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DECOMPOSITION OF THE EPOXIDES GLYCIDOL AND GLYCIDYL NITRATE

By

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) During chemical desensitization of GTN-containing waste streams with calcium hydroxide the epoxides glycidol and glycidyl nitrate are formed. The epoxide rings of both compounds are unstable to heat in aqueous solutions and open to form glycerol 1-mononitrate and presumably glycerol. These transformations are accelerated by microbiological activity. Glycerol 1-mononitrate is slowly denitrated to form glycerol. Glycidol and glycidyl nitrate cause base pair substitutions in the Ames test for mutagenicity while glycerol 1-mononitrate tests negative.		

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PREFACE

Direct discharge of glycerol trinitrate (GTN) or nitroglycerine-containing wastes into the environment is prohibited because GTN is toxic and sensitive to thermal and mechanical shock. GTN can be desensitized chemically using calcium hydroxide, but products of this reaction, the epoxides glycidol and glycidyl nitrate, must be assessed for their potential environmental hazard. This report describes the biodegradation of glycidol and glycidyl nitrate and the mutagenic properties of both compounds. This work was carried out under project number 13210502007. We thank John T. Walsh for his analysis of glycidol and Carmine DiPietro for his GC/MS analysis.

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DEGRADATION OF THE EPOXIDES GLYCIDOL AND GLYCIDYL NITRATE

INTRODUCTION

Wastewater contamination occurs during the manufacture and processing of glycerol trinitrate (GTN) or nitroglycerine. The solubility of GTN in water, its sensitivity to thermal and mechanical shock, and its toxicity preclude direct disposal of GTN containing waste streams into the environment.^{1,2} Microbial systems are effective in biodegrading GTN via successive denitration steps through glycerol dinitrate (GDN) and glycerol mononitrate (GMN) isomers with each succeeding step proceeding at a slower rate.³

Calcium hydroxide is used to chemically desensitize waste streams containing GTN.⁴ This treatment causes the disappearance of GTN, GDN, and GMN, but glycidol and glycidyl nitrate which are formed may also present potential

¹ US Army Medical Research and Development Command. 1975. Mammalian Toxicity of Munition Compounds: Phase I. Acute Oral Toxicity, Primary Skin and Eye Irritation, Dermal Sensitization and Disposition and Metabolism. Technical Report ADB01115D. Environmental Protection Research Division, Washington, DC.

² Wiley, B. Ed. 1977. Twenty-sixth Conference on Microbiological Deterioration of Military Materiel. Technical Report NATICK/TR-78/029. Food Sciences Laboratory, US Army Natick Research and Development Command, Natick, MA.

³ Wendt, T. M., J. H. Cornell, and A. M. Kaplan. 1978. Microbial Degradation of Glycerol Nitrates. Appl. Environ. Microbiol. 36: 693-699.

⁴ Wiley, B. Ed. 1979. Twenty-eighth Conference on Microbiological Deterioration of Military Materiel. Technical Report NATICK/TR-80/018. Food Sciences Laboratory, US Army Natick Research and Development Command, Natick, MA.

pollution problems. Glycidol and glycidyl nitrate contain the highly reactive epoxy group which tends to confer mutagenic properties.^{5,6}

Mammalian studies have demonstrated that glycidol is toxic^{7,8} and alkylates DNA.⁹ The purpose of this study is to determine the fate of glycidol and glycidyl nitrate when exposed to microbiological activity, thus indicating the feasibility of a biological approach to the decomposition of GTN chemical reaction products. An additional objective is to determine the mutagenic properties of both compounds.

MATERIALS AND METHODS

Media. Basal salts media contained 2.0 g $\text{NH}_4\text{H}_2\text{PO}_4$, 1.0 g K_2HPO_4 , 1.0 g KH_2PO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g CaCl_2 and 0.01 g NaCl per liter of distilled water. Glucose was added at 1.0 g per liter as indicated. The nutrient broth concentration was 4.0 g per liter. All media containing glycidol and glycidyl nitrate were filter-sterilized through filters of 0.2- μm pore size. Media for chemostat continuous cultures were autoclaved and after cooling glycidyl nitrate was added through sterile filters.

⁵ Hemminki, K. 1979. Fluorescence Study of DNA Alkylation Epoxides. *Chem. Biol. Interact.* 28: 269-278.

⁶ Wade, M. J., J. W. Moyer, and C. H. Hine. 1979. Mutagenic Action of a Series of Epoxides. *Mut. Res.* 66: 367-371.

