phenols was only a fraction above that of control values; picric acid was 1.4 times the endogenous rate.

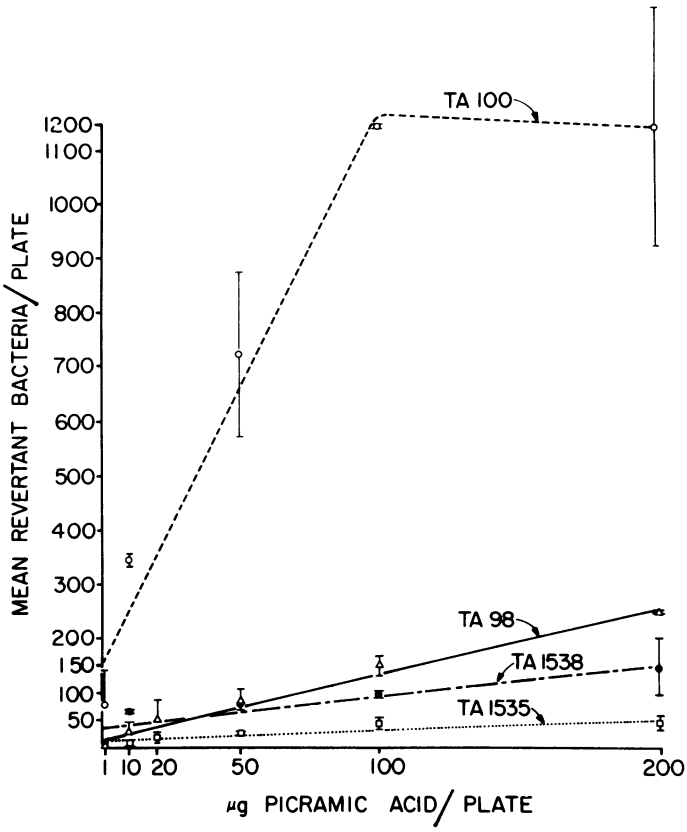

FIG. 2. *Mutagenic activity of picramic acid with S. typhimurium tester strains TA 1538 and TA 98, frame shift testers, and TA 1535 and TA 100, base substitution testers. (Each point represents the mean number for triplicate plates with vertical lines representing the standard deviation of the means. Lines drawn for each organism were determined by method of least squares. As computed by the Student's t test, the 95% confidence interval for the slope of each line is: for TA 100, b = 10.65 ± 2.45, r = 0.99; for TA 98, b = 1.18 ± 0.13, r = 0.99; for TA 1538, b = 0.56 ± 0.27, r = 0.90; and for TA 1535, b = 0.21 ± 0.03, r = 0.91.)*

Limited bioconversion of picric acid to picramic acid was accomplished in the present investigation by employing *P. aeruginosa*. The bacterial reduction of picric acid has been observed with *Mycobacterium avium* by Tsukamura (14). He described aerobic reduction of picric acid to picramic acid by whole cells as well as cell-free extracts of *M. avium* and suggested that the reduction of picric acid is enzymatically carried out through a reduced nicotinamide adenine dinucleotide-flavoprotein system in this organism.

In humans also, picric acid is reduced to picramic acid. In early studies (3), the urine of malingering individuals became a blood red color after consumption of picric acid. Analysis of urine demonstrated the presence of picramic acid. In perfusion studies with liver, kidney, and spleen, the liver was the site of strongest reduction of picric acid to picramic acid (6).

Although it was not detected in this investigation, the formation of dinitrophenols from microbial metabolism of picric acid seems plausible. Gundersen and Jensen (8) described the aerobic metabolism of picric acid and other organic nitro compounds by *Corynebacterium simplex* which was isolated from soil chronically exposed to organic nitro herbicides; biodegradation was indicated by the production of nitrite. Nitrite formation from these compounds requires the cleavage of carbon-nitrogen bonds, and thus the production of dinitrophenols could certainly be suggested, although they were not analytically demonstrated.

Employing the Ames mutagenicity assay, picramic acid and activated picric acid were found to be base substitution as well as frame shift mutagens. As discussed by McCann et al. (10), the base substitution mutant TA 100 and the frame shift mutant TA 98 have been made more

sensitive to certain mutagens than the parent tester strains TA 1535 and TA 1538. Apparently, picramic acid belongs to that class of mutagens that could be detected readily with a highly sensitive tester, as is manifested in the response of TA 100. It appears that picric acid belongs to the large class of compounds that are not mutagenic themselves but are metabolized by a host to form mutagens. Other typical examples of this group are the well-studied carcinogens aflatoxin B_1, benzo(α)pyrene, 2-acetylaminofluorene (2), and cycasin (methylazoxymethanol-β-D-glucoside) (13). Furthermore, it may be that picric acid is converted to picramic acid by the rat liver homogenate used in the Ames assay. This possibility was not investigated.

The results of this study suggest that picrates such as picric acid and ammonium picrate will persist in the environment; however, it is possible that the microflora at disposal sites may become acclimated to ammonium picrate and cause a slow reduction to picramic acid, a compound potentially more harmful than the parent picrate itself.

ACKNOWLEDGMENTS

We express our appreciation to Janet Coffey for her technical assistance, to John Blecka for his help in the identification of *P. aeruginosa*, and to William Coleman for his aid with nuclear magnetic resonance analyses.

This research was supported by the Naval Medical Research and Development Command and the Office of Naval Research.

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