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Toxicity and Mutagenicity of 2,4,6-Trinitrotoluene and Its Microbial Metabolites

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TNT (2,4,6-trinitrotoluene) of explosive grade is highly toxic to marine forms that included fresh water unicellular green algae (*Selenastrum capricornutum*), tidepool copepods (*Tigriopus californicus*), and oyster larvae (*Crassostrea gigas*), and mutagenic to *Salmonella typhimurium*. On the basis of mutagenic assays carried out with a set of histidine-requiring strains of the bacterium, TNT was detected as a frameshift mutagen that significantly accelerates the reversion rate of a frameshift tester, TA-98. In contrast, the major microbial metabolites of TNT appeared to be nontoxic and nonmutagenic.

Earlier we reported the isolation of pseudomonad-like organisms capable of degrading solubilized TNT (2,4,6-trinitrotoluene), thereby providing a potentially safe and economical procedure for disposal of TNT wastes (9). However, since TNT was not completely oxidized by the organisms, we have investigated whether TNT itself as well as the resultant metabolites posed health or ecological hazards. For these studies we have used as host models, *Selenastrum capricornutum* (fresh water unicellular green algae), *Tigriopus californicus* (salt water copepods), and *Crassostrea gigas* (oyster larvae) to observe toxic action of TNT and its degradative compounds, as indicated by changes in growth or death rates plus morphological alterations in *S. capricornutum*. Mutagenicity was assayed for by exposing histidine-requiring mutants of *Salmonella typhimurium* to the test substance and monitoring reversions to histidine independence (1).

MATERIALS AND METHODS

Algal, microcrustacean, and oyster larval cultures. *S. capricornutum* Printz, a fresh water unicellular green algae, was obtained from the National Eutrophication Research Program, Environmental Protection Agency, Pacific Northwest Water Laboratory, Corvallis, Ore. Cultivation was in the modified chemically defined medium of Guillard and Ryther (GR medium, Table 1) for 7 days at 25 C under 300-foot candlepower fluorescent lighting (4).

Tidepool copepods (*T. californicus*) were obtained from the California coastline and cultivated at 20 C in seawater enriched with GR medium (5 ml/liter of seawater). Oyster larvae (*C. gigas*) were obtained from Pigeon Point Research Center, Pescadero, Calif. These larvae, averaged about 225 μm in length, were 20 days old and were kept until use (within 96 h) in 20 C seawater containing, as feed, cultured phytoplankton (*Cyclotella nana*).

Toxicity assays of TNT and its metabolites. TNT of explosive grade was obtained from the Naval Weapons Station, Concord, Calif. TNT metabolites (Table 2), discussed previously (9), were prepared by chemical reduction of TNT according to procedures described by Sitzman (7). They were the generous gift of John C. Hoffsommer of the Naval Ordnance Laboratory, White Oaks, Silver Spring, Md.

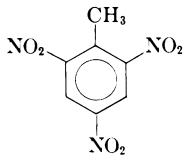
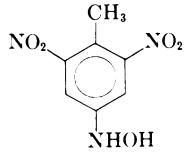
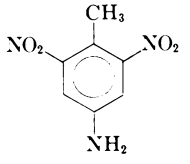
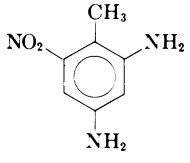
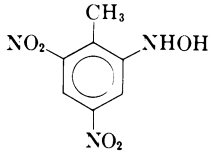
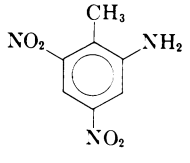
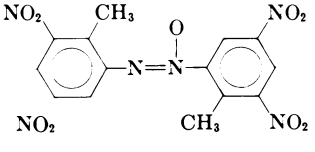
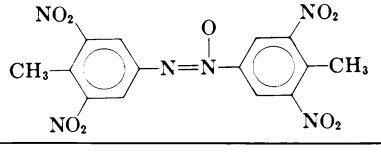
For toxicity evaluation with *S. capricornutum*, TNT at 100 mg/liter and separately each of the metabolites (ranging from 50 to 5 mg/liter, depending on the saturation solubility of each) was dissolved in demineralized water, to which was added the chemical components required for the GR medium mentioned above. Media containing lower con-

TABLE 1. *Composition of the modified Guillard-Ryther medium*[a]