⁷ Ivanov, N. G., L. V. Melnikova, A. M. Klyachkina, and A. L. Germanova. 1980. Study of Toxicity, Hazard, and Harmful Effects of Glycidol on Animals. *Gig. Tr. Prof. Zabol.* 3: 42-43.

⁸ Melnikova, L. V. and A. M. Klyachkina. 1979. Study of Glycidol Toxicity and the Direction of its Harmful Action on an Organism. *Toksikol. Nov. Prom. Khim. Veshchestv.* 15: 97-101.

⁹ See footnote 5.

Chemicals. Glycidol was purchased from Eastman Kodak Co., Rochester, NY. Freshly redistilled samples were used. Glycidyl nitrate was prepared according to the method of Ingham and Nichols.¹⁰ The identity and purity of the product were confirmed by IR, NMR and HPLC. Glycerol 1-mononitrate was prepared according to published procedures and its identity and purity confirmed by melting point, GC/MS, FTIR and HPLC.¹¹

Culture Conditions. Aerobic batch cultures were incubated in 250-ml Erlenmeyer flasks each containing 100 ml of media at 30°C on a New Brunswick G24 Environmental Incubator Shaker. Anaerobic batch cultures were incubated at 37°C in 250-ml Erlenmeyer flasks filled with media. New Brunswick Bio Flo Model C30 bench top chemostats for continuous cultures were maintained at room temperature under anaerobic conditions with a retention time of 7 days in a 1.4-liter reaction vessel.

Aerobic cultures were inoculated with activated sludge from the Marlboro Easterly sewage treatment plant (Marlboro, MA) and anaerobic cultures with digest from the Nut Island sewage treatment plant (Boston, MA).

High Performance Liquid Chromatography. Glycidyl nitrate and glycerol 1-mononitrate were determined on a DuPont 830 liquid chromatograph with a Perkin Elmer LC55 variable wavelength detector at 195 nm, and a Columbia Scientific Supergrator-2 programmable computing integrator. The mobile phase was acetonitrile/water (10v)/(90v). Culture media samples were clarified by centrifugation and filtered. Injections of 5 µl were made into a 25-cm x 4.6-mm

¹⁰ Ingham, J. D. and P. L. Nichols, Jr. 1954. Direction of Addition of the Nitrate Ion to an Unsymmetrical Oxide. *Am. Chem. Soc. J.* 76: 4477-4479.

¹¹ See footnote 1, p. 4.

DuPont Zorbax ODS reverse phase column at room temperature and 1200 psi (8.273×10^3 kPa). Retention times were about 6 min for glycerol 1-mononitrate and 22 min for glycidyl nitrate. The detection limit was 1 ppm ($\mu\text{g/ml}$).

Gas Chromatography. Analysis of glycidol was performed on a Perkin-Elmer Model 3920 gas chromatograph equipped with a flame ionization detector and a Spectrum Scientific Corp. Model 1021A electronic noise filter. Nitrogen carrier gas flowed at 30 ml per min through a stainless steel column (46 cm x 0.32 cm) packed with poropak Q-S, 80-100 mesh. Samples were clarified by centrifugation, filtered and 3- μl volumes injected directly on-column. The column temperature was 170°C and the detector was at 250°C . The detection limit was 0.6 ppm.

Extraction of Glycerol 1-mononitrate. A sample of the biologically mediated glycerol 1-mononitrate was prepared by continuous extraction of a sample of chemostat effluent for 24 hours with ether. The ether was evaporated and the residue examined by TLC, FTIR, and GC/MS. TLC was performed on silica gel plates without fluorescent indicator, developed in benzene/ethanol (95/5) sprayed with diphenylamine in ethanol (5%) and visualized under UV light for 5-10 min. The extracted glycerol 1-mononitrate had the same R_f as the synthetic standard and the FTIR and GC/MS traces of the sample and standard were identical.

Mutagenicity Testing. The Ames screening test for mutagenicity was performed with glycidyl nitrate, glycidol and glycerol 1-mononitrate according to stand-

and procedures.^{12,13} Five strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535, TA 1537, and TA 1538) were used to test concentrations from 5 µg to 5000 µg per plate with and without metabolic activation.

RESULTS

Aqueous solutions of glycidol and glycidyl nitrate were heat sensitive (Fig. 1). In a boiling water bath the half-life of glycidol was 52.5 min and that of glycidyl nitrate was 14.4 min. Under these conditions glycidol required about 3.2 times longer to decompose than glycidyl nitrate. As glycidyl nitrate decomposed, there was a corresponding formation of glycerol 1-mononitrate. The principal reaction involved the hydrolytic decomposition of glycidyl nitrate. This appears to be cleavage of the epoxide ring according to equation 1.



