Microbial Degradation of Glycerol Nitrates

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Received for publication 25 August 1978

The fate of glycerol trinitrate when exposed to microbial attack has been investigated. Contrary to some earlier reports, this compound was readily biodegraded by employing batch or continuous techniques under a variety of cultural conditions. Breakdown of glycerol trinitrate took place stepwise via the dinitrate and mononitrate isomers, with each succeeding step proceeding at a slower rate. After a residence time of 8 to 15 h, none of the glycerol nitrates could be detected in the effluent from a continuous-culture apparatus (chemostat) supplied with an influent containing 30 mg of glycerol trinitrate per liter.

Glycerol trinitrate (GTN) is manufactured in large quantities for use as a component of explosives and propellants and as a pharmaceutical compound (16). Industrial wastewater from the manufacture and processing of nitroglycerine poses difficult treatment and disposal problems because GTN and incompletely nitrated by-products comprising glycerol dinitrate (GDN) and glycerol mononitrate (GMN) isomers are relatively soluble in water. Their solubilities range from 1.8 g of GTN per liter to more than 700 g of GMN per liter at 20°C. The nitrated glycerols are toxic to a number of mammalian species in concentrations of 30 to 1,300 mg/kg for GTN, 800 to 2,300 mg/kg for 1,2-GDN, 500 to 800 mg/kg for 1,3-GDN, 900 to 6,000 mg/kg for 1-GMN, and greater than 5,000 mg/kg for 2-GMN (8, 16). GTN and GDN isomers are also sensitive to thermal and mechanical shock.

Chemical methods for treatment of GTN manufacturing and processing wastewaters, which may contain in excess of 2,000 mg of glycerol nitrates per liter, have usually been limited to digestion with strong caustic solutions or denitration with alkaline sodium sulfide. These methods are undesirable because of their relatively high cost and large consumption of chemicals, the presence of excess reactants which remain dissolved in the effluent, and the potential hazard of explosion resulting from the exothermic nature of the reactions. Evolution of toxic or offensive gases during the sulfide denitration (1) is a further disadvantage of this method.

Because of the widespread use of GTN as a pharmaceutical compound, it has long been known that in mammalian systems GTN is successively metabolized via the dinitrate esters, the mononitrate esters, and glycerol (10, 11). The metabolism of GTN in mammals is reported to be an enzymatic deesterification in the presence of glutathione which takes place principally in liver tissue (4, 5, 11, 12, 14), but may also occur in the blood (9, 10). In mammals, the enzymatic conversion of the trinitrate to the dinitrate is the most rapid reaction in the sequence, and the rates become progressively slower in the stepwise transformation of the dinitrate esters to mononitrate esters to glycerol (10, 11). It has also been reported that formation of glucuronide conjugates plays a major role in GTN metabolism in mammals (5).

Relatively little information appears in the literature concerning the biological treatment of GTN-bearing wastewater. One report mentions an activated sludge process which was apparently used for treatment of GTN manufacturing wastewater (7). However, it has frequently been reported that GTN is recalcitrant to biological attack, or is specifically nonbiodegradable (1, 13, 15). GTN was shown to exert a toxic effect on mixed microbial populations at concentrations in the range of 600 to 900 mg/liter (1, 17, 18) and was found to inhibit microbial denitration at concentrations as low as 100 to 150 mg/liter (18, 20).

In spite of reports to the contrary, recent work indicates that GTN undergoes biological modification by microorganisms (19). The object of this study was to determine whether GTN is, in fact, completely biodegradable, to clarify the rate and extent of breakdown under varying conditions, and to determine what, if any, intermediate compounds are formed. A further objective was to ascertain whether a biological process to treat wastewater containing glycerol nitrate esters is feasible from a technical point of view.

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MATERIALS AND METHODS

Media. The basal medium for batch shake-flask experiments consisted of 0.2 g of MgSO4·7H2O, 1.0 g of KH2PO4, 1.0 g of KHPO4, 0.02 g of CaCl2, and 0.05 g of FeC13 in 1 liter of distilled water adjusted to pH 7.0 before autoclaving. Where indicated, 1.0 g of glucose per liter and/or GTN (70 mg/liter of medium by dilution of a 980-mg/liter stock solution) was added.

Basal medium for continuous-culture, bench-scale, activated-sludge studies was composed of 2 g of nutrient broth (Difco) per liter of distilled water with GTN added prior to autoclaving at a concentration of 150 mg/liter of medium. The pH of the medium was adjusted to 7.0 by addition of 1 M KH2PO4 solution.

