APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1978, p. 949-954
0099-2240/78/0035-0949$02.00/0

Vol. 35, No. 5
Printed in U.S.A.

Microbial Transformation of ^{14}C-Labeled 2,4,6-Trinitrotoluene in an Activated-Sludge System†

DAVID F. CARPENTER,†† NEIL G. McCORMICK,* JOHN H. CORNELL, AND ARTHUR M. KAPLAN

Environmental Protection Group, U.S. Army Natick Research and Development Command, Natick, Massachusetts 01760

Received for publication 14 November 1977

The fate of ^{14}C-labeled 2,4,6-trinitrotoluene (TNT) in an activated-sludge system was investigated. No [^{14}C]TNT could be detected in the contents of an aerated reactor after 3 to 5 days of incubation. No significant $^{14}CO_2$ was formed, and the radioactivity was about equally divided between the floc and the supernatant. The radioactive carbon present in the microflora was mainly associated with the lipid and protein components, but the characteristic constituents of these compounds (e.g., fatty acids and amino acids) were not radioactive. The major part of the ^{14}C present in the lipid and protein fractions was found in precipitates that formed in both fractions. The solubility properties and infrared spectra of these precipitates suggested that they are macromolecular structures of the polyamide type formed by the reaction of TNT biotransformation products with lipids, fatty acids, and protein constituents of the microbial flora. This hypothesis is further supported by the correspondence of the infrared spectrum of the lipid precipitate with that of a model compound synthesized from TNT transformation products and lipid precursors. The resistance of these macromolecules to further biodegradation was paralleled by the reported resistance to microbial attack of polyamides containing similar linkages.

2,4,6-Trinitrotoluene (TNT) and related nitro aromatic compounds are difficult, if not impossible, to degrade completely (11, 23). The ability of biological systems to effect the disappearance of TNT has been investigated by numerous researchers. The initial reaction between a biological system and TNT is the stepwise reduction of the nitro groups to yield amino and hydroxylamino transformation products, which may then undergo additional reactions yielding azoxy compounds (2, 3, 6, 20). These reactions also occurred in vitro with homogenates of beef heart (22) or with cell-free extracts of *Escherichia coli* (17).

Microbiological systems have been evaluated for their potential value in eliminating TNT present in wastewaters from munitions-manufacturing facilities. Osmon and Klausmeier (13) found that inocula from a variety of sources were capable of eliminating TNT from a mineral salts medium supplemented with 1% yeast extract. Won et al. (23) observed that added organic nutrients stimulated the disappearance of TNT and its metabolites in cultures of *Pseudomonas*. However, Klausmeier et al. (7) reported that TNT at concentrations in excess of 50 mg/liter, in an organically rich medium, was inhibitory to gram-positive bacteria, actinomycetes, yeasts, and fungi. McCormick et al. (11) reported the products of stepwise reduction of TNT and other nitro aromatic compounds to transformed, but not degraded, amino aromatic compounds. Azoxy compounds, formed via coupling reactions between apparent intermediates, were also detected.

None of these studies has offered any evidence to indicate that the TNT aromatic nucleus is cleaved and ultimately degraded. To aid in assessing the biodegradability of TNT, the evolution of $^{14}CO_2$ from a system containing [^{14}C]TNT and sewage floc was monitored. Whereas only a trace of $^{14}CO_2$ was observed, approximately one-half of the added radioactivity became bound to the floc. The present study was conducted to determine the metabolic fate of the labeled TNT, i.e., whether significant biodegradation or biotransformation occurred, and how the activity became incorporated into the floc.

MATERIALS AND METHODS

Production of ^{14}C-labeled sewage sludge floc. Two different sludges were used in this study. Quali-

† Publication no. TP-1930 of the U.S. Army Natick Research and Development Command.