Comp	Concn
Macronutrients[b] (μg/liter)	
$NaNO_3$	25.5
K_2HPO_4	1.0
$MgCl_2 \cdot 6H_2O$	12.1
$MgSO_4 \cdot 7H_2O$	14.7
$CaCl_2 \cdot 2H_2O$	4.4
$NaHCO_3$	15.0
Micronutrients[b] (μg/liter)	
H_3BO_3	185.5
$MnCl_2$	264.2
$ZnCl_2$	33.0
$CoCl_2$	0.8
$CuCl_2$	0.1
$NaMoO_4 \cdot 2H_2O$	7.3
$FeCl_3$	96.0
$NaEDTA \cdot 2H_2O$	300.0

[a] pH = 6.8 to 7.2.
[b] In glass-distilled water.

TABLE 2. *TNT and its reduction products tested*

Comp	Structure	Stock concn (mg/liter)
TNT		100.0
2,6-Dinitro-4-hydroxyaminotoluene		5.0
2,6-Dinitro-4-aminotoluene		50.0
2-Nitro-4,6-diaminotoluene		50.0
2,4-Dinitro-6-hydroxyaminotoluene		5.0
2,4-Dinitro-6-aminotoluene		50.0
2,2′,4,4′-Tetranitro-6,6-azoxytoluene		5.0
2,2′,6,6′-Tetranitro-4,4′-azoxytoluene		5.0

centrations of each of the test substances were prepared by making appropriate dilutions using the GR medium as diluent. Sterilization was by filtration through 0.22-μm membrane filters. Assays were carried out in triplicate 50-ml samples contained in 125-ml Erlenmeyer flasks. Inoculation was with 0.5 ml of an algal culture diluted to give a final viable concentration of 3×10^4 cells/ml. Incubation was for 7 days under light as mentioned above. Algal cell yields, in terms of dry weight postincubation and morphological alterations, were used as the measure of relative toxicity.

In titrations with *T. californicus*, 100 copepods were screened from culture aquaria, rinsed with fresh seawater, and then placed in each 50-ml triplicate flask of TNT or metabolite substrate, prepared as described above except that seawater was used as the diluent or solvent for the test substances. Incubation was for 3 days at 20 C. Relative toxicity was evaluated in terms of mortality response.

Similar procedures and interpretation were followed in titrations using *C. gigas* as the host indicator. In this system, however, larvae in groups of 200 were exposed at 20 C for 96 h to the various concentrations of the test substances.

Mutagenesis assays. Ames' assay system, using histidine-requiring strains of *S. typhimurium*, was used for determining mutagenicity (1). These strains of organisms with known genetic makeup and with a loss of excision repair system for deoxyribonucleic acid are capable of detecting and differentiating mutagens that cause base substitution of an adenine-thymine base pair or those that cause shifts in the reading frame of messenger ribonucleic acid. The technique, essentially is a pour plate procedure; the tester strain, 0.1 ml (12-h nutrient broth culture), was mixed with 2.0 ml of sterile soft agar (0.6% agar + 0.5% NaCl + 0.5 mM L-histidine + 0.5 mM biotin) containing a given concentration of the compound to be assayed. The mixture was poured onto the surface of minimal agar plate of the following composition, in g/liter: $MgSO_4 \cdot 7H_2O$, 0.2; citric acid $\cdot H_2O$, 2.0; K_2HPO_4, 10; and $NaNH_4HPO_4 \cdot 4H_2O$, 3.5 (8). Incubation was at 37 C for 48 h. For TNT activation studies 0.5 ml of phenobarbitol-induced rat liver microsomal enzymes, prepared according to procedure described by Ames for enhancing detection sensitivity of the system (2), was added into the soft agar overlay before pouring. The number of revertant colonies formed in these and control plates, examined in triplicate, were recorded. Reversion frequency as is referred to here represents the difference between the average number of mutagen-induced revertants and that spontaneously produced in the controls.

RESULTS AND DISCUSSION

No toxicity was observed in assays with fresh water algae, oyster larvae, and copepods exposed to graded concentrations of each of the TNT breakdown products over the observation period given for each of the organisms. In marked contrast, clear-cut toxicity was noted in the TNT-supplemented cultures. For example, in the case of *S. capricornutum*, TNT at as low as 2.5 mg/liter suppressed growth significantly (Table 3), evoked chlorosis as judged by visual comparison with the controls, and produced a dominant population (80 to 90%) of ballooned, extensively granulated cells (Fig. 1).