Basal medium for chemostat continuous-culture experiments consisted of 11 mg of KH2PO4, 0.7 mg of NaCl, 3.6 mg of MgSO4·7H2O, 0.57 ml of ethanol, 0.07 ml of activated-sludge, and 2.86 mg of NH4H2PO4 per liter of distilled water at pH 6.8. GTN was added to the basal medium for chemostat experiments at a concentration of 30 mg/liter of medium. This medium was selected to simulate as nearly as possible the composition of wastewater known to occur in GTN manufacturing and processing operations.

Culture conditions. Batch shake-flask experiments were performed using 100 ml of medium in 250-ml Erlenmeyer flasks incubated at 30°C with shaking at 225 rpm in a New Brunswick G24 Environmental Incubator Shaker. Activated-sludge continuous-culture experiments were run at room temperature in an apparatus consisting of two 2,000-ml reagent bottles, with tubulations for overflow at 1,500 ml, connected in series with a final 500-ml conical sedimentation vessel for solids recovery and return to the first reagent bottle in the series. Both reagent bottles were aerated by means of a fritted glass sparger with air flowing at 3.2 liters/min. The reagent bottles were agitated by means of air-driven magnetic stirrers. Detention time in the system was approximately 84 h, with influent flow rate controlled by means of a peristaltic tubing pump (Sage Instruments Tubing Pump model 375). Continuous-culture studies were also performed in a chemostat (New Brunswick, Bioflow model C30) with agitation at 400 rpm, aeration at 0.8 liter/min, and the temperature controlled at 30°C. Detention time in the chemostat was 8 to 15 h, with a working volume of approximately 350 ml.

Inocula. Inocula for the batch shake-flask culture experiments were obtained by inoculating nutrient broth with fresh activated sludge from a local domestic sewage treatment plant, incubating at 30°C for 18 h, centrifuging and washing three times in sterile buffer solution (0.312 mM KH2PO4 solution), and resting for 12 h at 4°C in the sterile phosphate buffer solution. Inocula for the continuous-culture experiments were obtained by adding 10 ml of aerated activated sludge from a domestic sewage treatment plant to the reaction vessels (the tubulated reagent bottles in the case of the activated-sludge system, and the culture vessel of the chemostat).

Pure cultures. The cultures used in the pure-culture experiments were obtained by streaking chemostat culture vessel contents onto nutrient agar plates, picking isolated colonies of differing colonial morphology, and subculturing. Purity of the cultures was confirmed by subculturing and microscopic examination of Gram-stained smears from each culture. No attempt was made to identify isolated organisms. As great a diversity as possible of cultural and microscopic morphological representatives was used in pure-culture studies. The eight pure cultures included three short gram-negative rods (cultures 1, 3, and 7), three very short, ovoid gram-negative rods (cultures 4, 5, and 6), one gram-negative rod of intermediate length (culture 2), and one large gram-negative rod exhibiting bipolar gram-positive staining (culture 8).

Chemical analytical methods. Samples of microbial growth medium were analyzed for glycerol nitrate ester content by first extracting the ester with organic solvents, followed by thin-layer chromatography (TLC) and/or high-performance liquid chromatography (HPLC) as later described. Extraction of 25 ml of spent medium with three 10-ml portions of dichloromethane in a separatory funnel very efficiently extracted GTN and the two GDN isomers but left the two GMN isomers in the aqueous phase. Extraction with ethyl ether in a liquid/liquid continuous extractor for 24 h removed all the glycerol nitrate esters. Using either method, extracts were concentrated by evaporation of solvent, at room temperature for ether and at 40°C for dichloromethane. Qualitative estimation of the nitrate esters in samples was done by spotting 10 to 30 µl of the concentrate on silica gel TLC sheets (Eastman Chromogram 13179, without fluorescent indicator). The sheets were developed in a chamber (Gelman model 51325-1) using benzene-ethanol (95/5, vol/vol) as the liquid phase. The spots were visualized by spraying the sheets with a fine mist of diphenylamine in ethanol (5%), followed by exposure to UV light (germicidal lamp) for 5 to 10 min. Quantitative analyses for GTN and the GDN isomers were performed by HPLC using a liquid chromatograph (Varian 4100) in conjunction with a silica column (Varian Micropak Si, 10 µm) 50 cm in length with 2.2 mm ID, a variable wavelength detector (Varian Variscan 635), and a strip chart recorder (Varian A-25). Peak areas were obtained by use of an electronic digital integrator. The liquid phase consisted of hexane-isopropanol (95/5, vol/vol). Analyses were conducted at 220 nm, with a slit width of 2 µm, a liquid phase flow of 30 ml/h, and a back pressure of 1,700 lb/in2.

