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NITROGLYCERIN BIODEGRADATION: THEORETICAL THERMODYNAMIC CONSIDERATIONS

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ABSTRACT

Biochemical transformation of nitroglycerin (glycerol trinitrate, GTN) has been broadly established, yet its mineralization as a sole carbon and energy source has not. This manuscript examines whether there are thermodynamic reasons why GTN can not serve as a sole carbon and energy source for microbial growth based on hypothetical pathways for biochemical transformation, and considers the stoichiometric limitations for the removal of the released nitrite or nitrate ions during GTN transformation. The results of these analyses indicate that growth on GTN as sole carbon and energy source is thermodynamically feasible under both aerobic and anoxic conditions, and that maximal nitrite and

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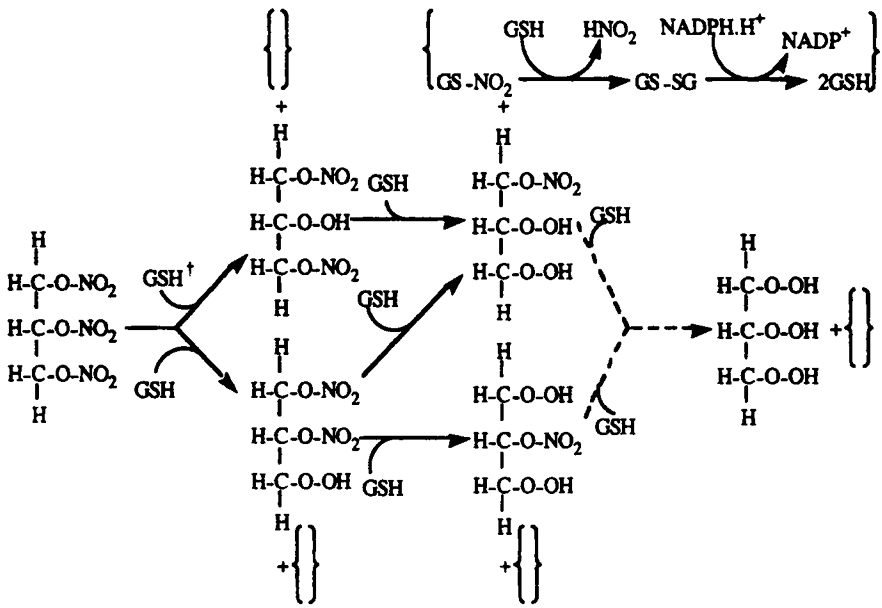
nitrate removal is achievable under anoxic conditions when denitrification is incomplete.

INTRODUCTION

Transformation of GTN in mixed microbial cultures was reported as early as 1978 by Wendt *et al.*[1] in aerobic batch and continuous reactor systems in the presence of other carbon sources such as glucose, nutrient broth, or ethylether and ethanol. They observed complete conversion of all nitrate esters in chemostats fed 30 mg/L of GTN and demonstrated the sequential formation of di- and mononitrate in batch assays. Pesari and Grasso[2] measured transformation of GTN in a sequencing batch reactor with aerobic and anoxic cycles treating a wastewater containing up to 170 mg/L of GTN and ethylacetate. They maintained an ethylacetate COD/GTN ratio of > 7 in their work to ensure that sufficient energy was available for GTN transformation. GTN removal was fastest during the aerobic phase, but also occurred during the anoxic phase. These authors were unable to retrieve enrichment cultures which could transform GTN as sole carbon and energy source in batch assays.

Biochemically more detailed work on GTN transformation has been done with fungal cultures: *Geotrichum* sp. and *Phanerochaete* sp.[3,4] In assays with resting cells pregrown on complex media, complete conversion of the trinitrate to di- and mononitrates was observed.[3,4] Regioselectivity of the enzymes involved was implicated.[5] The initial release of nitrite rather than nitrate suggested that

cleavage of the nitrate esters was reductive, not hydrolytic, and the involvement of a glutathione-S-transferase was suggested.[3,4] Glutathione-S-transferases are known to be involved in GTN transformation in mammalian systems[6] and are also known to act as dehalogenases with activity towards dichloromethane and pentachlorophenol in bacteria.[7,8] Based on the work by Wendt *et al.*[1] and Servent *et al.*,[3] the following pathway can be proposed for microbial transformation of GTN to glycerol.



