Thiol Synthesis by Halophilic Bacteria Indigenous in a Coastal Lagoon

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The presence of beds of sulfide muds among the sediments underlying the waters of the saline lagoon which forms the major waterway along the central east coast of Florida was established by Nevin et. al. (1973), and the observations extended to the sediments of a tributary, Banana Creek, by Beazley et. al. (1974). These authors demonstrated a relationship between the sulfide mud beds, the transport of nutrients, and the numbers of certain anaerobic bacteria. In the present work, preliminary observations indicated the disappearance of H_2S from the water column just above the muds, but no measurable increase in sulfur oxides could be demonstrated. Therefore, investigation into the probable fate of H_2S was undertaken.

METHODS AND MATERIALS

Lagoonal water samples were collected in sterile 500 ml glass bottles. Sterile 125 ml Nalgene bottles containing 12.5 ml of nutrient broth (Difco) were filled with freshly collected samples. The medium was pre-prepared according to label directions with lagoonal water and sterilized before use. Parallel enrichment cultures were incubated at 37° C for 48 hours under either aerobic or anaerobic conditions. A disposable gas pack (BBL) equipped with and H_2, CO_2 generator envelope was used to establish anaerobosis.

Pre-incubation, post incubation and control bottles of enriched lagoonal water were studied. The control consisted of filtered sterilized (pre-sterilized 0.4 µ membrance filters: Millipore Corp.) enriched water.

After incubation precent transmission at 460 µ was determined with a Bausch & Lomb Spectronic 20 colorimeter, then the cell crop was harvested by centrifugation (International Centrifuge Model HT, head number 856) at 9,000 rpm for five minutes.

Acid liberated H_2S was trapped in 0.1 N $CdCl_2$ at pH 1-2, and established by modified iodometric titration (STANDARD METHODS, 1971). Sulfite ions were also determined iodometrically in an acidified sample (STANDARD METHODS, 1971), and when necessary corrected for sulfide concentration by difference. Sulfate ions were separated by barium precipitation (STANDARD METHODS, 1971) then quantities were estimated turbidimetrically. A photometric standard curve at 420µ was used for direct readings between 0 and 3 gm/l.

Qualitative tests used regularly to identify suspect compounds were: the malachite green test for sulfites (FEIGL, 1958), the mecuric chloride-litmus paper tests for thiosulfates (FEIGL, 1958), and the iodine-azide test for reduced sulfur (FEIGL, 1958).

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Thiols were identified with an alkaline solution of cupric chloride and hydroxylamine hydrochloride (FEIGL, 1966); primary and secondary thiols by heating with NH_4OH and lead acetate paper (FEIGL, 1966).

Ether extractions were performed by vigorously shaking approximately 100 ml of ether and 300 ml of the supernatant culture medium after centrifugation for 10 minutes. The water layer was then drawn off and the ether layer decanted into a clean beaker. The ether was allowed to evaporate, leaving a residue suspended in a small amount of water. Infrared analysis of the residue was carried out using a Perkin-Elmer 614 grating infrared spectrophotometer and a 1.5 g KBr pellet.

RESULTS

Sulfide production was noted in the enrichments under both aerobic and anaerobic conditions, however only small amounts (0.004-0.02 mg/l) were recovered.

TABLE I. Increase (mg/l) in iodometrically titratable material upon addition of sulfur containing compounds to aerobically incubated enrichments of lagoonal water.

	initial	final	net increase
amino acid enrichments [1]			
control (nutrient broth)	11.0	16.0	
cysteine	11.0	23.0	7.0
methionine	11.0	24.0	8.0
cystine	11.0	21.0	6.0
sulfide enrichments [2]			
control (unenriched water)	2.0	3.9	1.9
Na_2S and nutrient broth	2.0	19.7	15.8
nutrient broth	1.7	12.0	8.1

[1] The amino acids were added to cultures which had been incubated at 37° C for 48 hours. They were then reincubated for 12 hours.

[2] The inorganic enrichment was added to the culture initially and incubated for 48 hours at 37° C.

The amounts of another iodometrically titratable material increased markedly under anaerobic conditions. This material was estimated as sulfite as a matter of convenience. Surface water samples incubated anaerobically yielded about 3 times (16.75 mg/l) that of identical samples, collected three to six inches above the sediments, yielding greater amounts than did the surface water samples under either condition,and again about 2.5 times more was produced anaerobically (18.30 mg/l) than aerobically (7.25 mg/l).More complete identification of the iodometrically titratable material was then undertaken.

The qualitative iodine-azide reaction for sulfur, present as sulhydryl, disulfide or thiosulfate was positive, whereas qualitative tests for sulfite, metabisulfite, bisulfite and thiosulfate were negative. The iodine-azide reactions was also positive when cystine and cysteine were tested, therefore each of these amino acids was added singly to a grown nutrient broth enriched culture (48 hours incubation, aerobic conditions). Methionine was included as a matter of interest. Each of the three amino acids served as substrate for the production of additional iodometrically titratable material by the mixed bacterial flora, as did Na_2S when added to a freshly prepared nutrient both enrichment and incubated aerobically. These data are summarized in Table I.