Standard solutions of GTN in dichloromethane were prepared by extracting a standard aqueous solution of GTN (980 mg/liter) with an equal volume of dichloromethane. Standards of lesser strength were prepared by diluting the aqueous standard 10- or 100-fold and extracting with a volume of dichloromethane equal in volume to the diluted aqueous standard. Standards for the two GDN isomers and the two GMN isomers were prepared by synthesizing and purifying these compounds according to the methods described by Dunstan et al. (2, 3). A number of calibrating runs were made with GTN standards prior to each series of analyses to evaluate the performance of the chromatograph and to obtain values for calculating concentrations of the various components of the samples. An unsuccessful attempt was made to chromatograph aqueous samples using a 60-cm, bonded octadecysilane column (reversed phase) with water as the
liquid phase. The two GDN isomers were not resolved using this procedure, and there was interference from other metabolites in the samples taken after exposure to microbial attack. Resolution of the various fractions was not improved by adding an additional 60-cm length of column.

Very few special precautions were required for handling of GTN, which was obtained as a water solution of the pure compound. The most concentrated solution was approximately 50% of the 20°C saturation strength. This solution was maintained at 10°C or above to avoid precipitation of pure GTN from solution. During concentration of solvent extracts containing GTN or the GDN isomers, care was taken to avoid evaporation of all the solvent. Concentrated solvent samples containing GTN or the GDN isomers were stored at 4°C, care being taken to avoid subjecting the samples to freezing temperatures.

RESULTS

The results of analyses on samples from batch shake-flask experiments containing 67 to 68 mg of GTN per liter (with mixed cultures) indicated that a breakdown or biotransformation of GTN had taken place. Flasks which contained GTN, glucose, mineral salts, and a mixed culture of organisms showed a 53.6% decrease in GTN concentration after incubation with shaking for 5 days. A sterile control showed a 9.4% decrease in GTN after incubation, but the decrease in a nonsterile control with no glucose nutrient was only 3.1%. GTN was found not to be a suitable carbon source, nor, based on earlier studies, does it serve as a suitable nitrogen source for freshly isolated mixed cultures of organisms from domestic sewage activated sludge. The small decrease in GTN seen in the sterile control may have been due in part to a low level of chemical decomposition of GTN under the conditions of the experiment.

Figure 1 shows a typical TLC separation of dichloromethane extracts from a GTN-containing medium before and after exposure to a mixed culture in a bench-scale activated sludge process. The influent concentration was 150 mg of GTN per liter, and the detention time was about 84 h. Spots 1 to 4 were made from extracts of the influent, contents of the first of the two reaction vessels in series, contents of the second reaction vessel, and effluent from the sedimentation vessel, respectively. The identities of the various compounds separated and visualized by this technique were determined by standards run concurrently (spot 5). The progressive breakdown of GTN via the two GDN isomers through the stages of the bench-scale process is apparent.

Figures 2 and 3 show results of HPLC analyses on dichloromethane extracts of samples from the activated sludge system prepared in the same way as those samples on the TLC plate shown in Fig. 1. In chromatogram 1 of Fig. 2 a large peak for GTN is found in the extract of the sterile influent to the activated sludge process. Chromatogram 2 of Fig. 2 shows the results of analysis of the contents of the first of the two reaction vessels in the biological process. In that stage the GTN peak is reduced, and prominent

Fig. 1. TLC separation of dichloromethane-extracted samples of (1) influent, (2) first-stage contents, (3) second-stage contents, (4) effluent from bench-scale activated-sludge process. (5) Standards of nitrate esters extracted.
FIG. 2. HPLC analyses of influent and first-stage contents samples from a bench-scale, two-stage activated-sludge process.

FIG. 3. HPLC analyses of second-stage and effluent samples from a bench-scale, two-stage activated-sludge process.

1,3-GDN and 1,2-GDN peaks are detected. Chromatogram 1 of Fig. 3 shows the results of HPLC analysis of dichloromethane extracts of the contents of the second reaction vessel of the biological process. In this vessel GTN and GDN isomers are all diminished from those seen in the analysis of the contents of the proceeding stage. Analysis of the effluent from the process (chromatogram 2, Fig. 3) shows nearly undetectable peaks for the GDN isomers and a very
small residual peak at the retention time for GTN. TLC analysis of the same samples showed no GTN in the effluent, with a sensitivity nearly equal to that of the HPLC analysis used here. This observation suggests that some constituent in the spent medium, not related to the glycerol nitrates, may have interfered with the HPLC analysis leading to somewhat erroneously high results for GTN in the effluent from the activated sludge process.