†† Present address: Baxter Travenol Laboratories, Morton Grove, IL. 60053.

tatively, similar results were obtained with the two sludges. Activated-sludge sample (experiment I) was supplied by L. Kaplan and J. Hoffsommer (Naval Surface Weapons Center, Silver Spring, Md.). It was produced in a bench-scale reactor containing sewage sludge, corn steep liquor, and 100 μCi of uniformly labeled [^{14}C]TNT in a total volume of 150 ml. The system was incubated aerobically for 3 days at room temperature. The second sample (experiment II) was prepared in our laboratory. Modifications were made by substituting sweet dairy whey for corn steep liquor and by adding 200 μCi of ring-labeled [^{14}C]TNT to the reaction. The system was inoculated with activated sludge obtained from a domestic sewage treatment plant and incubated for 5 days at room temperature. The effluent gas stream from each system was drawn through $Ba(OH)_2$ solutions to trap the evolved $^{14}CO_2$. At the end of the experiment, HCl was added to lower the pH and recover all of the $^{14}CO_2$. The $BaCO_3$ was dissolved in dilute HCl, and the radioactivity was determined. The pH of the reaction mixture did not deviate from neutrality during the course of the experiment.

Chemical extraction. The labeled sludge (ca. 10 g, wet weight) was subjected to a modified Sutherland and Wilkinson sequential extraction procedure (19) shown in Fig. 1. The lipid extraction was accomplished by heating the residue from the cold 0.6 M (10%) trichloroacetic acid extract in the presence of ethanol-diethyl ether (1:1) at 50°C for 20 min. Protein was extracted from the hot 0.3 M trichloroacetic acid extract residue by treatment with 10 N NaOH at 4°C for 18 h. All centrifugations were done in a Sorvall RC-2B centrifuge at 10,000 × *g* for 10 min at 4°C.

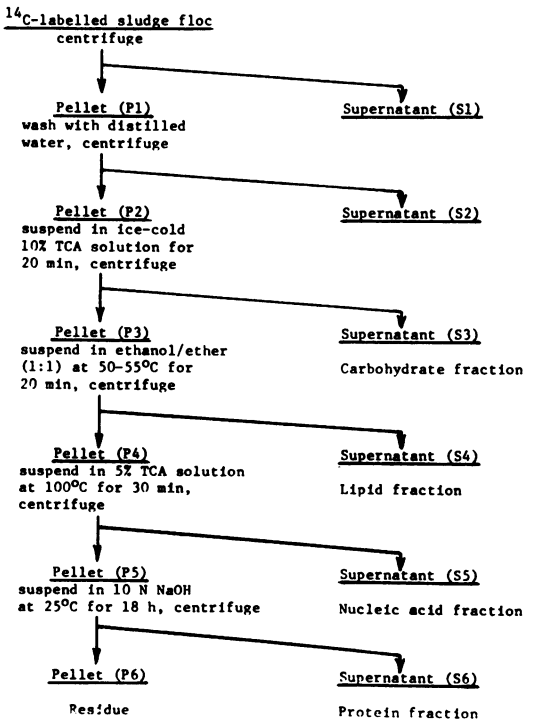

FIG. 1. *Fractionation scheme used to separate the ^{14}C-containing activated-sludge floc into various components.*

Lipid analysis. The lipid fraction (S4) was taken to dryness in a rotary vacuum evaporator and extracted with two 10-ml portions of acetone (Fig. 2). The supernatant contained the neutral lipids (S4a). The residue was extracted with chloroform-methanol (1:1) to provide a supernatant solution containing most of the phosphatides (S4b). Thin-layer chromatography (TLC) of the neutral lipid fraction was conducted on either activated (105°C for 30 min) or unactivated silica gel sheets (Eastman Chromagram, silica gel without fluorescent indicator; Eastman Kodak, Rochester, N.Y.). Activated sheets were developed with hexane-ether-acetic acid (90:15:1); unactivated sheets were developed in chloroform-methanol-water (65:20:2). Visualization was accomplished with 2,7-dichlorofluorescein (Applied Sciences, State College, Pa.). Fatty acid methyl esters (FAME) were produced by transesterification in BF_3-methanol (Applied Sciences), according to the method of Morrison and Smith (12). Gas chromatography (GC) of the FAME was conducted on an F & M model 720 gas chromatograph. A stainless-steel column (6 mm OD by 180 cm), packed with acid-washed, silanized 60/80-mesh Chromasorb G coated with 5% DEGS (diethyleneglycol succinate, Hewlett-Packard), was used. The oven was programmed from 140 to 210°C at 10°C/min and held at 210°C for 10 min. The carrier (helium) flow rate was 75 ml/min. The injection port was held at 250°C; the detector port was held at 230°C. The FAME were collected in a glass U-tube immersed in an acetone-dry ice bath, and the amount of radioactivity was determined. The FAME standards were obtained from Applied Sciences.

