Metabolism of Trimethylamine, Choline, and Glycine Betaine by Sulfate-Reducing and Methanogenic Bacteria in Marine Sediments†

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The response of methanogenesis and sulfate reduction to trimethylamine, choline, and glycine betaine was examined in surface sediments from the intertidal region of Lowes Cove, Maine. Addition of these substrates markedly stimulated methanogenesis in the presence of active sulfate reduction, whereas addition of other substrates, including glucose, acetate, and glycine, had no effect on methane production. Sulfate reduction was stimulated simultaneously with methanogenesis by the various quaternary amines and all other substrates examined. Incubation of exogenous trimethylamine, choline, or glycine betaine with either bromoethane sulfonic acid or sodium molybdate was used to establish pathways of degradation of the substrates. Methanogenesis dominated the metabolism of trimethylamine, although limited nonmethanogenic activity, perhaps by sulfate-reducing bacteria, was observed. Acetate was oxidized primarily by sulfate reducers. Both choline and glycine betaine were fermented stoichiometrically to acetate and trimethylamine; apparently, neither substrate could be utilized directly by methanogens or sulfate reducers, and the activities of fermenters, methanogens, and sulfate reducers were all required to effect complete mineralization. These observations support the hypothesis that the presence of quaternary amines can mediate the coexistence of sulfate reduction and methanogenesis in marine surface sediments; they also implicate methanogens in the nitrogen cycle of marine sediments containing quaternary amines.

To understand the factors regulating the dynamics of methylamines, and therefore the controls of methanogenesis in surface marine sediments, one must understand the patterns of metabolism of methylamine precursors. Two potential precursors of TMA are CHO and GBT. CHO, which is widely distributed in membrane lipids and is undoubtedly a component of the sediment biota, is readily fermented to TMA and acetate by clostridia (3) and SRB (7, 9). GBT is a common solute used in osmoregulation by bacteria (8, 15, 25), algae (2), many marine invertebrates (1, 14, 23), and vertebrates (34). Significantly, many benthic organisms and sediment infauna contain GBT. The wide distribution and high concentrations of GBT support a role for this compound as a major source of TMA. Observations by Naumann et al. (18) on GBT fermentation by Clostridium sporogenes have verified that GBT can be degraded to TMA anaerobically. However, the pathways of metabolism and the organisms involved in GBT degradation in marine sediments have not been described.

Results of a study of CHO and GBT metabolism in intertidal sediments from a mud flat are reported here. In these sediments, both CHO and GBT are fermented to TMA and acetate. Neither substrate appears to be used directly by SRB. The TMA formed from the fermentation of CHO and GBT is rapidly converted to methane in the presence of active sulfate reduction; acetate from CHO and GBT is oxidized simultaneously by SRB.

MATERIALS AND METHODS

CHO and GBT metabolism were investigated in sediments obtained from the intertidal region of Lowes Cove, Maine. Some general aspects of these sediments have been previously described (11). Sediments were collected with 10-cm (outer diameter) coring tubes from areas which appeared devoid of large fragments of seaweed detritus. The cores were sectioned in an anaerobic glove bag containing 100% deoxygenated N₂. The 0- to 10-cm depth interval was slur-
ried (1:1) with deoxygenated seawater; 9-ml samples of the slurry were dispensed into Hungate pressure tubes (Belco Glass, Inc.) containing a gas phase of 100% deoxygenated N₂. Effects of substrate addition on methanogenesis were determined by injecting into each sealed tube 1.0 ml of a solution containing TMA, CHO, GBT, glucose, acetate, glycine, or proline. Final concentrations of all added substrates were 1 mM. In addition, some sediment samples were supplemented with either bromomethane sulfonic acid (BES) or sodium molybdate at final concentrations of 100 and 20 mM, respectively; BES concentrations of <100 mM were not always completely effective. Methane production from unamended controls or sediments plus substrate was determined by sampling the tube headspace with a needle and syringe and analyzing methane with a Varian 1440 gas chromatograph fitted with a flame ionization detector and a stainless steel column (1 m by 3 mm [outer diameter]) containing Porapak Q (Waters Associates, Inc.). The column was operated at 70°C with a nitrogen carrier flow rate of 20 ml/min.