To determine whether GTN or its metabolites were being concentrated in the sludge, samples of solids from the activated-sludge process were sonically disrupted until no intact cells could be found microscopically, and each sample was extracted with dichloromethane. None of the glycerol nitrates could be detected in the extracts from the sonically disrupted solids, suggesting that there was no significant retention of GTN or its metabolites by the activated-sludge floc.

A series of controls was run to evaluate the efficiency of extraction of GTN from sonically disrupted activated sludge. GTN was added to distilled water, to a 1% suspension of activated-sludge solids, and to a 1% suspension of activated-sludge solids which was subsequently sonically disrupted. There was no difference in extraction efficiency between the distilled water and the two controls containing activated-sludge solids.

HPLC analyses were run daily over a 2-week period on the various stages of the activated-sludge process. The influent concentration of GTN was 150 mg/liter. The analytical results indicated an average GTN reduction in the first reaction vessel of 66.1% (range of 21.0 to 91.8%) and an average reduction of GTN and the GDN isomers taken together of 49.4% (range of 6.2 to 77.9%). Average GTN reduction in the second reaction vessel was 85.3% (range of 49.6 to 97.6%) and 68.2% for GTN with the GDN isomers (range of 18.6 to 90.4%). The effluent from the process showed a 92.2% reduction in GTN (range of 86.1 to 99.8%) and a 77.4% reduction in GTN with GDN isomers (range of 60.5 to 89.6%).

Figure 4 shows TLC results from an experiment in which a chemostat received the chemostat basal medium containing 30 mg of GTN per liter. The TLC was spotted with concentrates from the influent, culture vessel contents, and effluent from the chemostat. The two GDN isomers and GMN isomers were included as reference standards. Under these conditions, the chemostat basal medium provided the necessary environment for the bacteria to completely degrade the GTN at the influent concentration, as shown by the absence of any detectable nitrate ester at spot 4 on the TLC.

Figure 5 shows a TLC plate spotted with ether extracts of shake-flasks which had been inoculated with pure single cultures of bacteria isolated from the chemostat. Extracts from eight cultures were spotted, numbered 1 to 8, with standards for GTN, both GDN isomers, and the 1-mononitrate isomer. The same intermediates were present in each of the pure cultures as resulted from the degradation of GTN by mixed cultures, showing variation only in concentration. One culture (spot 1) showed a faint spot.

![TLC separation of ether-extracted samples](http://aem.asm.org/)

**Fig. 4.** TLC separation of ether-extracted samples of (1) influent to chemostat systems, (2) contents of chemostat having no added nutrient, (3) effluent from chemostat having no added nutrient, and (4) standard compounds.
which corresponded to none of the standards used. This compound did not appear in any of the other analyses and has not been identified. Under the TLC conditions we employed, the 1 and 2 GMN isomers have the same Rf and were not resolved by modifying the solvent system or use of alumina plates.

**DISCUSSION**

The microbially mediated breakdown of GTN occurs in a stepwise fashion involving all of the mono- and diesters. The metabolic pathways we postulate for the microbial degradation are similar to those described earlier as occurring in mammalian systems. Based upon all the data available to date, the pathway of breakdown of GTN appears to follow the scheme presented in Fig. 6. No attempt was made to determine glycerol concentration in the spent medium. The arrows indicating breakdown of the mononitrate esters to glycerol are dashed to indicate that it is the probable metabolite.

**Fig. 5.** Thin-layer separation of ether-extracted samples from pure shake-flask cultures containing 3 mg of GTN per liter (1 to 8) and (9) standard compounds.

**Fig. 6.** Possible breakdown routes of GTN in microbial systems.
The fact that the di- and mononitrate esters of glycerol can be found in spent media from both the continuous and batch processes suggests that each succeeding step in the breakdown sequence is slower than the one preceding.

This study has clearly shown not only that GTN is subject to rapid and complete breakdown by microorganisms, but that it degrades relatively quickly under conditions similar to those which would be encountered in actual field conditions, i.e., after 8 to 15 h of detention time in a continuous-culture apparatus (chemostat) under aerobic conditions with no solids recycling. Solids-recycling processes such as the conventional activated-sludge process are not likely to see widespread use in treating GTN manufacturing wastewaters from government-owned facilities due to problems encountered with solids retention (6). The composition of the influent to the chemostat system was designed to simulate as nearly as possible the concentrations of components which would be seen by microorganisms in water treatment plants where waste effluents from propellant processing and manufacturing operations would be treated. The effluent from the chemostat showed no evidence of any nitrate esters. The present work suggests that a microbiological treatment process for GTN-bearing wastewater using wastes normally present at GTN manufacturing and processing plants is feasible. Such a biological process would avoid a costly physical-chemical treatment, which is also environmentally objectionable and potentially hazardous.

LITERATURE CITED