Protein analysis. The protein fraction (S6) was neutralized with 6 N HCl and dialyzed against 25 volumes of 0.01 M NH_4HCO_3 at 4°C for 24 h. The protein was precipitated by the addition of trichloroacetic acid to a final concentration of 0.3 M at 0°C. The suspension was centrifuged, and the pellet was washed twice with ice-cold 0.3 M trichloroacetic acid, dissolved in 5 ml of 0.01 N NaOH, and dialyzed in 500 volumes of 0.01 M NH_4HCO_3 at 4°C for 24 h. The dialyzed protein was shell frozen and lyophilized to remove all traces of ammonia. Protein content was determined by the method of Lowry et al. (9), using bovine serum albumin as standard. Amino acid analyses were conducted in a Beckman amino acid analyzer after acid hydrolysis with *p*-toluenesulfonic acid (8). TLC of the amino acids was conducted on unactivated silica gel sheets. The developing solvent was *n*-butanol-acetic acid-water (4:1:1). The chromatograms were visualized with ninhydrin.

Carbohydrate analysis. Carbohydrates were evaluated by TLC and liquid chromatography (Waters Associates ALC-100) by the method of Palmer (14). The thin-layer chromatograms were visualized with alkaline silver oxide. Total carbohydrate was determined by the anthrone method (5).

Nucleic acid analysis. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) content were determined by the diphenylamine and orcinol methods,

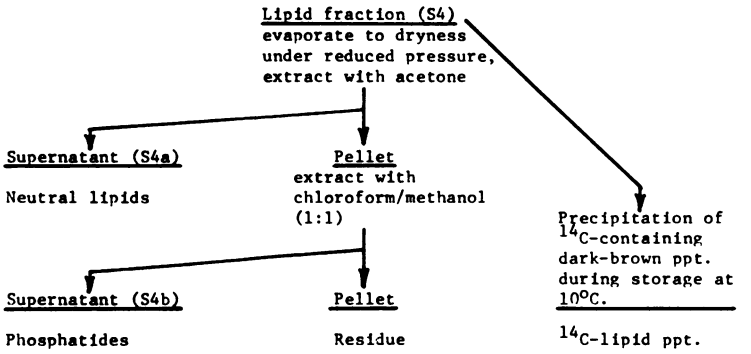

FIG. 2. *Fractionation of lipid fraction (S4).*

respectively (5). Salmon sperm DNA (Calbiochem, A grade) was used for the DNA standard. RNA was obtained from Schwartz Bioresearch.

Determination of radioactivity. Levels of radioactivity were determined by placing samples in 10 ml of Aquasol scintillation cocktail (New England Nuclear Corp., Boston, Mass.). Counting was conducted in a Beckman LS-233 liquid scintillation system, equipped with external standardization.

IR analysis. A model 521 Perkin-Elmer infrared (IR) spectrophotometer was used to obtain spectra of isolated materials, using KBr pellets. Interpretation was made by reference to Dyer (4) and Pasta and Johnson (15).