Glycidyl nitrate was not photosensitive (Fig. 2). There was no significant difference in the rate that glycidyl nitrate hydrolyzed in samples kept at room temperature in the light or dark for 30 days. Glycerol 1-mononitrate formed at equivalent rates.

Glycidol disappeared from filter-sterilized solutions at room temperature, but the rate of decomposition was accelerated by microbiological activity (Fig. 3). In sterile aerobic batch culture experiments, about 36% of the

¹² Ames, B. N. 1979. Supplement to the Methods Paper. University Calif., Berkeley. 1-10.

¹³ Ames, B. N., J. McCann, and E. Yamasaki. 1975. Methods for Detecting Carcinogens and Mutagens with the *Salmonella* Mammalian-Microsome Mutagenicity Test. Mut. Res. 31: 347-364.

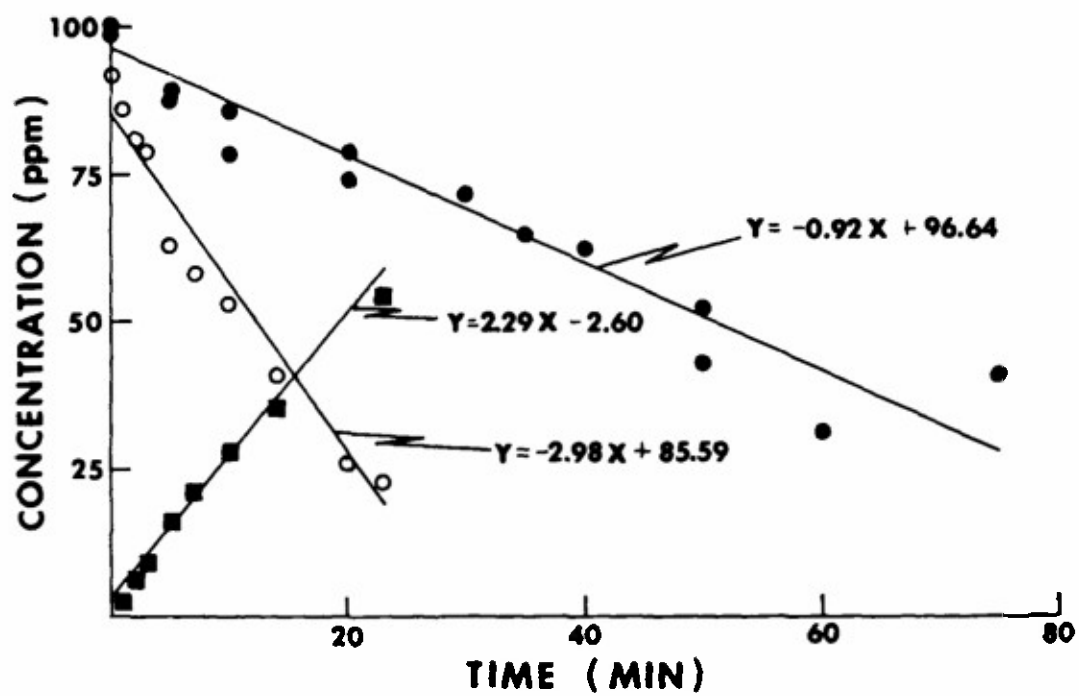


Figure 1. Effects of a boiling water bath on stability of glycidol (●) and glycidyl nitrate (○) and the formation of glycerol 1-mononitrate (■) from glycidyl nitrate.

