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Products of Mercury Demethylation by Sulfidogens and Methanogens

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Experiments with molybdate, a specific inhibitor of sulfidogens, established the predominant role of these bacteria as methylators of mercury in anoxic aquatic sediments (Compeau and Bartha, 1985, 1987). Similar experiments indicated that sulfidogens and methanogens are also the principal demethylators of methylmercury in such sediments (Oremland et al., 1991). Somewhat paradoxically, these investigators found that $^{14}CO_2$ was the principal product of $^{14}CH_4Hg^+$ demethylation in strictly anoxic aquatic sediments, while aerobic incubations yielded principally $^{14}CH_4$. These results led to the suggestion of sulfidogen and methanogen involvement in the oxidative demethylation of mercury. Direct mercury demethylation experiments using pure cultures of sulfidogens and methanogens conducted within the same study remained inconclusive due to the marginal mercury demethylation activity of these cultures. Nevertheless, the report left the reader with the impression that mercury demethylation by methanogens and sulfidogens is likely to be an oxidative one, yielding $^{14}CO_2$ as the product of the methyl group. The high mercury demethylation activities of two *Desulfovibrio* and one methanogen strain reported by Pak and Bartha (1998) allowed us a direct reexamination of the methylmercury demethylation products generated by pure cultures of sulfidogens and methanogens.

MATERIALS AND METHODS

We synthesized $^{14}CH_3HgI$ from $^{14}CH_3I$ (50 mCi mmol^{-1}, ICN, Irvine, CA). Of this highly volatile compound, 1.0 mCi (2.86 mg) was combined with 142 mg unlabeled CH_3I in 2ml dry diethyl ether and 25 mg magnesium metal. The mixture was refluxed for 1 h to prepare a Grignard reagent (Thayer, 1988). Subsequently, 454 mg HgI was added and the mixture was refluxed for an additional hour. At this time, the reaction mixture was transferred into a small separatory funnel and extracted first with 10 ml 0.1 N HCl, followed by 10 ml distilled H_2O. The ether phase was subsequently dried with anhydrous Na_2SO_4, filtered, and evaporated, leaving 167 mg white crystalline material (50% yield) with 0.043 mCi/mmol specific activity. The purity of the product (> 99%) was established by gas chromatography (no detectable peak besides $^{14}CH_3HgI$) and by melting point (145-147 °C), identical to the melting point of a commercial CH_3HgI standard (American Tokyo Kasei Inc., Portland, OR).

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The relatively low radiochemical yield of the synthesis (21%) was explained by the low amount (2.86 mg) of the volatile starting material and the unavoidable losses in its transfer and handling.

The *Desulfovibrio desulfuricans* strains "LS" and "ND 132" and *Methanococcus maripaludis* ATCC No. 4300 (ATCC, 1996) were cultivated under strict anaerobic conditions using Hungate techniques (Hungate, 1969). The sulfidogens were grown in Postgate's low sulfate medium D (Postgate, 1984) modified to render it even lower in sulfate (Pak and Bartha, 1998). Serum bottles (160 ml) contained 125 ml of this medium. These bottles were inoculated by 10 ml of a 24 h culture suspension pre-grown on the same medium, increasing the liquid volume to 135 ml. *M. maripaludis* was pre-grown in ATCC Medium 1439 (American Type Culture Collection, 1996) and 10 ml suspension was inoculated into 100 ml of the same medium. The remaining headspace of the 160 ml serum bottle was flushed with a mixture of 80% H_2 and 20% CO_2. To all cultures, $^{14}CH_3HgI$ was added at 0.5 µg/ml final concentration. The bottles were turned upside down to prevent any escape of gas from the headspace and the cultures were incubated for 7 days in the dark at 37 °C and with slow shaking. After gas analysis, the culture medium was analyzed for residual methylmercury by the procedure of Longbottom et al. (1973).