Synthesis of simulated lipid residue. Glycerol (10 mmol) and 2,4-diaminotoluene (5 mmol) were dissolved in 25 ml of a solution of chloroform and pyridine (1:1). Sebacyl chloride (10 mmol) and palmitoyl chloride (20 mmol) were dissolved in 25 ml of chloroform, and the solution was added slowly to the pyridine solution with swirling and cooling. An exothermic reaction took place that gave a homogeneous solution. After standing for 64 h, the reaction mixture was poured into water. An emulsion formed that separated into three layers when centrifuged. The middle semisolid layer was separated, thoroughly mixed with chloroform, and recentrifuged. This procedure was repeated until the middle layer remained constant in volume. The waxy middle layer was collected by filtration, washed with methanol, and dried under reduced pressure for 24 h, yielding 1.4 g of a grayish powder.

RESULTS

Less then 0.5% of the radioactivity added to the system was present in the metabolically produced $^{14}CO_2$ from either experiment (3 day or 5 day). The distribution of radioactivity in the cellular fractions obtained from the extraction procedure is indicated in Table 1. Most of the radioactivity was associated with the lipid fraction, with a smaller amount found in the protein fraction.

The lipid fraction (S4) was fractionated further as shown in Fig. 2. The acetone-soluble, neutral lipid fraction (S4a) was chromatographed on silica (TLC) and developed with the hexane-ether-acetic acid solvent system (Fig. 3A). The area corresponding to the triglycerides (R_f = 0.63) contained negligible levels of radioactivity, whereas more than 95% of the applied ^{14}C remained at the origin. This radioactive material at the origin was eluted from the chromatogram and rechromatographed in chloroform-methanol-water (Fig. 3B). Under these conditions almost 95% of the applied radioactivity was localized in an area at $R_f \geq 0.75$. This area also reacted with dichlorofluorescein reagent to give a visible color. A portion of the material from this area of a chromatogram was eluted and converted to the FAME, which were separated and collected by GC. The fatty acid esters, consisting principally of palmitate, oleate, and palmitoleate, contained practically no ^{14}C. The bulk of the radioactivity remained near the head of the GC column and was not eluted under the conditions used.

The phosphatide fraction (Fig. 2, S4b), analyzed in the same way, gave results very similar to those obtained from the lipid fraction; i.e., virtually no radioactivity was found in the

TABLE 1. *Distribution of ^{14}C between various floc fractions*

Treatment	Fraction obtained	Radioactivity (% of total)	
		Expt 1[a]	Expt 2[b]
	Pellet (P1)[c]	50.0	52.4
Water wash	Supernatant (S2)	1.5	2.4
Cold 10% TCA[d]	Carbohydrate (S3)	0.8	4.7
Ethanol-ether	Lipid (S4)	30.6	20.5
Hot 5% TCA	Nucleic acid (S5)	1.0	1.9
10 N NaOH	Protein (S6)	7.8	15.8
None	Residue	6.8	5.9

[a] Incubated for 3 days.
[b] Incubated for 5 days.
[c] The floc pellet was obtained by centrifugation of the reactor contents. The balance of the radioactivity was present in the supernatant (S1).
[d] TCA, Trichloroacetic acid.

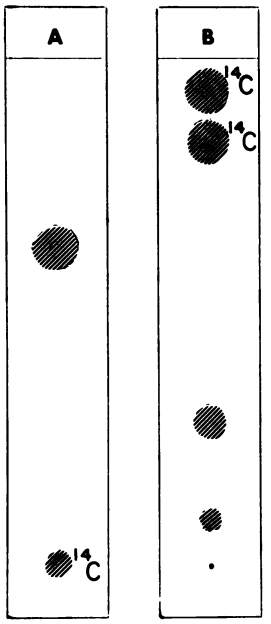

FIG. 3. *TLC of ^{14}C-containing acetone-extracted lipid fraction. Chromatogram A was developed in hexane-ether-acetic acid. Chromatogram B was developed in chloroform-methanol-water. Cross-hatched areas represent compounds visualized with 2,7-dichlorofluorescein. Areas labeled "^{14}C" represent areas containing the major portion of the applied radioactivity.*

FAME, whereas the major part of the ^{14}C was retained on the column.