Continued qualitative testing indicated the production of either a primary or secondary thiol.

Ether extraction yielded an infrared assayable material,which provided a partial identification of the enrichment produced thiol. A grating infrared curve yielded strong peaks at 1600 cm^{-1} indicating a benzene ring. Meta substitution on the ring was evidenced by peaks at 680 cm^{-1} and 450 cm^{-1}. The -SH group was identified by weak peaks at 2550 cm^{-1} and 900 cm^{-1} as were CH_3-C and $-CH_2-$ groups by strong peaks at 910 cm^{-1}, and 2850 cm^{-1}, 1375 cm^{-1}, 1525 cm^{-1}. The significant groups on the scan were identified from Dyer's (1965) tabulations.

DISCUSSION

The occurrence of organic intermediates in the sulfur cycle has been proposed on several occasions; Peck (1962) in reviewing the literature suggested that at some oxdiative level, inorganic sulfur was incorporated into an organic molecule. Lees (1960), believed that an organic acceptor stripped the -SH groups from the sulfur compounds in the medium and Vogler's (1942) work indicated that Thiobacillus thiooxidans synthesized an organic storage product from CO_2 during sulfur oxidation. No references, however, have been found concerning the production, identification or function of such organic sulfur compounds, especially in lagoonal-marine environments.

In the present work, an iodometrically titratable material was produced by a mixed bacterial flora which developed in enrichment cultures of lagoonal water. Production of the material was augmented by the addition of any of a variety of sulfur compounds to the enrichment, and tentative identificaton of the compound was undertaken.

The "cell free" supernatants of freshly grown enrichments were known to react with iodine, and also produced gas when mixed with an iodine-azide reagent indicating sulfur present as sulfhydryl, disulfide or thiosulfate groups (FEIGL, 1958). Dithionates and polythionates do not react with iodine (KARCHMER, 1970 and STARKEY, 1934) and were ruled out on this basis. The malachite green test for sulfite, bisulfite and metabisulfite groups and the mercuric chloride test for thiosulfate (FEIGL, 1958) were negative.

Having eliminated the most probable inorganic sulfur compounds as the iodometrically titratable material, attention was directed toward commonly occuring organic sulfur compounds. Cystine and cysteine reacted in the iodine-azide test, but methionine did not, thus emphasizing the greater probability of either a sulfhydryl (-SH) group or a disulfide (-S-S-)group in the bacterial product.

Upon reaction with $Hg(NO_3)_2$ (KARCHMER, 1970) the bacterial product yielded a black precipitate. Cysteine, but not cystine, did also, increasing the probability that a thiol (-SH) group was part of the compound. Thiols are known to react with iodine (KARCHMER, 1966 and RAYLAND, 1970)and qualitative tests of culture supernatants for thiol groups, using an alkaline solution of cupric chloride and hydroxylamine (FEIGL, 1966) were strongly positive as were alkaline decompostion tests (FEIGL, 1966) for primary and secondary thiols.

Infrared analysis suggested the most probable chemical structure of the compound to be one of those presented in figure 1. The scan indicated that the sulfydryl and methyl side chains on the benzene ring were in the meta position with respect to each other. The methylene group(s) is most probably located subterminally on either of the meta positioned chains described since no third substitution was indicated. These thiols are remarkably similar in structure to metathioanisole, and may eventually prove to be close analogues. The benzene moiety in the natural compound is probably derived from tannins and/or lignins which accumulate in the waters when plant tissues undergo degradation. It is possible then, that thiol synthesis serves two purposes; as a storage depot for reduced sulfur, since the amount produced under anaerobic conditions is 2 to 3 times that found under aerobic conditions,and; as a means of detoxifying the potentially bactericidal natural phenolics.

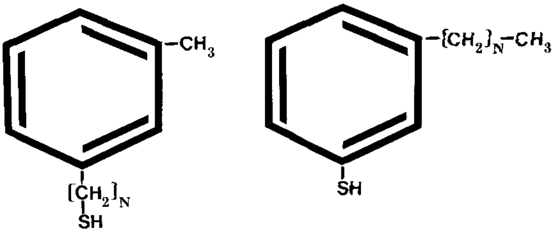

Figure 1. The infrared scan suggested that one of the above is the most probable structure of the thiol.

The thiol was produced whenever recognized inorganic intermediates other than sulfate in the sulfur cycle were added to enrichment cultures. Further, the reactions leading to thiol production are most probably mediated by heterotrophic organisms since the methods used were selective for heterotrophs, whereas those leading to sulfate production are more probably mediated by autotrophic organisms (Peck, 1962 and TRUDINGER, 1965).

The failure to demonstrate hydrogen sulfide in water samples particularly those collected above sulfide mud beds is probably resolved since small amounts (1-2 mg/l) of thiol were encountered in all of the unenriched control cultures. Following storms and periods of high winds a noticeable stench, particularly along the shore lines of the subject lagoon, attests to the rapid increase in amount and volatilization of the thiol. The storm induced increase is readily explained as a result of the turnover of the water column, disturbance of the bottom muds, and a concomitant increase in the precursor nutrients in the water, in effect an enrichment. The volatile thiol is then formed by bacterial action.

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