

## Metabolic Disposition of 2, 4, 6-Trinitrotoluene

WILLIAM D. WON, ROBERT J. HECKLY, DONALD J. GLOVER, AND JOHN C. HOFFSOMMER

*Naval Biomedical Research Laboratory, School of Public Health, University of California, Berkeley, California 94720 and Naval Ordnance Laboratory, White Oak, Silver Spring, Maryland 20910*

Received for publication 1 November 1973

Three pseudomonas-like organisms have been shown to metabolically oxidize 2, 4, 6-trinitrotoluene (TNT). Capability for this oxidative dissimilation varied with each organism. Of the three, isolate "Y" was the most proficient, isolate "I" was less, and isolate "II" was the least. For accelerated TNT degradation, addition of glucose or a nitrogenous substance was essential. Complete dissimilation within 24 h by isolate "Y" cultures supplemented with 0.5% yeast extract is presumed since no TNT was detectable.

Disposal of waste 2, 4, 6-trinitrotoluene (TNT) becomes a particularly difficult problem in those operations such as shell loading, which use large volumes of hot water to wash off residual explosives. Relatively large volumes of water are required because TNT is only slightly soluble in water (100  $\mu\text{g}/\text{ml}$ ). The practice has been to discharge the water which is practically saturated with TNT into small drainage ditches; this water may then flow into a small holding lagoon before being discharged into local streams. TNT is toxic to certain fish at concentrations greater than 2  $\mu\text{g}/\text{ml}$  (2) and, in humans, TNT has been shown to cause liver injury and marked changes in the hematopoietic system producing anemia (3). Accordingly, it is indisputable that treatment of TNT wastes is important if these are to be discharged into receiving water, particularly when recreational or potable water is involved. However, chemical or physical treatment of the waste is costly and inefficient.

The results of studies on microbial degradation of TNT, described in this report, indicate that biological treatment can be an effective and efficient method for disposing of TNT dissolved in water before it is discharged from the processing plant area.

### MATERIALS AND METHODS

**Organisms.** Three yellow pigment-producing organisms were isolated from mud and water samples (obtained from the U.S. Naval Ammunition Depot at McAlester, Okla.) using the culture enrichment technique where about  $35 \times 10^{-5}$  M of TNT (explosive grade obtained from Naval Weapons Station, Concord, Calif.) was included in a basal salt solution consisting of  $86 \times 10^{-3}$  M NaCl,  $4 \times 10^{-3}$  M  $\text{MgSO}_4$ , and 0.02 M sodium-potassium phosphate (pH 7.0). These organisms were motile, slender, gram-negative

rods, producing acid in peptone water containing one of the following: glucose, glycerol, galactose, fructose, sucrose, or xylose. No reaction was observed in peptone-lactose, -sorbitol, -mannitol, or -starch. Litmus milk was not coagulated. However, one of these organisms was capable of reducing nitrates, whereas another was capable of forming indole. Optimal temperature for growth was about 30 C. Tentatively, these organisms were regarded as belonging to the genus *Pseudomonas* and designated as isolate "Y" (nitrate reducer), "I" (indole former), and "II". Stock cultures were maintained in peptone-glucose broth at 4 C, subculturing in freshly prepared media at monthly intervals. Although parallel studies were conducted with all three strains, only data pertaining to isolate "Y" are presented.

**Growth and oxidation experiments.** Cultures were grown in 100 ml of TNT-enriched basal medium in 250-ml Erlenmeyer flasks at 30 to 32 C on a rotary shaker having a throw of about 3.5 cm at 200 rpm. In media supplemented with yeast extract (0.5%) sterilization was effected by saturated steam at 15 psi for 15 min. Sterile glucose was introduced aseptically, when called for, into sterile media to yield a 0.1% concentration. Viability was determined by preparing 10-fold serial dilutions in 1% peptone and plating 0.1 ml of appropriate dilutions on nutrient agar containing 0.5% glucose. Inoculated plates were incubated for 24 h at 35 C.

For  $\text{O}_2$  consumption determination, cells from 24-h peptone-glucose broth cultures were sedimented at  $3,000 \times g$  for 20 min at 5 C followed with resuspension to 0.1 of the original volume in physiological saline yielding approximately  $4 \times 10^{10}$  viable cells/ml. Measurements of the  $\text{O}_2$  uptake were made at 35 C by using a Warburg respirometer according to standard procedures (4). The cell substrate mixture consisted of 0.5 ml of cell suspension and 1.0 ml of TNT solution (80  $\mu\text{g}$ ). For pH adjustment, dilute NaOH or HCl was added to the mixture.