A portion of the protein fraction (S6) was subjected to acid hydrolysis, and the component amino acids were estimated by TLC. All of the normally occurring amino acids were present, but there was no correlation between ninhydrin-positive areas and radioactive areas of the chromatogram. The identities and relative amount of the amino acids were confirmed by amino acid analysis of the protein hydrolysate. The cumulative results of the analyses demonstrated that the amino acids did not contain ^{14}C.

The carbohydrate fraction (S3) was separated into several components by TLC. None of the bands gave a typical carbohydrate reaction when the chromatogram was visualized with alkaline silver oxide.

The nucleic acid fraction (S5) was subjected to hydrolysis followed by TLC analysis. Ribose and deoxyribose were present, but no radioactivity was associated with areas of the chromatogram containing these pentoses.

The various fractions were stored at 10°C in the dark after analysis. Under these conditions, small amounts of a dark-brown precipitate formed in each fraction. The precipitate that separated from the lipid fraction (S4) was isolated (^{14}C-lipid ppt.) and found to contain more than 90% of the ^{14}C present in the fraction. Most of the radioactivity in the protein fraction (S6) was contained in a precipitate that similarly separated out from that fraction (^{14}C-protein ppt.).

The IR spectrum of the ^{14}C-lipid ppt. exhibited absorption bands at 1,650 and 1,530 cm^{-1} (Fig. 4a). These bands are characteristic of secondary amides. Peaks representing ester carbonyl (1,725 cm^{-1}) and aliphatic hydrocarbon (2,910 and 2,850 cm^{-1}) groups were also present, a finding not unexpected in a substance possessing lipoidal character.

The same bands were observed in the IR spectrum of the ^{14}C-protein ppt. (Fig. 4b). However, in this case, the amide band was far stronger than the ester band, as would be expected for a substance containing peptide linkages.

A model compound was synthesized that incorporated moieties of the types suggested by the chemical and spectroscopic evidence (i.e., glycerol, mono- and dibasic fatty acids, and aromatic amines). Since the reaction mixture contained mono-, di-, and trifunctional components, the reaction took place in a random fashion with the formation of oligomers, high-molecular-weight polymers, and cross-linked fractions. The IR spectrum of the insoluble portion of this product (Fig. 4c) was similar to the spectra of the biologically derived precipitates obtained from the floc. All three spectra possessed bands characteristic of amide, ester, and aliphatic hydrocarbon groups.

DISCUSSION

The finding that an activated-sludge system acting on [^{14}C]TNT for 3 to 5 days produced only very low levels of $^{14}CO_2$ suggests that the aromatic ring was not cleaved. In addition, the radioactivity present in the lipid fraction derived from the biomass was not incorporated into individual triglycerides or phosphatides. The ^{14}C present in these fractions remained on the column after GC, a property consistent with the behavior of a polymeric material. Likewise, the radioactivity present in the protein fraction was not incorporated into individual amino acids. When the carbohydrate was analyzed by TLC, an atypical carbohydrate reaction was observed after the plates were treated with alkaline silver oxide reagent. One could speculate that the low levels of radioactivity found in the carbohydrate fraction were due to conjugates formed between [^{14}C]TNT transformation products and carbohydrates, but no studies were carried out to elucidate this point.