Effects of the addition of a selected substrate on sulfate reduction were determined by adding acetate, GBT, CHO, or TMA to sediment slurries as described above. In addition, the slurries were amended with 1 μCi of 35SO₄²⁻ (carrier-free; New England Nuclear Corp.). Rates of sulfate reduction in the slurries were assayed by acidifying triplicate samples for each substrate after incubation for periods of up to 7 days. H₂³⁵S volatilized by acidification was flushed from the tubes with deoxygenated N₂ and trapped in a series of three scintillation vials, each of which contained 7.5 ml of 3% zinc acetate; 7.5 ml of Aqueous Counting Scintillant (Amersham-Searle, Inc.) was added to the vials for radioassay.

TMA and acetate production were assayed as described above in sediments that were supplemented with 1 mM (final concentration) CHO or GBT and either BES (final concentration, 100 mM) or sodium molybdate (final concentration, 20 mM). TMA produced in each treatment was measured by collecting 1-ml samples of interstitial water from triplicate samples for each time point. Each sample of interstitial water was acidified with 100 μl of 1 N HCl and supplemented with 20 mg of (NH₄)₂SO₄. The resulting solutions were evaporated to dryness in 10-ml serum vials. After the vials were sealed with Teflon-faced septa, 1 ml of 2 N KOH was injected into each vial to volatilize the TMA. The TMA content of the gas phase of the vials was determined by injecting a 0.5-ml sample of the headspace into a Varian 3700 gas chromatograph fitted with a flame ionization detector and a glass column (2 m by 6 mm [outer diameter]) containing Carbopak B–4% Carbowax 20M–0.8% KOH (Supelco, Inc.). The column was operated at 90°C with a nitrogen carrier flow rate of 30 ml/min. Acetate production was monitored by collecting samples of interstitial water as above, adding 10 μl of 1 N KOH, and evaporating the solutions to dryness in 10-ml serum vials. The acetate in the dried residues was distilled under vacuum after acidification with 200 μl of 1 N H₃PO₄ as described by Christiansen and Blackburn (6). Samples (1 μl) of distillate were injected into a Varian 3700 gas chromatograph fitted with a glass column (2 m by 6 mm [outer diameter]) containing SP-1200 (Supelco). TMA recoveries with the above technique were >95%, and sample were not corrected for this efficiency. Recoveries of acetate were about 85%, and samples were corrected for this value.

Ammonia production from TMA, CHO, and GBT (final concentrations 1 μmol/ml) was assayed by using sediment slurries as described above. Sediments plus substrates were incubated at ambient laboratory temperatures for up to 7 days. The slurries were centrifuged, and 100-μl samples of the supernatant were collected for ammonia analysis by the method of Koroleff (14). Control slurries to which no substrates were added were used to determine ammonia production from endogenous substrates.

All of the preceding assays and treatments made use of at least triplicate samples. Typically, coefficients of variation for the replicate samples of any given treatment at any of the incubation periods assayed were less than 20%. The slurries used were all incubated in darkness at 20°C without shaking.

**RESULTS**

The addition of TMA, CHO, or GBT to sediment slurries resulted in a marked stimulation of methane production (Fig. 1). The response to TMA was, however, more rapid than that for CHO or GBT. Methane accumulated rapidly in sediments containing TMA for a period of ca. 48 to 72 h, with lower rates of accumulation for the following 72 to 120 h. At the termination of the experiment, the observed methane concentrations accounted for ca. 58% of the theoretical yield, assuming a 2.25:1 molar conversion of TMA to methane. Methane production in the presence of added CHO underwent a brief lag, after which rates of methane accumulation paralleled those for TMA (Fig. 1). The total concentration of methane at the termination of the experiments was also ca. 58% of that expected on the basis of potential TMA production, assuming that no other methanogenic precursors from CHO metabolism were utilized. A somewhat longer lag was observed for stimulation of methanogenesis by GBT. Significant increases occurred only after 48 to 72 h of incubation, and rates of methane accumulation were less than those when either TMA or CHO was used. The final concentation of methane was about 42% of that expected on the basis of potential TMA production, again assuming that no other methanogenic precursors formed from GBT were utilized.