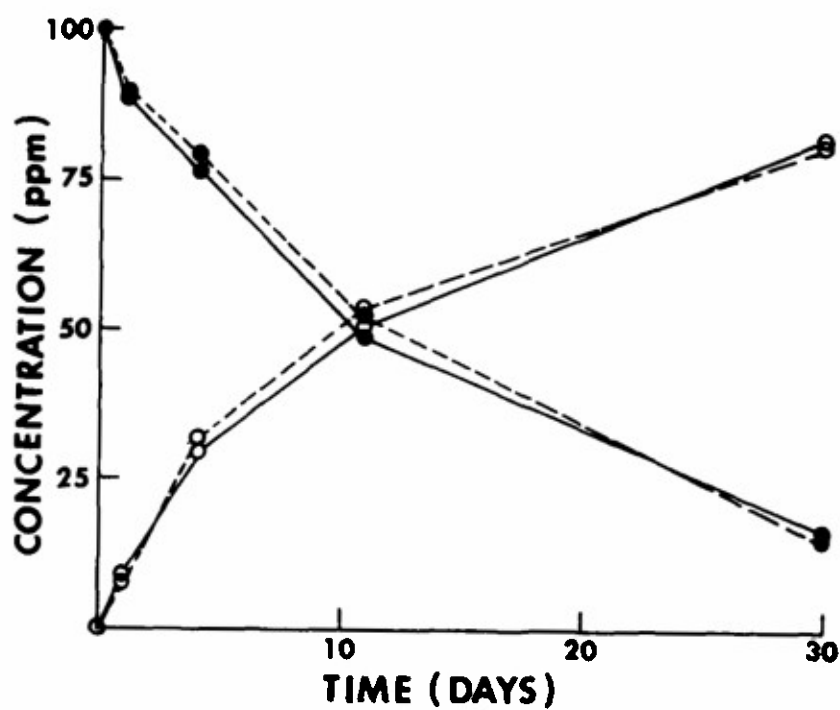


Figure 2. Photosensitivity of glycidyl nitrate (●) at room temperature in the light (—) and dark (---) and the formation of glycerol 1-mononitrate (○).

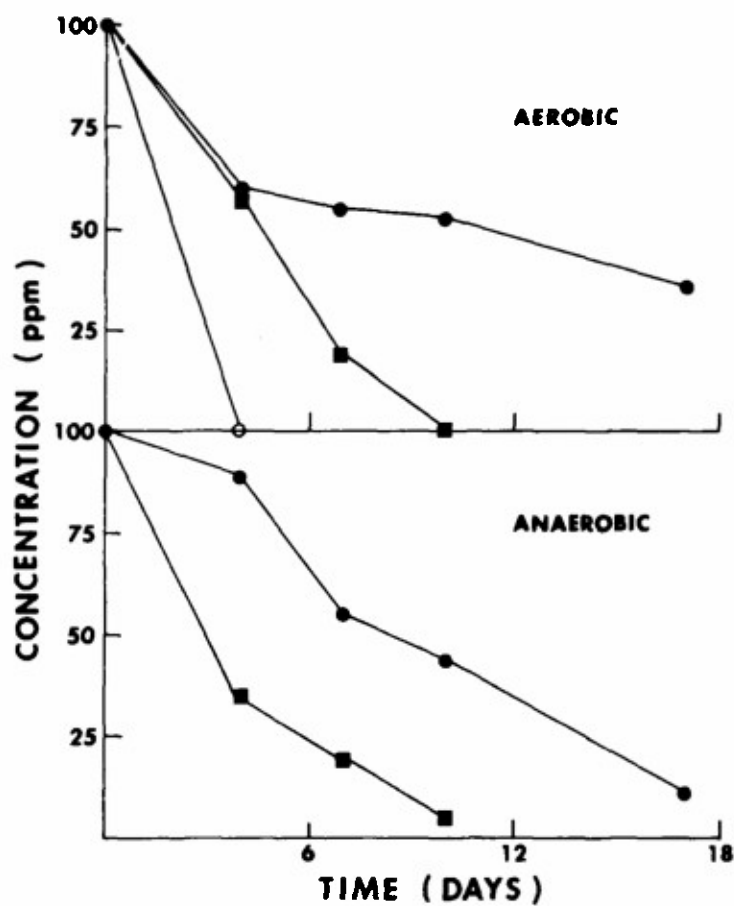


Figure 3. Decomposition of glycidol in batch cultures under aerobic and anaerobic conditions with filter sterilized (●), nutrient broth (○) and basal salts (■) media.

initial 100 ppm glycidol remained after 17 days. Under sterile anaerobic conditions, 11% of the glycidol remained. Under aerobic conditions in inoculated nutrient broth cultures, glycidol was below the limits of detection after about 4 days. As the sole source of carbon, levels of glycidol were below detection limits in 10 days under both aerobic and anaerobic conditions.

Under batch culture conditions the epoxide ring of glycidyl nitrate opened even at low temperatures (Fig. 4). At 3°C 47% of the initial 90 ppm of glycidyl nitrate remained after 28 days in sterile nutrient broth flasks. There was no acceleration of decomposition in inoculated flasks at 3°C. Similar results were obtained for sterile control and inoculated flasks with basal salts media with and without glucose. There were no differences in the levels of glycerol 1-mononitrate that formed in sterile and inoculated flasks in the three media.