Biological activity was stopped in all bottles by the injection of 10 ml 2 N HCl. This addition was designed also to drive out any $^{14}CO_2$ from solution into the gas phase. A 20 gauge syringe needle connected with a stainless steel stopcock (Popper and Sons, Hyde Park, NY) to a 60 ml disposable syringe (Beckton and Dickinson, Franklin Lakes, NJ) was charged with 0.5 ml 3% KOH and inserted through the stopper into the headspace of the culture bottles. The gas concentrated in the headspace was allowed to expand into the syringe, and the remaining gas in the headspace was pushed into the syringe by injecting, also by syringe, more water into the culture bottle until no gas headspace remained. The stopcock was closed and the $^{14}CO_2$ was allowed to be absorbed by the KOH for 30 min. Subsequently, the KOH solution was transferred to a scintillation vial with 10 ml Scintiverse counting fluid (Fisher, Springfield, NJ) and counted. Subsequently, 10 ml toluene-based Betaflour counting fluid (National Diagnostics, Atlanta, GA) was introduced into the same syringe, allowed to absorb any $^{14}CH_4$ in the syringe for 30 min and counted. Counting efficiencies for Betafluor and Scintiverse were compared by adding to them equal amounts of $^{14}CH_3HgI$ and counting the samples. Counts were corrected for background and for efficiency by the external standard ratio method.

RESULTS AND DISCUSSION

An initial attempt to analyze the headspace gas of the culture bottles by a radioactive gas analyzer (RAGA, Varian Model 3300) was unsuccessful because of the low specific activity of the $^{14}CH_3HgI$. Better results were obtained by absorbing selectively $^{14}CO_2$ and $^{14}CH_4$ from the entire headspace volume of the culture bottles, after transferring these gases into a large-volume disposable syringe (Table 1). No measurable radioactivity was trapped by KOH in the form of $^{14}CO_2$, but $^{14}CH_4$

absorbed in the toluene-based Betafluor counting fluid gave counts that corresponded reasonably well to the amounts of $^{14}CH_3HgI$ decomposed by each of the bacterial cultures, especially when considering the limited trapping efficiency of toluene for methane. As decomposition product of $^{14}CH_3HgI$, $^{14}CO_2$ was conclusively ruled out for the two sulfidogens and the one methanogen cultures tested.

Methane as the decomposition end product of methylmercury has been demonstrated in several studies on aerobes and facultative anaerobes (Robinson and Tuovinen, 1984, Moore et al., 1990). In all cases studied to date involving more than 100 mercury-resistant organisms, mercury has been shown to be converted to a volatile form, Hg°, and simultaneous CH_4 production from methylmercury was also demonstrated. The detoxification of organomercurials occurs in two steps: the action of the organomercurial lyase enzyme that releases CH_4 is followed by the reduction of Hg^{2+} to Hg° by the mercuric reductase enzyme (Fox and Walsh, 1982). Organomercuric lyase catalyzes the protonolytic cleavage of carbon-mercury bonds to yield the parent hydrocarbon and inorganic Hg^{2+}. Hg^{2+} then becomes a substrate for mercuric reductase, which catalyzes the two-electron reduction of mercuric ions to elemental mercury at the expense of NADPH. Although the organomercurial lyase-mercuric reductase detoxification system has been extensively studied (Robinson and Tuovinen, 1984, Moore et al., 1990), the presence of this system in sulfidogens and methanogens has not been documented to date.

Table 1. Products of $^{14}CH_3HgI$ decomposition in cultures of three strictly anaerobic bacteria[1].

Bacteria	$^{14}CH_3HgI$ Decomposed (μg/ml)	(dpm)	(%)	$^{14}CH_4$ Trapped (dpm)	(%)	Trapping Efficiency (%)
D. desulfuricans LS	0.29	7830	58	3257	24	41
D. desulfuricans ND	0.24	6480	48	2053	15	32
M. maripaludis	0.23	5060	46	4599	42	91

1) All cultures received 0.5 µg/ml $^{14}CH_3HgI$. $^{14}CH_3HgI$ decomposition was measured by gas chromatography and the corresponding dpm was calculated only. Because of different culture volumes, the added radioactivity to *D. desulfuricans* cultures was 13,500 dpm, to *M. maripaludis* 11,000dpm. No radioactivity above background was trapped in the form of $^{14}CO_2$ and, therefore, $^{14}CO_2$ trapping is not included in the Table.