In contrast to the results from the methylated amines, no other substrate additions stimulated methanogenesis (Fig. 2). Methane production in sediments containing 1 mM (final
The recovery followed by aate during incubation of proline, concentrations in these -35SO42(1) methanol (concentration) glucose, glycine, or proline, or no exogenous substrates. CTRL, Control.

Addition of glucose, acetate, CHO, GBT, and TMA at 1 mM (final concentrations) stimulated sulfate reduction in sediment slurries, as indicated by the recovery of added 35SO42- as H235S (Fig. 3). The extent of stimulation by these substrates varied somewhat during the first 24 to 72 h of incubation, but by the end of the experiment the greatest stimulation was observed for acetate, CHO, and GBT, followed by glucose and TMA. Addition of 1 mM (final concentration) methanol resulted in a stimulation intermediate between those of glucose and TMA (data not shown). The recovery of radiolabeled sulfide and assays of sulfate concentrations in these sediment slurries (final concentrations, ca. 20 mM) indicated that sulfate depletion did not occur during any of the experiments reported here.

Patterns of CHO and GBT metabolism were determined by incubation of these substrates with an inhibitor of methanogenesis (100 mM BES) or sulfate reduction (20 mM sodium molybdate). The formation of TMA and acetate from the added substrates was then assayed for 7 days. In sediments containing BES, the concentration of added TMA decreased slightly from an initial value of 850 nmol/ml during a 7-day incubation period (Fig. 4a). The observed decreases could have been due to nonmethanogenic metabolism or to adsorption to the sediment. In contrast, TMA accumulated in sediments containing BES and either CHO or GBT (added to a final concentration of 1,000 nmol/ml). Accumulation occurred after a lag of 24 h for CHO and 48 h for GBT, with maximum TMA concentrations reaching 775 and 555 nmol/ml for the two respective substrates. No changes in TMA concentrations were noted for unamended controls; TMA accumulation in sediments containing BES only was ca. 20 nmol/ml.

In sediments containing 20 mM sodium molybdate, the concentration of added TMA decreased rapidly from 850 to ca. 35 nmol/ml within 5 days, reaching <20 nmol/ml by 7 days (Fig. 4b). TMA accumulated transiently in sediments containing molybdate and either CHO or GBT. The lag in...
accumulation of TMA from these substrates in the presence of molybdate was similar to that in sediments containing BES (Fig. 4a and b). However, in the presence of molybdate, both maximum and final TMA concentrations were higher for GBT than for CHO additions. The TMA concentration decreased slightly from initial levels (ca. 5 nmol/ml) in controls containing only molybdate; loss of added TMA in uninhibited controls and sediments containing molybdate was similar (data not shown).

Patterns of acetate production in sediments containing BES and either CHO or GBT (Fig. 5a) were similar to those of TMA in sediments containing molybdate (Fig. 4b). Acetate accumulated transiently and decreased after 72 h; maximum concentrations were higher in sediments containing CHO than in those containing GBT (603 versus 517 nmol/ml, respectively), although final concentrations were greater for sediments containing GBT than for those containing CHO (233 versus 77 nmol/ml, respectively). Acetate in controls containing only BES showed a small, transient increase after 72 h and a return to initial concentrations (30 nmol/ml) by 168 h.

Incubation of sediments with molybdate resulted in a continuous increase in acetate concentrations in all treatments (Fig. 5b). Acetate concentrations in controls containing only molybdate increased from ca. 250 nmol/ml at 24 to 700 nmol/ml at 168 h; acetate concentrations in sediments containing CHO or GBT increased from 180 and 310 nmol/ml to 1,420 and 1,240 nmol/ml, respectively, over the same interval. Initial acetate concentrations (time zero) were ca. 10 nmol/ml for all treatments (data not shown).