**Analytical procedures.** Samples for analysis were prepared by centrifugation of the culture suspensions to remove bacterial growth, after which, 0.3 ml of reagent grade benzene was added to 10 or 20 ml of

centrifugate depending on the level of TNT concentration contained in screw-capped culture tubes (16 by 150 mm). Extraction was carried out by shaking at room temperature for 5 min in a clinical reciprocating shaker at 160 strokes per min through a distance of 2 inches.

Routinely, TNT was assayed by using a Hewlett-Packard F and M gas chromatographic unit (Model 700) fitted with a flame ionization detector. The column (0.32 mm by 30.5 cm) packing was Chromosorb G (80/100 mesh) coated with 2% OV-101, operating at 190 C. The injector temperature was set at 200 C, and the detector was set at 290 C. The flow rate of the carrier gas ( $N_2$ ) was 15 ml/min. For definitive analyses, particularly the 2,6-dinitro-4-aminotoluenes (4-Am) and the 4,6-dinitro-2-aminotoluenes (2-Am) were analyzed with a Hewlett-Packard research gas chromatograph with a high-temperature nickel-63 electron capture detector. Optimum conditions for these analyses employed a glass column (4 ft by  $\frac{1}{4}$  inch) packed with 6.7% Apiezon M liquid phase on Diatoport S 60/80 mesh solid support maintained isothermally at 180 C with a flow rate of 168 ml/min, argon/methane, 95/5 (vol/vol), and a detector temperature of 275 C pulsed at 150-microsecond intervals. Injection port temperatures were 195 to 200 C. Chromatographic areas of eluted compounds as well as their retention times were measured with an Infotronics automatic digital integrator, model CRS-208. Under these conditions, the retention times of 2-amino-4,6-dinitrotoluene (2-Am) and 4-amino-2,6-dinitrotoluene (4-Am) were 635 and 881, respectively, and the relative responses on a gram basis were 0.677 and 0.603, respectively. Ortho-dinitrobenzene (J. T. Baker Chemical Co.) was used as an internal standard in both unknown and standard solutions. Analyses were made by direct comparison of integrated areas of unknown solutions to known standard concentrations after normalization of internal standard integrated areas. The 2-Am 4-Am, and other reference materials, were prepared at the Naval Ordnance Laboratory.

Nitrite and nitrate ion concentrations in aqueous solutions were measured by a recently developed method which will be reported more fully (D. J. Glover and J. K. Hoffsomer, *J. Chromatogr.*, 1974, in press). Briefly, the nitrite or nitrate was converted to nitrobenzene which was then analyzed by vapor phase chromatography.

For thin-layer chromatographic analysis, 10-ml samples of the cultures were extracted twice with 5 ml of ethyl ether for 10 min each. The combined extracts were evaporated to dryness and redissolved in 10  $\mu$ liters of acetone. The entire sample, and 2  $\mu$ liters of a reference solution containing 10  $\mu$ liters each of the TNT derivatives per ml of acetone, was spotted on a silica gel sheet (20 by 20 cm) (Polygram Sil G, Brinkman Instrument Inc., Burlingame, Calif.). This was subsequently developed twice at 20 C by the ascending method with a solvent system comprised of toluene/benzene/hexane (10:10:5 by volume) for a distance of 10 cm (total time 30 min). Spots of intermediates were visualized under ultraviolet illumination. Identity of the spots was confirmed by extraction with benzene with analysis carried out in a

Varian MAT-III gas chromatograph-mass spectrometer unit.

## RESULTS AND DISCUSSION

The present studies show that pseudomonad isolates can degrade TNT. Results of respirometric studies indicate that TNT was a biologically oxidizable substrate. Furthermore, as shown in Fig. 1, oxidation proceeded without a lag. The  $O_2$  consumption by isolate "Y" with TNT during a 4-h period was 23% greater than the endogenous uptake. In contrast, the  $O_2$  consumption with TNT by other similar isolates ranged from 0 to 7.5% above endogenous  $O_2$ . Gas uptake was negligible in substrate controls without cells. In all instances, pH in the region of 6 to 7.2 was found optimal for maximal  $O_2$  uptake and for survival at 30 C. Acidic solutions (pH 2 to 3.5) were very unfavorable since these culture suspensions appeared to be sterile after 24 h. At pH values greater than 7.7, viability was also adversely affected. Moreover, alkaline media induced pleomorphism in cell population and about 30% of the cells in 48-h cultures at pH 7.7 formed small, atypical colonies about 0.5 mm in diameter.