In the two systems testing higher forms in the natural food chain, namely, oyster larvae and copepods, exposure to concentrations of TNT greater than 5.0 mg/liter likewise resulted in drastic increase in death rates (Table 4). The test organisms, particularly *C. gigas*, showed signs of relaxed ciliary activity followed by complete arrest and cessation of locomotion and feeding as early as 24 h after initial exposure to TNT concentrations greater than 10 mg/liter.

With respect to potential mutagenic activity of TNT metabolites, revertant colony counts of tester strains of *S. typhimurium* were essentially similar to the controls; this suggests that TNT intermediates are nonreactive and nonmutagenic.

In the case of TNT, however, at concentrations over 10 μg/ml of overlay toxic effects in terms of growth inhibition were noted. At lower concentrations (0.5 to 10 μg/ml of overlay), our data indicated TNT is a frameshift mutagen characterized by a linear mutagenic response curve. The direct correlation between TNT concentration and reversion rate extended to 10 μg/ml, above that concentration, as indicated, TNT was inhibitory in that no growth developed at 48 h. At 72 h, besides a few (<15) sporadic revertant-like colonies, there was development of a lawn consisting almost entirely of tiny (<0.5 mm), rough, dry-appearing colonies not at all like those of the controls or test plates containing TNT at or below the 10-μg level. In contrast, no such mutagenic activity was detected in cultures inoculated with base substitution tester strains, even when they were cultivated in systems supplemented with microsomes to activate TNT. In experiments using frameshift testers, frameshift mutation was induced only in cultures inoculated with strain TA-98, a histidine-requiring frameshift

TABLE 3. *Influence of TNT on growth of S. capricornutum*

TNT (mg/liter)	Mean dry wt (mg/50 ml)
100.0	0
50.0	0
25.0	0
10.0	0
5.0	2.8
2.5	6.4
1.0	12.8
0	14.6