Results of the substrate addition-inhibition experiments are summarized in Table 1, which shows net acetate and TMA production from CHO and GBT (after subtraction of concentrations in controls) in the various treatments at the end of the incubations. The ratio of acetate production in sediments containing molybdate to TMA production in sediments containing BES is used to examine the stoichiometry of end product formation in the absence of the processes primarily responsible for their consumption. These ratios are ca. 1.0 (Table 1) for both CHO and GBT.

An analysis of ammonia concentrations in sediment slurries after incubation for 7 days with TMA, CHO, or GBT (final concentration, 1 μmol/ml) (Table 2) indicated that there were similar levels of ammonia formation for TMA and CHO and lower values for GBT. Total ammonia production followed previous patterns for methanogenesis (Fig. 1) as well as acetate and TMA production from the added substrates (Table 1). In addition, the time courses of both ammonia and methane production from TMA were similar; each end product accumulated after a 24- to 48-h lag and reached relatively constant values within 96 to 108 h (data not shown). Observed ammonia production also agreed reasonably with expected production based on acetate and TMA formation in sediments containing CHO or GBT (Figs. 4 and 5; Table 1). Observed ammonia production from TMA was somewhat less than expected, based on TMA uptake in sediments containing molybdate (Fig. 4b). Ratios of observed to expected ammonia levels were ca. 0.69, 0.95, and 0.88 for TMA, CHO, and GBT, respectively. Differences between observed and expected ammonia production may be attributed to the use of different sediment slurry samples for the ammonia production and the inhibitor assays.

**DISCUSSION**

Quaternary amines are a relatively common component of the marine biota, occurring in almost all taxa (34). Compounds such as CHO and its derivatives are ubiquitous components of the polar membrane lipids of most organisms. However, since concentrations of CHO are usually rea-
between TMA and acetate production (Table 1), the low levels of butyrate production, and the absence of dimethylamine also tend to eliminate the pathways described for *Eubacterium limosum* (17). At present the reductant(s) for and pathways of GBT degradation in Lowes Cove sediments are unclear. It seems clear, however, that GBT is not metabolized directly by methanogens (addition of BES does not inhibit GBT hydrolysis [Fig. 4 and 5]) or SRB.

It is also noteworthy that neither dimethylamine nor methylamine were observed as intermediates in this study. Both of these amines were assayed simultaneously with TMA and with lower limits of detection of ca. 1 μmol/liter. This absence contrasts with observations by others who have reported the sequential production of dimethylamine and methylamine from TMA, CHO, or GBT (7, 10, 18). The absence of these intermediates is in agreement with the results of Neill et al. (19) on the degradation of radiolabeled CHO in the sheep rumen. Differences among these studies are not easily reconciled, but they may be due to differences in the concentrations of substrates to which the methanogenic populations were exposed. For example, the observations of Fiebig and Gottschalk (7), Hippe et al. (10), and Naumann et al. (18) were based on the addition of 50 mM TMA, CHO, or GBT; on the other hand, Neill et al. (19) examined CHO at in situ levels, whereas maximum concentrations of 1 mM were used in the study reported here.

In Lowes Cove sediments, added CHO was metabolized more rapidly and to a greater extent than GBT. This pattern was supported by the production of methane, TMA, acetate, and ammonia, all of which were greater for CHO (Fig. 1, 4, and 5; Tables 1 and 2). In contrast, the rate and extent of CHO metabolism were similar to those for added TMA with respect to both methane and ammonia production, although CHO degradation proceeded after a brief lag.