At 30°C under aerobic conditions no detectable glycidyl nitrate remained after 28 days in sterile and 14 days in inoculated nutrient broth flasks. During the same time period glycerol 1-mononitrate reached a level of 90 ppm in the sterile nutrient broth, while in the inoculated broth the level peaked at 42 ppm and then gradually declined with time. In basal salts media with and without glucose, no evidence was found for an accelerated rate of decomposition of glycidyl nitrate due to microbiological activity. The concentration of glycerol 1-mononitrate decreased slightly in inoculated flasks with basal salts with and without glucose after about 14 days, while in sterile controls there was no decrease from the 90 ppm to 100 ppm levels reached. At 37°C under anaerobic conditions no detectable glycidyl nitrate remained after 14 days in sterile and 7 days in inoculated nutrient broth flasks. The concentration of glycerol 1-mononitrate rose to 90 ppm in the sterile flasks and 58 ppm in inoculated flasks, decreasing to below 10 ppm in the latter. There was no significant

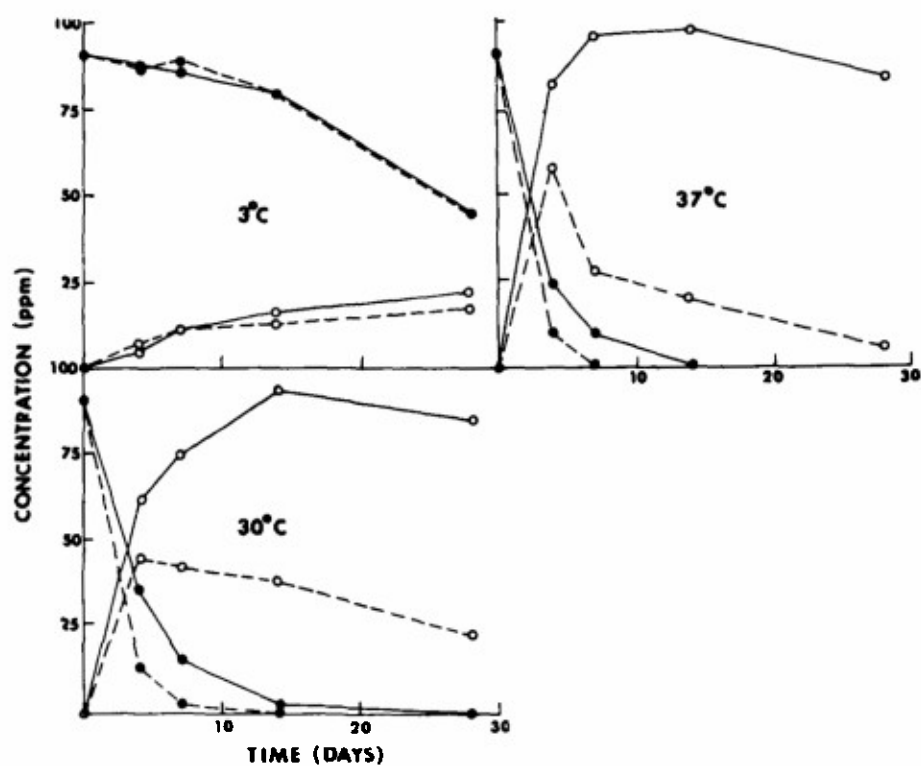


Figure 4. Decomposition of glycidyl nitrate (●) in batch cultures and the formation of glycerol 1-mononitrate (○) in filter sterilized nutrient broth (—) and inoculated nutrient broth (----).

difference in the rates of disappearance of glycidyl nitrate and glycerol 1-mononitrate between control and inoculated flasks in basal salts media with or without glucose.

Figure 5 illustrates the data from a continuous culture system maintained in a chemostat under anaerobic conditions at room temperature. The glycidyl nitrate concentration of the filter sterilized media in the reservoir decreased from 100 ppm to 7 ppm during 22 days. During the same time the concentration of glycerol 1-mononitrate in the reservoir rose to 100 ppm. The decomposition of glycidyl nitrate in the culture vessel that was originally 100 ppm was accelerated by microbial activity; by 6 days less than 5 ppm remained. During this period the level of glycerol 1-mononitrate reached a maximum of 50 ppm in the culture vessel. There was no detectable glycidyl nitrate in the product reservoir and levels of glycerol 1-mononitrate ranged from 15 ppm to 30 ppm.

Samples from the culture vessel were used for the extraction and analysis of glycerol 1-mononitrate formed in the biological systems. The structure of the product and its identity with the synthesized standard was confirmed by TLC, FTIR, and GC/MS.