†GSH = γ-L-glutamyl-L-cysteinylglycine

FIGURE 1.
Initial Hypothetical Pathway in the Biotransformation of GTN

GTN is first transformed to a mixture of glycerol dinitrate esters. The conversion is assumed to involve the transfer of two glutathiones per nitrate group removed.[3,9] The subsequent removal of an additional nitrate group occurs via a

similar mechanism, although that enzyme may be different from the GTN transforming enzyme.[5] A final nitrate removing step results in the formation of glycerol. The initial steps in GTN transformation may take place in the periplasm or cytoplasm; this has not been examined. Periplasmic activity would require the presence of glutathione and glutathione regenerating systems in the periplasmic space and these have been identified in *Escherichia coli*.[11, 12] On the other hand, cytoplasmic transformation of GTN would require its transport across the cell membrane. GTN is only moderately hydrophylic and facilitated diffusion across the membrane appears feasible, although the facilitator uptake system for the structurally similar glycerol may not be functional due to steric hindrances. Evidence for cytoplasmic activity against ethylene glycol dinitrate, the two carbon analog of GTN, has been reported (Tan-Walker, 1987[10] as reported in [5]).

Thus far, microbial transformation of GTN when present as a sole carbon source has not been observed. All reports to date have reported GTN transformation either by resting cells pregrown on other carbon sources or by cells grown in continuous culture degrading other substrates.[1-5] The question then arises whether this is the result of limitations in the experimental approaches employed or the result of inherent properties of GTN that render it unable to serve as a growth substrate.

In support of the first possibility is the fact that microbial GTN transformation has been measured with very different and biogenic compounds[3,4] as the main carbon source, ethylacetate[2], glucose, nutrient broth, ethylether and

ethanol[1]. Furthermore, in these reports, the attempt to isolate enrichment cultures on GTN as sole carbon source has involved aerobic batch type experiments. Development of enrichment cultures is typically more successful when done under continuous reactor conditions where the steady low substrate mixed culture environment provides a more efficient selective pressure;[13] in addition different biochemical environments for enrichments should be explored.[14]

THERMODYNAMIC BASIS FOR GTN BIOTRANSFORMATION

To consider the second possibility, it is instructive to evaluate whether thermodynamic considerations suggest a limitation in GTN's degradation as a sole carbon and energy source. A compound which can only be degraded co-metabolically is one which does not yield net energy to the transforming cell, which therefore requires other primary substrates to sustain cell growth and maintenance.[15] Thus, the energy-requiring steps in GTN transformation should be identified, and their energy sink compared with the energy available in subsequent enzymatic reactions.

Assuming the biochemical pathway proposed above for the transformation of GTN to glycerol and established microbial metabolic pathways for growth on glycerol, a probable pathway for GTN mineralization can be hypothesized and its thermodynamic feasibility explored.

Thermodynamic calculations involve computation of the available Gibbs free energy when glycerol is transformed to CO_2 and the comparison of that available energy to the Gibbs free energy required to convert GTN to glycerol. If more energy is required to transform GTN to glycerol than is available in the oxidation of glycerol to CO_2, growth is thermodynamically impossible. On the other hand, if the GTN-to-glycerol transformation requires less energy than is available in the glycerol-to-CO_2 oxidation, growth on GTN as sole carbon and energy source is thermodynamically feasible. Subsequently, the net available Gibbs free energy available per electron equivalent of GTN is calculated and compared with the generally accepted minimum value of 10 kJ/equivalent needed to drive ATP synthesis (This assumes a 1/1 stoichiometry between H^+ extruded per e transferred in the electron transport chain and a ratio of 3 H^+ required per ATP synthesized). Such calculations must be performed with the terminal electron acceptors O_2, NO_2^- and NO_3^- to evaluate the feasibility of GTN serving as carbon and energy source under aerobic and anoxic conditions. With glutathione-S-transferase being the nitrite releasing enzyme the following overall reaction stoichiometry per nitrite group released can be written as:

$$\overset{|}{\underset{|}{C}}-ONO_2 + 2\ GSH + NADPH.H^+ \Rightarrow \overset{|}{\underset{|}{C}}-OH + HNO_2^- + NADP^+ + 2\ GSH$$

Where GSH refers to glutathione. This illustrates that 1 mole of $NADPH.H^+$ is required per mole of nitrite released. $NADPH.H^+$ synthesis can occur via only a few mechanisms, of which formation from $NADH.H^+$ by transhydrogenation is

likely[16]. $NADH.H^+$ itself is formed in oxidative dehydrogenation reactions in the metabolism of the glycerol product of GTN. The first occurrence is at the level of glyceraldehyde-3-P dehydrogenase:[16]

$$\text{glyceraldehyde-3-P} + NAD^+ + Pi \Rightarrow \text{1,3-bis-P-glycerate} + NADH.H^+$$

This reaction is thermodynamically unfavorable; a drain in free energy for this reaction is approximately 20 kJ/mole.[17]