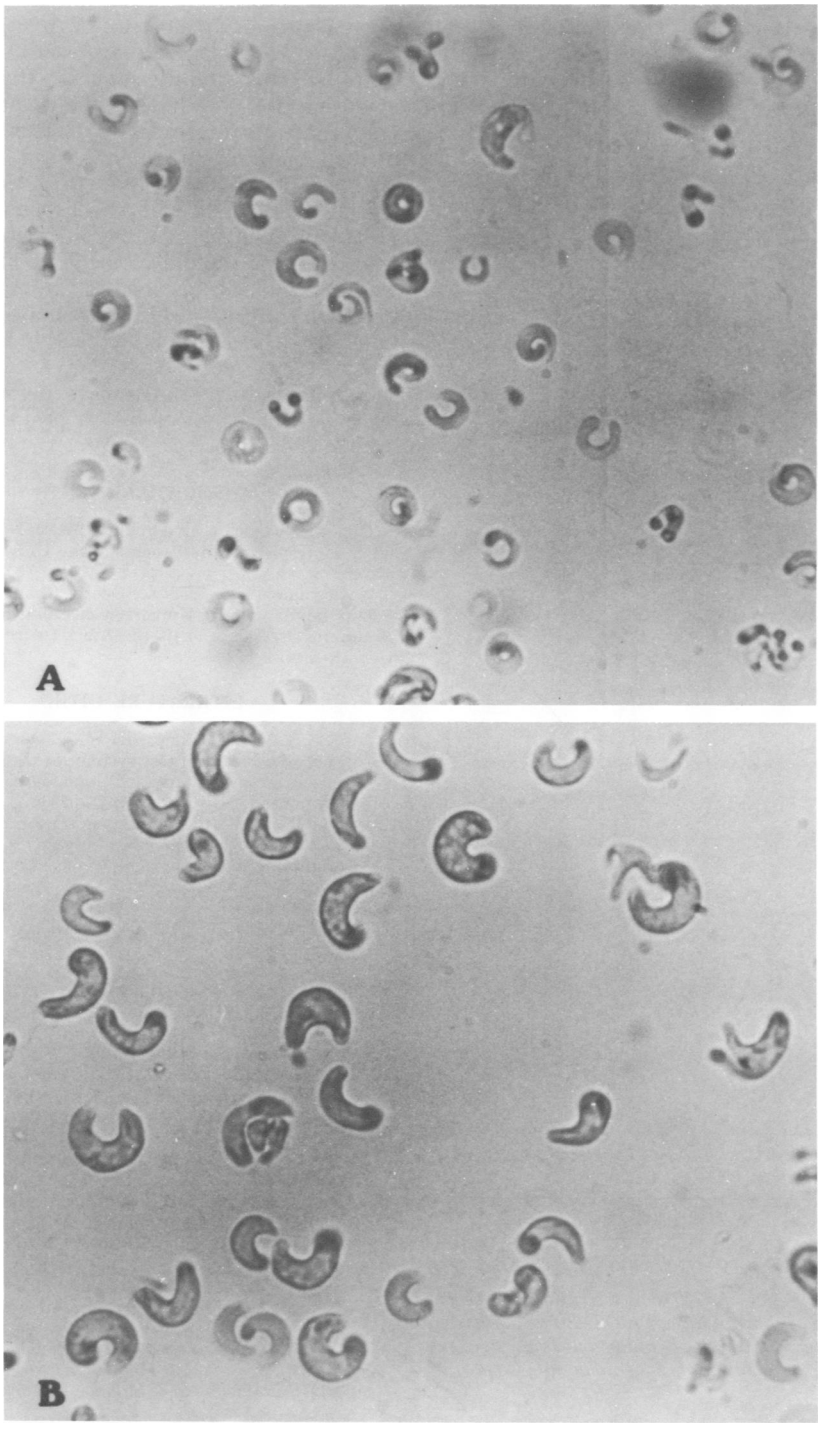


FIG. 1. *Morphological reaction of S. capricornutum to TNT (2.5 mg/liter) after 72-h exposure period. (A) Untreated control cells. (B) Ballooned, granulated cells induced by TNT. Approximately ×400.*

mutant made more sensitive to certain mutagens by introducing an antibiotic-resistant transfer factor plasmid (pKM101) into the strain by mating strain TA-1538 with donor strain TA-2000 (5, 6). A typical TNT induction of reversion in strain TA-98 is presented in Fig. 2.

TABLE 4. *Toxicity of TNT following 3-day exposure of T. californicus and 4-day exposure of C. gigas*

TNT (mg/liter)	Mortality	
	No. of dead/no. exposed	
	T. californicus	*C. gigas*
100	100/100	200/200
50	100/100	200/200
25	100/100	200/200
10	80/100	163/200
5	44/100	0/200
2.5	18/100	0/200
1.0	0/100	0/200
Control	0/100	0/200

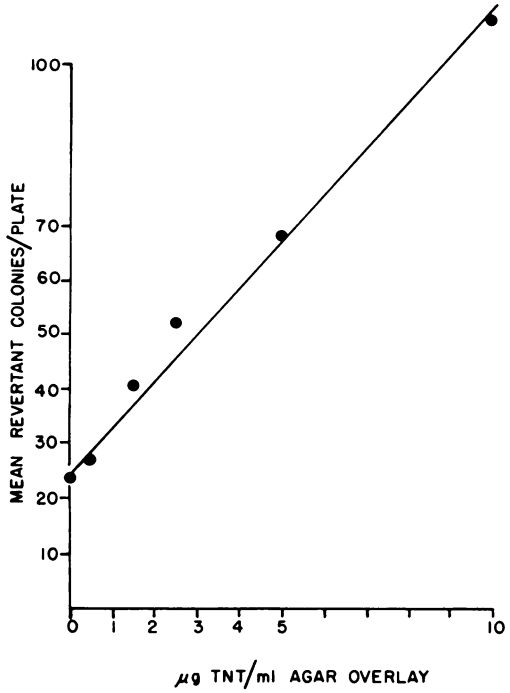


FIG. 2. *Influence of TNT concentrations on reversion rate of S. typhimurium TA-98. Each point represents the mean of triplicate plate counts. At concentration above 10 μg/ml there was no visible growth in 48 h.*

In instances where TNT was activated with microsomal enzyme, strain TA-98 showed no increased reversion rate over that seen in the controls. This finding suggests that enzymic oxidation of TNT in vitro yields nonreactive, nonmutagenic intermediates. If this be the case in vivo as well, since it has been established that the hepatobiliary system is the primary site of metabolism of hydrocarbons and since identical, major nonmutagenic metabolic products have been found in urine of TNT factory workers and of experimental animals (3), the health hazard of TNT per se may be of little importance. Exceptionally, carcinogenicity or mutagenicity and toxicity might occur when the hepatobiliary function is defective or is overwhelmed by excessive, or prolonged, exposure to the explosive.

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