Cultivations at pH 6.5 to 7.2 appeared to effect TNT oxidation more efficiently than those of lower or higher pH values. As in the respirometric studies, utilization of TNT varied with the isolates. Isolate "Y" oxidized or degraded TNT most effectively, whereas isolate "II" was the least effective. A typical course of TNT degradation by isolate "Y" in the TNT basal media containing glucose is shown in Fig. 2. The reduction in the number of viable cells was probably a result of acid production from the sugar. Chromatographic analysis revealed two intermediate products of TNT dissimilation whose retention times were identical, respectively, to that of 2-Am and that of 4-Am. However, the full significance of these products remains to be determined since only a small

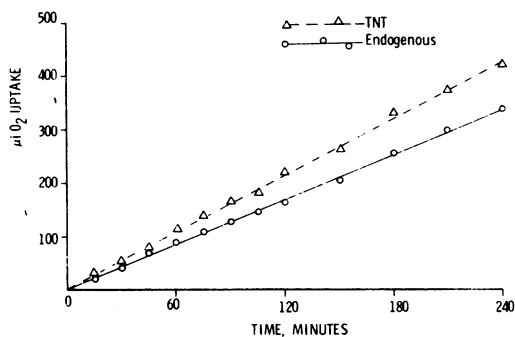


FIG. 1. Effect of TNT on oxygen uptake by "Y" at 35 C, pH 6.0.

portion of the TNT nitrogen can be accounted for in these and those of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  ions.

On the other hand, when analyses were made with thin-layer chromatography, additional well-defined intermediates were visible under ultraviolet illumination. Figures 3, 4, and 5 represent the thin-layer chromatograms of extracts from isolate "Y" cultures in the basal TNT medium that was enriched with 0.5% glucose or 0.5% yeast extract after selected

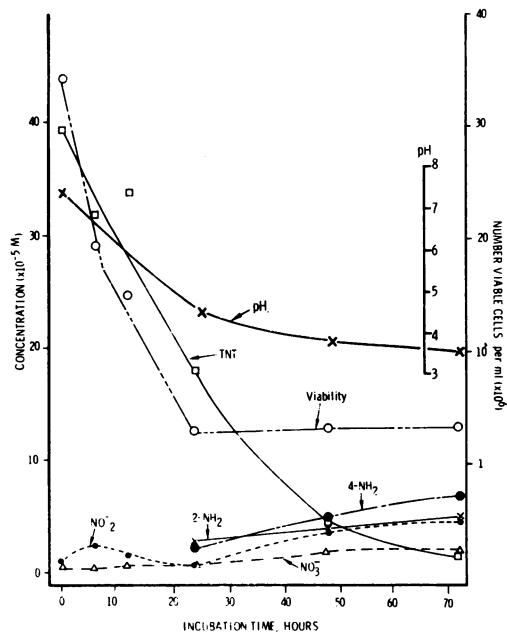


FIG. 2. Degradation of TNT by isolate "Y" in basal medium supplemented with  $5 \times 10^{-3}$  M glucose. 4-NH<sub>2</sub> = 4-amino-2,6-dinitrotoluene. 2-NH<sub>2</sub> = 2-amino-4,6-dinitrotoluene.

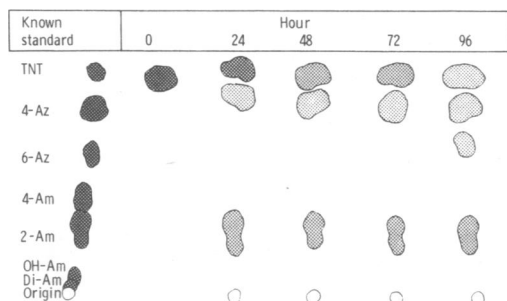


FIG. 3. Ascending thin-layer chromatographic separation of TNT metabolites in isolate "Y" cultures. TNT =  $\alpha$ -trinitrotoluene; 4-Az = 2,2',6,6'-tetranitro-4-azoxytoluene; 6-Az = 2,2',4,4'-tetranitro-6-azoxytoluene; 4-Am = 4-amino-2,6-dinitrotoluene; 2-Am = 2-amino-4,6-dinitrotoluene; OH-Am = 2,6-dinitro-4-hydroxylaminotoluene; DiAm = Nitrodiaminotoluene.

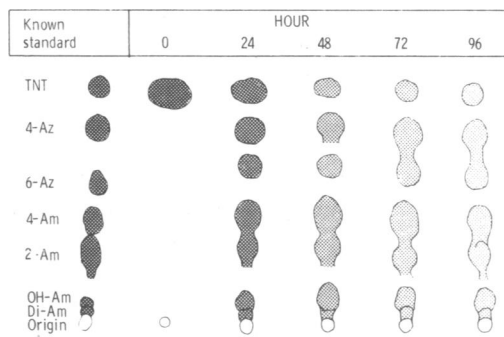


FIG. 4. Ascending thin-layer chromatographic separation of TNT metabolites in isolate "Y" cultures supplemented with 0.5% glucose. TNT =  $\alpha$ -trinitrotoluene; 4-Az = 2,2',6,6'-tetranitro-4-azoxytoluene; 6-Az = 2,2',4,4'-tetranitro-6-azoxytoluene; 4-Am = 4-amino-2,6-dinitrotoluene; 2-Am = 2-amino-4,6-dinitrotoluene; OH-Am = 2,6-dinitro-4-hydroxylaminotoluene; DiAm = Nitrodiaminotoluene.