The subsequent transhydrogenation can be represented as

$$NADH.H^+ + NADP^+ \Rightarrow NAD + NADPH.H^+$$

This reaction is thermodynamically unfavorable as well, and is driven by the proton motive force of the cell.[16] The actual energy requirement is not well known, and will be highly dependent on the actual intracellular concentration of the products and reactants. As a first approximation, hydrolysis of one ATP can be assumed, which corresponds to approximately 32 kJ/mole.[17] In sum, the total Gibbs free energy spent on the generation of 1 mole of $NADPH.H^+$ is approximately 52 kJ. This energy drain is required to permit the removal of one nitrate moiety from GTN. This drain can be expressed in the amount of energy drained per available electron in glycerol, wherein the available electrons are calculated as presented by Heijnen *et al.*[18] Because GTN contains 3 nitrate groups, and the removal of each demands 1 mole of GSH and therefore 1 mole of

NADPH.H$^+$, 3* 52 kJ or 156 kJ of energy are required to convert GTN to glycerol. This value can then be converted to an average quantity of energy drained per equivalent of available electron in glycerol (with the degree of reduction of 4.67 and 3 moles of C/mole of glycerol [18]): 156/ (4.67 * 3) = 11.2 kJ. (or 0.116 eV, with $\Delta G = - nF\Delta E$, where F = Faraday constant = 96.6 kJ/V/mole, and E = potential in V). The Gibbs free energy available in the electron transfer from the electron donor (glycerol) to the terminal electron acceptor (O_2, NO_2^-, or NO_3^-) must be adjusted by subtraction of that value to calculate the net available energy for growth:[18,19]

$$\Delta G_{e,av}^{o1} = \Delta G_{eD}^{o1} - \Delta G_{eA}^{o1} - \Delta G_{e^-}^{o1}$$

Where ΔG_{eD}^{o1} and ΔG_{eA}^{o1} refer to the available Gibbs free energy per electron in the electron donor and electron acceptor reaction, respectively, while $\Delta G_{e^-}^{o1}$ refers to the average energy per electron used to convert GTN to glycerol, and the resultant net available energy for growth per electron is represented by $\Delta G_{e,av}^{o1}$. The results of such analysis are shown in Table 1.

Table 1. also considers the use of NO_3^- as a terminal electron acceptor, because, in mixed microbial communities, nitrifying organisms may convert the NO_2^- to NO_3^-, which can subsequently be available as electron acceptor for the GTN transforming strains. Several redox reactions are considered with NO_2^- and NO_3^- because the actual electron redox reaction used may differ.[17] The results in Table 1 clearly illustrate that with the assumed pathway for GTN transformation

growth on GTN as a sole carbon source should be feasible with any of the examined terminal electron acceptors including the poorest electron acceptor redox couple, NO_2^-/NO.

TABLE 1.
Relevant Thermodynamic Properties of Electron Donor and Electron Acceptor Couples and The Available Gibbs Free Energy per Electron

Redox Pair	Degree of Reduction* (γ_i)	ΔG_e^{o1}, (kJ)	$\Delta G_{e,av}^{o1}$ (kJ)	$\Delta G_{e,av}^{o1} > 10$ kJ?
$Glycerol/HCO_3^-$	4.67	37.8		
O_2/H_2O	-4	-78.8	105.4	Yes
NO_3^-/NO_2^-	-2	-41.8	68.4	Yes
NO_2^-/NO	-1	-33.8	60.4	Yes
NO_3^-/N_2	-5	-72.4	99.0	Yes
NO_2^-/N_2	-3	-93.7	120.3	Yes

*γ_i = The degree of reduction of a compound describes its electron content. For compounds which serve as electron donors, it reflects the number of electron equivalents which can be removed per mole of carbon when the compound is entirely mineralized. For a compound which serves as electron acceptor, it refers to the number of electron equivalents that can be consumed per mole of compound in the pertinent terminal electron acceptor reaction[18].

, ΔG_e^{o1} refers to ΔG_{eD}^{o1} or ΔG_{eA}^{o1} depending on the considered redox pair

STOICHIOMETRY OF NITRO-N-REMOVAL

Because release of NO_2^--N or NO_3^--N, together termed nitro-N, in the environment is undesirable, complete treatment of GTN should include the removal of the generated NO_2^- or NO_3^- ions. The biological removal of NO_2^- or NO_3^- can take place via two mechanisms. First, the nitro-N can be removed by incorporation into biomass. Removal of nitro-N through cell synthesis is limited