The Ames testing revealed the mutagenic potential of glycidol and glycidyl nitrate (Fig. 6). Both compounds caused significant increases in the rates of back mutation over controls with or without metabolic activation. Significant increases were evident without activation at levels of 50 μ g to 5000 μ g per plate of glycidol with strains TA 1535 and TA 100, and 500 μ g to 5000 μ g per plate of glycidyl nitrate with TA 100 and 50 μ g to 5000 μ g per plate with TA 1535. Results presented in Figure 6 are without metabolic activation. With activation the number of revertant colonies increased in most cases, although the lower limits for positive findings remained unchanged. The levels of back mutation are higher for glycidol than glycidyl nitrate in all cases.

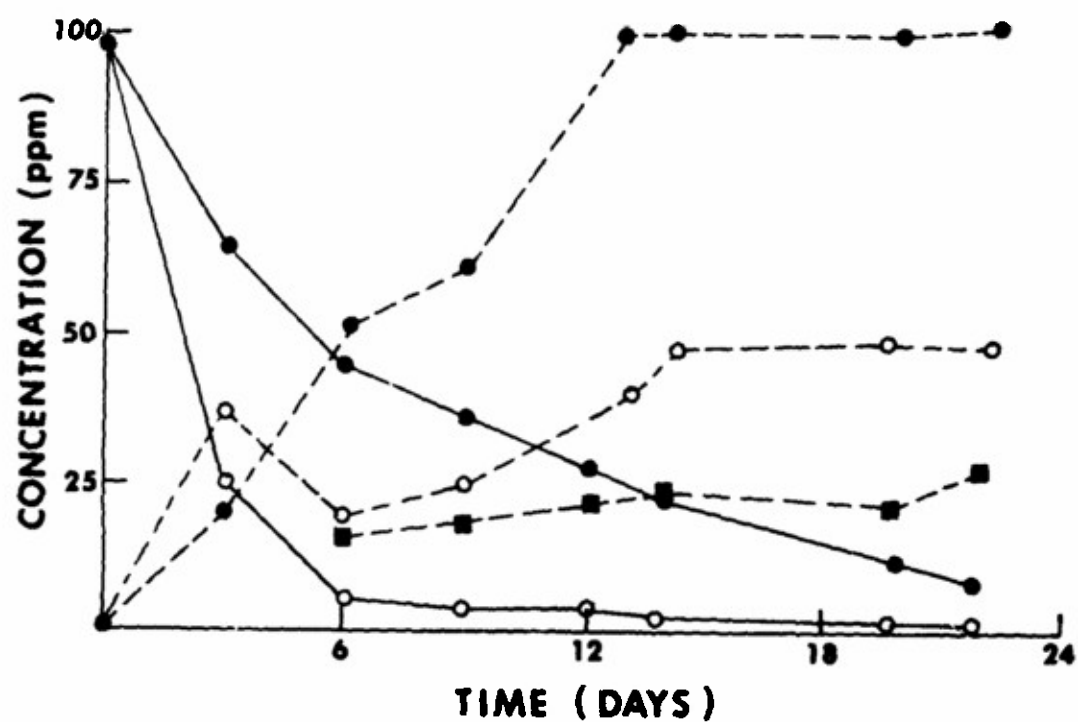


Figure 5. Disappearance of glycidyl nitrate (—) and the formation of glycerol 1-mononitrate (---) under chemostat continuous culture in the sterile medium (●), culture vessel (○) and product reservoir (■).