Reasons for the slower metabolism of added GBT are not clear. One explanation may be that GBT hydrolysis is dependent to a greater extent than CHO hydrolysis on the availability of a suitable reductant. Oxidation of the hydroxyl function of CHO could provide sufficient reducing equivalents for the reductive hydrolysis of the quaternary amino function without the need for additional inputs. Hydrolysis of added GBT would presumably require the availability of 2 mol of H₂ per mol of GBT. Interestingly, the addition of both GBT and various sources of reducing equivalents, including glucose, alanine, leucine, or valine, did not stimulate GBT metabolism. This result suggests that populations of clostridia capable of using GBT as a Stickland reaction oxidant are not active in situ or that GBT is degraded by a different mechanism than reported by Naumann et al. (18). Results of the inhibition experiments (Fig. 4 and 5; Table 1) indicate that both CHO and GBT are fermented to TMA and acetate in a 1:1 stoichiometry. This pattern is consistent with data from various pure cultures (7, 18), although ethanol has been reported as an end product of CHO at low sulfate concentrations (7). Ethanol may have been produced as a transient intermediate during this study; however, the close correlation between acetate and TMA production (Table 1) precludes any significant accumulation. Further work is necessary to determine the importance, if any, of ethanol as a fermentation intermediate.

Complete degradation of both CHO and GBT in surface marine sediments appears to require both SRB and methanogens and results in the formation of CO₂, methane, and ammonia. The acetate formed from initial CHO or GBT fermentation is oxidized primarily to CO₂ by SRB in agreement with previous studies on the fate of acetate (11, 12, 16,

### Table 2. Observed ammonia production in sediments containing TMA, CHO, or GBTs

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concen (nmol/ml) (±1 SE)</th>
<th>Observed</th>
<th>Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMA</td>
<td>660.0 (33.0)</td>
<td>556.3 (7.7)</td>
<td></td>
</tr>
<tr>
<td>CHO</td>
<td>695.4 (37.9)</td>
<td>732.7 (10.3)</td>
<td></td>
</tr>
<tr>
<td>GBT</td>
<td>470.2 (29.9)</td>
<td>536.5 (3.2)</td>
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</tbody>
</table>

* Sediments were incubated anaerobically for 7 days at ambient laboratory temperature. Expected values of ammonia production were calculated from total TMA uptake in sediments containing molybdate (Fig. 4b) and from the extent of CHO and GBT metabolism measured in the inhibition experiments (Fig. 4 and 5; Table 1), assuming 1 mol of ammonia produced per mol of substrate metabolized.

* Final concentration, 1 μmol/ml.
The TMA formed during fermentation is mineralized primarily by methanogens to CO₂, methane, and ammonia (11, 20). The degradation of both acetate and TMA occur simultaneously as suggested by the time courses of sulfate reduction and methanogenesis with added CHO and GBT (Fig. 1 and 3). This observation indicates that coexistence of SRB and methanogens can be mediated by the presence and metabolism of complex quaternary amines commonly found in the marine biota. In addition, the production of ammonia in approximately stoichiometric amounts with TMA utilization (Table 2) by methanogens suggests that methanogenesis may contribute to the nitrogen cycle in those marine systems (e.g., salt marshes) in which quaternary amines occur in significant concentrations (5).

Finally, it should be noted that TMA metabolism in marine sediments may not be entirely due to the activity of methanogens. In fact, several lines of evidence support a limited role for SRB. First, ratios of ¹³CH₄/¹⁴CO₂ from [¹³C]TMA in the presence of active sulfate reduction are significantly less than the theoretical value of 3; inhibition of sulfate reduction by sodium molybdate results in a marked increase in ratios to ca. 3 (11). The observed changes in ratios are consistent with a nonmethanogenic oxidation of about 20% of the TMA. Second, added TMA decreases slightly in sediments containing BES to inhibit methanogenesis (Fig. 4a); the measured decrease could be due in part to slow metabolism by SRB. Third, the addition of TMA stimulates sulfate reduction in sediment slurries (Fig. 3); although TMA has not been described as a substrate for pure cultures, SRB can utilize other methylated compounds such as methanol (24; unpublished data). Further work is necessary to establish the possible utilization of TMA by SRB unequivocally. However, it is interesting to speculate that methanogens are able to outcompete SRB for at least one substrate used by both organisms.

**LITERATURE CITED**