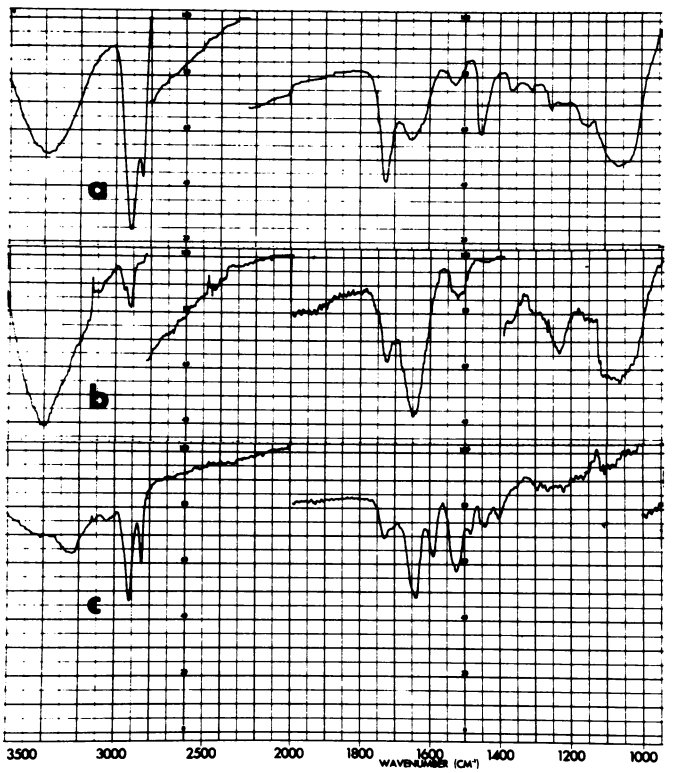

FIG. 4. *IR spectra of biologically and synthetically derived precipitates: (a) ^{14}C-lipid ppt.; (b) ^{14}C-protein ppt.; (c) precipitate derived from chemical interaction between 2,4-diaminotoluene, glycerol, palmitoyl chloride, and sebacyl chloride.*

During storage of the lipid and protein fractions, small amounts of insoluble materials formed that contained the bulk of the radioactivity present in these fractions. The IR spectra of these precipitated materials indicated that they contained aliphatic, ester, and amide groups in varying proportions. The presence of amide linkages in the lipid-derived precipitate was unexpected, as was the finding of ester linkages in the precipitate from the protein fraction. The properties and spectra of these precipitates suggested that they were cross-linked polymers formed primarily by the reaction between the constituents of a particular cellular fraction and the biotransformation products of TNT.

The evidence suggests that the radioactive residue that precipitated from the lipid fraction was the result of a reaction between fatty acids, glycerides, and TNT bioreduction products. Likewise, the ^{14}C-containing precipitate obtained from the protein fraction probably was formed by reaction of TNT bioconversion products with carboxylate groups in the protein fraction. The similarity in spectral and solubility properties between the synthesized model compound and the biologically mediated [^{14}C]TNT-lipid precipitate leads us to conclude that aromatic amines arising from the biotransformation of TNT may undergo condensation reactions with the carboxylate groups of cellular components, thus leading to the formation of polyamides. Coupling reactions have been reported in which phenolic and naphtholic intermediates of pesticides and industrial pollutants were polymerized to dimers, trimers, tetramers, and pentamers by phenol oxidase from the fungus *Rhizoctonia praticola* (18). The reaction of reduced biotransformation products from TNT and 2,4-dinitrotoluene to form glucuronide and amide linkages has been reported (2, 10).

Although a major factor in the resistance of TNT to cleavage of the aromatic ring appears to be the resistance of the highly substituted molecule to enzymatic hydroxylation, it can be postulated that one reason for the failure of TNT biotransformation products to be further biodegraded is the formation of polyamides that are resistant to microbial action. Resistance of these polymers to further microbial transformation would not be surprising based on the recalcitrance of some alkyl and aryl polyamides to microbial degradation (16, 21).

Generally, when considering pollution abatement processes, one is accustomed to think of the final product or products as simpler compounds rather than as products of a more complex nature. Our findings support other reports (1, 10, 18) suggesting that polymer formation may be a more common occurrence in the biotransformation reactions of aromatic compounds than otherwise suspected. In any investigation of the fate of organic compounds subjected to biological treatment processes, the possibility of polymer formation should be considered. This is of particular concern when the polymers formed may be persistent in the environment and of unknown impact on ecosystems.

ACKNOWLEDGMENTS

We wish to acknowledge the aid of D. E. Remy for assistance in conducting and interpreting the IR analyses, and the aid of L. Holmes for conducting the amino acid analyses. Thanks are due to H. S. Levinson for valuable suggestions in reviewing the manuscript.

The paper reports research undertaken at the U.S. Army Natick Research and Development Command.