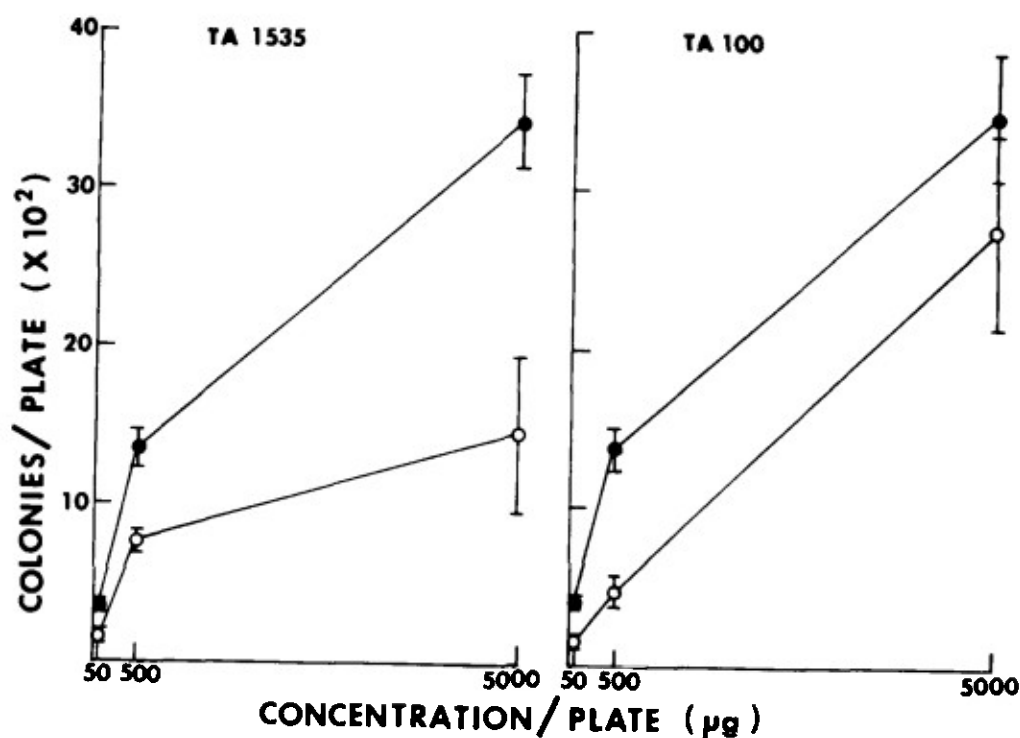


Figure 6. Ames test results illustrating dose response curves with glycidol (●) and glycidyl nitrate (○) without metabolic activation. Revertant colonies per plate $\pm$ 1 S. D. Background controls for TA 100 and TA 1535 were 133 ± 34 and 24 ± 8 without activation. Not shown are values for 5 μ g per plate which were not significant based on the criteria of a threefold increase in numbers of revertant colonies per plate over background. All values shown are significant except for 50 μ g per plate glycidyl nitrate with TA 100.

None of the other three strains tested showed significant increases in rates of back mutation. Glycerol 1-mononitrate tested negative with all five strains examined.

DISCUSSION

Glycidol and glycidyl nitrate chemically decompose in aqueous solutions even at low temperatures. Glycidyl nitrate disappears about three times more rapidly than glycidol under the conditions studied. Glycidyl nitrate was not photosensitive. The epoxide ring of glycidyl nitrate opens to form glycerol 1-mononitrate. Glycidol would be expected to form glycerol, but this aspect of the reaction was not studied. The effects of temperature of the epoxide rings are significant even at room temperature as seen with sterile control flasks (Figs. 3, 4, 5).

With glycidol present, even as the sole carbon source, there is a significant acceleration in the rate of epoxide scission due to microbiological activity under both aerobic and anaerobic conditions. Whether this accelerated effect is through primary activity directly on the compound or through secondary effects such as microbial mediated changes in the media remains to be shown.

The gas chromatographic method developed for analysis of glycidol was subject to interference with anaerobic nutrient broth culture samples. A spectrophotometric method for the colorimetric determination of glycidol was found unacceptable due to interferences from nutrient broth and alkaline pH's.¹⁴

¹⁴ Laryukhina, F. Z. and N. V. Chechkova. 1978. Colorimetric Determination of Glycidol in the Air of Industrial Premises. *Neftepererab, Neftckhim*, 3: 35-36.

In nutrient broth batch cultures at 30°C and 37°C there was evidence for an accelerated rate of transformation of glycidyl nitrate to glycerol 1-mononitrate due to microbial activity. This increase was over that due to chemical hydrolysis alone. This conclusion is supported by the absence of a difference in the rate of transformation of sterile and inoculated media at 30°C where microbiological activity is minimized. Even at this temperature glycidyl nitrate undergoes some chemical hydrolysis.

In continuous culture there was a more pronounced acceleration of the transformation of glycerol 1-mononitrate due to microbiological activity than in batch cultures. Importantly, chemostats were maintained at room temperature to prevent differential heating of any one component of the system, which would have produced significant differences in rates of chemical hydrolysis.

Glycerol 1-mononitrate is slowly metabolized in batch and continuous cultures. In continuous culture with a 7-day retention time influent, glycerol 1-mononitrate attained a maximum concentration of about 100 ppm; in the culture vessel and spent media, it was considerably reduced. Presumably, with longer retention times or more optimized conditions, complete disappearance of this ester could be achieved. Glycerol 1-mononitrate is probably transformed by microbiological activity to glycerol through a denitration step as are other nitrate esters.^{15,16} No evidence was found for other nitro compounds by TLC or HPLC at 195 nm and 254 nm. ¹⁴C-labelled glycerol was found in urine from rats treated with glycerol 1-mononitrate 1,3-¹⁴C.¹⁷ Glycerol would be meta-

¹⁵ See footnote 3, p. 4.

¹⁶ See footnote 4, p. 4.