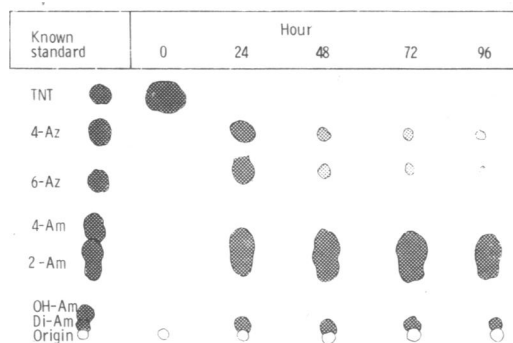


FIG. 5. Ascending thin-layer chromatographic separation of TNT metabolites in isolate "Y" cultures supplemented with 0.5% yeast extract. TNT =  $\alpha$ -trinitrotoluene; 4-Az = 2,2',6,6'-tetranitro-4-azoxytoluene; 6-Az = 2,2',4,4'-tetranitro-6-azoxytoluene; 4-Am = 4-amino-2,6-dinitrotoluene; 2-Am = 2-amino-4,6-dinitrotoluene; OH-Am = 2,6-dinitro-4-hydroxylaminotoluene; DiAm = Nitrodiaminotoluene.

periods of incubation. It is evident that TNT disappeared most rapidly in cultures supplemented with yeast extract (Fig. 5). At 24 h the TNT concentration was reduced from 100  $\mu\text{g}/\text{ml}$  to less than 1  $\mu\text{g}/\text{ml}$ , whereas a substantial amount of TNT persisted up to 96 h in the corresponding control and glucose-supplemented cultures (Fig. 3 and 4). Unidentifiable benzene soluble products were either absent or not detectable by this procedure. Apparently, as indicated in Fig. 5, TNT was metabolized to yield (in descending order) 2,2',6,6'-tetranitro-4-azoxytoluene (4-Az), its isomer 2,2',4,4'-tetranitro-6-azoxytoluene (6-Az), 4,6-dinitro-2-aminotoluene (2-Am), 2,6-dinitro-4-hydroxyl-

aminotoluene (OH-Am), and nitrodiaminotoluene (DiAm). After depletion of TNT (24 h) the azoxy compounds are shown to degrade gradually, approaching complete disappearance at 96 h. On the other hand, these organisms seemed incapable of oxidizing the intermediates 2-Am and DiAm, the levels of these appearing to remain constant throughout the 96-h observation period. Other potential intermediates 4-Am, an isomer of 2-Am, and OH-Am were not demonstrable in the chromatograms. Based on what has been reported on the oxidation of TNT in other biological systems (1) the metabolic pathway leading to the formation of these products may be assumed that at the initial stage  $\alpha$ -TNT was reduced to form 2,6-dinitro-4-hydroxylaminotoluene or to its isomer, 2,4-dinitro-6-hydroxylaminotoluene, each of which was further reduced to yield the respective 4-amino and the 2-amino compounds. These, in turn, were further reduced, forming the end product DiAm. According to Channon et al. (1), the azoxy compounds may not be the products of direct TNT metabolism. It is possible that these substances were formed from the coupling reactions of the corresponding hydroxylamines.

Observations from this work have far-reaching possibilities in the solution of TNT waste disposal problems. Further studies on the metabolism of these and other isolates, including artificially induced mutants, may lead to a more rapid and complete TNT degradation process.

#### ACKNOWLEDGMENTS

This work was sponsored by the Naval Ordnance Systems Command through a contract between the Regents of the University of California and the Office of Naval Research.

Our sincere thanks to Cheryl Wang for her skillful technical assistance, and to Eillis Pardee, NAD, McAlester, Okla., for assistance in obtaining soil and water samples from which the organisms were isolated.

#### LITERATURE CITED

1. Channon, H. J., G. T. Mills, and R. T. Williams. 1944. The metabolism of 2:4:6-trinitrotoluene ( $\alpha$ -TNT). *Biochem. J.* **38**:70-85.
2. Mathews, E. R., E. H. Tex, R. L. Taylor, and J. F. Newell. 1954. Laboratory studies pertaining to the treatment of TNT wastes. Paper presented to Division of Water, Sewage and Sanitation Chemistry, 126th Meeting, American Chemical Society, New York, N.Y.
3. Sax, N. I. 1957. *Dangerous Properties of Industrial Materials*. Reinhold Publ. Corp., New York, N.Y.
4. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1957. *Manometric and biochemical techniques*, 3rd ed., Burgess Publishing Co., Minneapolis, Minn.