LITERATURE CITED

1. **Baird, R., L. Carmona, and R. L. Jenkins.** 1977. Behavior of benzidine and other aromatic amines in aerobic wastewater treatment. J. Water Pollut. Control Fed. **49**:1609–1615.
2. **Channon, H. H., G. T. Mills, and R. T. Williams.** 1944. The metabolism of 2,4,6-trinitrotoluene (α-TNT). Biochem. J. **38**:70–85.
3. **Dale, H. H.** 1921. The fate of TNT in the animal body. Med. Res. Counc. G.B. Spec. Rep. Ser. **58**:53–61.
4. **Dyer, J. R.** 1965. Applications of absorption spectroscopy of organic compounds. Prentice-Hall, Englewood Cliffs, N.J.
5. **Herbert, D., P. J. Phipps, and R. E. Strange.** 1971. Chemical analysis of microbial cells, p. 209–344. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 5B. Academic Press Inc., New York.
6. **Jensen, H. L., and G. Lautrup-Larson.** 1967. Microorganisms that decompose nitro-aromatic compounds, with special reference to dinitro-*o*-cresol. Acta Agric. Scand. **17**:115–126.
7. **Klausmeier, R. E., J. L. Osmon, and D. R. Walls.** 1974. The effect of trinitrotoluene on microorganisms. Dev. Ind. Microbiol. **15**:309–317.
8. **Liu, T. Y., and Y. H. Chang.** 1971. Hydrolysis of proteins with p-toluenesulfonic acid. J. Biol. Chem. **246**:2842–2848.
9. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
10. **McCormick, N. G., J. H. Cornell, and A. M. Kaplan.** 1977. Identification of biotransformation products from 2,4-dinitrotoluene. Appl. Environ. Microbiol. **35**: 945–948.
11. **McCormick, N. G., F. E. Feeherry, and H. S. Levinson.** 1976. Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds., Appl. Environ. Microbiol. **31**:949–958.
12. **Morrison, W. R., and L. M. Smith.** 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron trifluoridemethanol. J. Lipids Res. **5**:600–608.
13. **Osmon, J. L., and R. E. Klausmeier.** 1972. The microbial degradation of explosives. Dev. Ind. Microbiol. **14**:247–252.
14. **Palmer, J. K.** 1975. A versatile system for sugar analysis via liquid chromatography. Anal. Lett. **8**:215–224.
15. **Pasta, D. J., and C. R. Johnson.** 1969. Organic structure determination. Prentice-Hall, Englewood Cliffs, N.J.
16. **Rogers, M. R., and A. M. Kaplan.** 1971. Effects of *Penicillium janthinellum* on parachute nylon—is there microbial deterioration? Int. Biodeterior. Bull. **7**:15–24.
17. **Saz, A. K., and R. B. Slie.** 1954. The inhibition of organic nitro reductase by aureomycin in cell-free extracts. II. Co-factor requirement for the nitro-reductase enzyme complex. Arch. Biochem. Biophys. **51**:5–16.
18. **Sjoblad, R. D., and J. M. Bollag.** 1977. Oxidative coupling of aromatic pesticide intermediates by a fungal phenol oxidase. Appl. Environ. Microbiol. **33**:906–910.
19. **Sutherland, I. W., and J. F. Wilkinson.** 1971. Chemical extraction methods in microbial cells, p. 345–383. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 5B. Academic Press Inc., New York.
20. **Takahashi, H., S. Taniguchi, and F. Egami.** 1963. Comparative biochemistry, vol. 5. Academic Press Inc., New York.
21. **U.S. Army Natick Research and Development Command.** 1973. Twenty-first conference on microbiological deterioration of military material, p. 37–49. U. S. Army Natick Research and Development Command, Natick, Mass.
22. **Westfall, B. B.** 1954. The reduction of symmetrical trinitrotoluene by a succinic dehydrogenase preparation. J. Pharmacol. Exp. Ther. **79**:23–26.
23. **Won, W. D., R. J. Heckly, D. J. Glover, and J. C. Hoffsommer.** 1974. Metabolic disposition of 2,4,6-trinitrotoluene. Appl. Microbiol. **27**:513–516.