¹⁷ See footnote 1, p. 4.

bolized to carbon dioxide and water through cleavage of carbon-carbon bonds, or to any of a number of one, two, and three-carbon compounds.

The pathways of microbial metabolism of GTN chemical transformation products are shown in Fig. 7. No attempt was made to identify glycerol in any effluent. The transformation from glycidyl nitrate to glycerol 1-mononitrate to glycerol proceeds more slowly with each succeeding step. The steps from glycidol to glycerol and from glycidyl nitrate to glycerol 1-mononitrate occur spontaneously in aqueous solutions above freezing, but are accelerated through microbiological activity.

Glycidol and glycidyl nitrate gave positive results in the Ames test producing base pair substitutions, while the transformation product, glycerol 1-mononitrate, tested negative. Our results corroborate those of other investigators who have examined glycidol in the Ames test with an incomplete battery of *Salmonella* strains.^{18,19} As with GTN, glycerol 1-mononitrate produces toxicity in mammals.²⁰

Biological treatment of a waste stream originally containing GTN, which on chemical treatment with calcium hydroxide became contaminated with epoxides, may be a feasible approach to alleviating this pollution problem. Both glycidol and glycidyl nitrate, the principal products, are mutagens and potential environmental hazards. These contaminants disappear during biological treatment through cleavage of the epoxide rings and mutagenic activity is lost.

¹⁸ Stolzenberg, S. J. and C. H. Hine. 1979. Mutagenicity of Halogenated and Oxygenated Three-Carbon Compounds. 5: 1149-1158.

¹⁹ See footnote 6, p. 5.

²⁰ See footnote 1, p. 4.

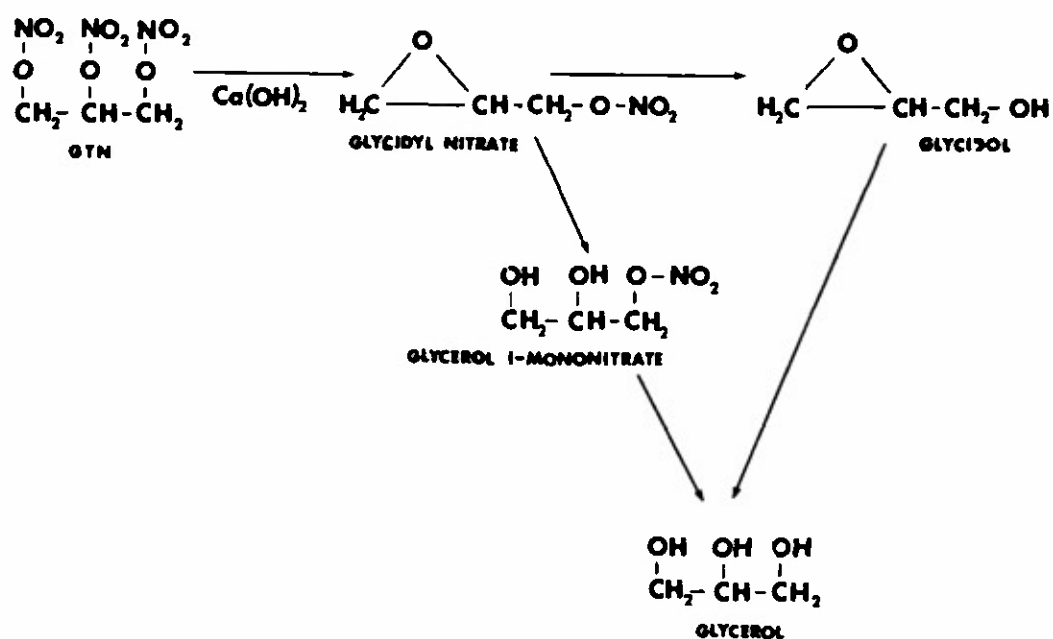


Figure 7. Scheme for chemical and biological degradation of GTN.

Glycerol 1-mononitrate, although toxic, is biodegraded. A biological treatment process could be optimized to accelerate the transformation rates under less energy rich media conditions.

CONCLUSIONS

The use of calcium hydroxide to chemically treat waste streams containing glycerol trinitrate may be a useful approach toward alleviating environmental hazards associated with nitroglycerine. The glycidol and glycidyl nitrate formed by this reaction are chemically/physically and biologically degraded to glycerol (from glycidol) and glycerol 1-mononitrate and glycerol (from glycidyl nitrate). Although glycidol and glycidyl nitrate are mutagenic in the Ames test, mutagenic activity is lost once the epoxide ring is cleaved.

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