by the typical elemental composition of the bacterial cell because, unlike for P and C, intracellular storage polymers containing large molar ratios of N are not known.[22] Thus, a pseudo-chemical formula for bacterial biomass, $C_5H_7O_2N$ or $C_{60}H_{87}O_{23}N_{12}P$,[21] permits calculation of N-removal based on cell synthesis. Second, under anoxic conditions, NO_2^- or NO_3^- can serve as terminal electron acceptors, permitting the removal of more nitro-N per unit of substrate carbon consumed. The above thermodynamic analysis suggests that GTN should be able to serve as a sole carbon and energy source under both aerobic and anoxic conditions. Furthermore, there is thus far no evidence for the involvement of a step requiring molecular oxygen in the degradation of GTN. As a result, anoxic conversion of GTN appears feasible. The same thermodynamic approach used above allows one to estimate the stoichiometry of bacterial growth with knowledge about the electron donor (its carbon chain length and degree of reduction), the pertinent electron acceptor reaction, the nitrogen source used, and a consideration of energy dissipation in growth and maintenance related functions.[18,19] NO_2^--N or NO_3^--N removal accompanying growth on GTN or glycerol can then be calculated as Y_{DA}, the moles of terminal electron acceptor (NO_2^- or NO_3^-) used per mole of electron donor consumed. Figure 2 illustrates Y_{DN} the ratio of NO_2^--N or NO_3^--N to organic substrate which can be removed under various scenarios, expressed in units of mole of N/C-mole of substrate. The different terminal acceptor couples presented in Table 1 were examined. In addition, we examined the cases where the N-source for biomass synthesis was either ammonia-N or nitrate-N to quantify the effect of assimilative NO_3^-

reduction on N removal. Furthermore, cases were examined where growth was on GTN or on its putative transformation product, glycerol.

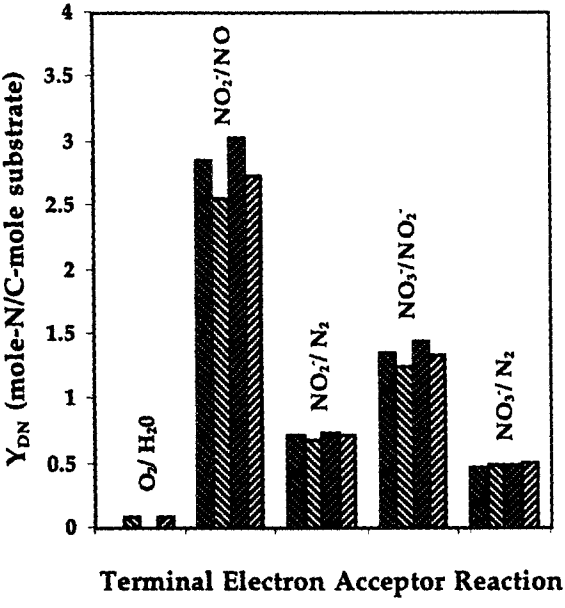


FIGURE 2.
The Stoichiometry of Nitro-N Removal per Unit of Growth Substrate Removed for Different Terminal Electron Acceptor Reactions

Glycerol (Diagonal downward to the right) and GTN (Diagonal downward to the left) were considered as growth substrates, while NH_4^+ (Bold diagonals) or NO_2^- and NO_3^- (Thin diagonals) were considered as nitrogen sources. Y_{DN} measures the nitro-N removed per unit of growth substrate removed (mole-N/C-mole of growth substrate).

Figure 2 illustrates several key points. First, the choice of either ammonia-N or nitrate-N as the N source has only a small impact on the potential nitro-N removal efficiency. Second, when denitrification is complete, i.e., when NO_2^- or NO_3^- are reduced to N_2, denitrification does not suffice to remove all nitro-N available, because the N/C mole ratio in GTN is 1 and additional carbonaceous

substrate would be needed to achieve complete nitro-N removal. Third, incomplete denitrification, i.e. the production of NO_2^- from NO_3^- or the formation of NO from NO_2^- allows much higher nitro-N removal efficiencies. The production of NO_2^- from NO_3^- is clearly undesirable, as N is not removed form the aqueous waste stream. If formation of NO from NO_2^- were the main electron acceptor reaction, GTN would contain insufficient NO_2^- to provide terminal electron acceptor, and additional electron acceptor would be required. Finally, under aerobic conditions, the removal of nitro-N is small and depends on the incorportion of N into new biomass. Clearly, the actual terminal electron acceptor reaction will be a key in the ability of the system to efficiently transform GTN and remove nitro-N.

SUMMARY

This manuscript examined and confirmed the thermodynamic feasibility of GTN mineralization as a sole carbon and energy source under both aerobic and anoxic conditions. This feasibility corroborates the need to pursue continued development of GTN mineralizing enrichment cultures, preferably using continuous culturing methods with exposure to aerobic and anoxic biochemical environments. In addition, it was shown that removal of released nitrite is most efficiently achieved under anoxic conditions, furthering the interest in developing anoxic enrichment cultures for GTN mineralization. Eventually the development of enrichment cultures will allow one to study the biochemical basis of GTN mineralization, specifically the nitrate releasing steps, in more detail, and allow

one to acquire the kinetic information on GTN transformation that, in addition to the thermodynamic and stoichiometric information, is necessary for optimal design of biochemical GTN treatment processes.

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