Oremland et al. (1991) have shown that the products of $^{14}CH_3HgI$ methylmercury decomposition in sediments were $^{14}CO_2$ and $^{14}CH_4$. The products of mercury demethylation in strictly anaerobic sediments were principally $^{14}CO_2$ with lesser amounts of $^{14}CH_4$. Aerobic demethylation in estuarine sediments produced only $^{14}CH_4$, while aerobic demethylation in freshwater sediments produced small amounts

of both $^{14}CH_4$ and $^{14}CO_2$. Two cultures of sulfate-reducing bacteria, *Desulfovibrio gigas* and *D. africans*, produced trace amounts of $^{14}CH_4$ from $^{14}CH_3HgI$, while a culture of a methylotrophic methanogen formed traces of $^{14}CO_2$ and $^{14}CH_4$ when grown on trimethylamine in the presence of $^{14}CH_3HgI$. The experiments on pure cultures of sulfidogens and methanogens were not conclusive in this study, because the quantity of $^{14}CH_3HgI$ decomposed by the cultures was in the range of the radiochemical impurities present in the $^{14}CH_3HgI$ (~ 2%). Oremland et al. (1991) were clearly puzzled by the apparent paradox that in strictly anoxic sediments the product of methylmercury demethylation was the oxidized $^{14}CO_2$, and in more oxidized sediments it was the reduced $^{14}CH_4$. Because of their inconclusive results on pure cultures of sulfidogens and methanogens, it was not possible for them to determine with certainty whether the production of $^{14}CO_2$ was in fact a metabolic feature of either the sulfidogens or methanogens, though the results of the inhibitor studies on the sediments strongly suggested that it was. The results presented here make it clear that this is not the case. The tested sulfidogens and methanogen used the methylmercuric lyase system, releasing the methyl group of monomethylmercury as methane. Nevertheless, the specific inhibitors of sulfidogens and methanogens (molybdate and BES) completely or near-completely prevented $^{14}CO_2$ evolution from $^{14}CH_3HgI$ in anoxic sediments (Oremland et al., 1991). This indicates that the producers of $^{14}CO_2$ do not oxidize the methyl group of $^{14}CH_3HgI$ directly, but rather oxidize the methane released by the former microorganisms. There is strong evidence that anaerobic methane oxidation does occur (Niewohner et al., 1998) and appears to be coupled to sulfate reduction, though the organisms or consortia responsible for this process remain to be characterized. The available evidence indicates that in anoxic sediments methylmercury is decomposed principally by sulfidogens and methanogens that release the methyl group as methane. This is subsequently oxidized by anaerobic methanotrophs to CO_2.

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REFERENCES

American Type Culture Collection (1996) Catalogue of bacteria and bacteriophages. 19th Ed., American Type Culture Collection, Rockville, MD

Compeau GC, Bartha R (1985) Sulfate reducing bacteria: Principal methylators of mercury in anoxic estuarine sediments. Appl Environ Microbiol 50:498-502

Compeau GC, Bartha R (1987) Effect of salinity on mercury-methylating activity of sulfate-reducing bacteria in estuarine sediments. Appl Environ Microbiol 53:261-265

Hungate RE. (1969) A roll tube method for cultivation of strict anaerobes. *Zn:* Norris JR, Ribbons DW (eds). Methods in microbiology Vol 3B. Academic Press, New York p 117

Longbottom JE, Dressman RC, Lichtenberg JJ (1973) Metals and other elements: gas chromatographic determination of methylmercury in fish, sediment, and water. J Assoc Off Anal Chem 56: 1297-1303

Moore MJ, Distefano MD, Zydowsky LD, Cummings RT, Walsh CT (1990) Organo mercurial lyase and mercuric ion reductase: Nature's mercury detoxification catalysts. Act Chem Res 23:301-308.

Niewohner C, Hensen C, Kasten S, Zabel M, Shulz HD (1998) Deep sulfate reduction completely mediated by anaerobic methane oxidation in sediments of the upwelling area off Namibia. Geochim Cosmochim Acta 62:455-464.

Oremland RS, Culbertson CW, Winfrey MR (1991) Methylmercury deposition in sediments and bacterial cultures: Involvement of methanogens and sulfate reducers in oxidative demethylation. Appl Environ Microbiol 57:130-137

Pak KR, Bartha R (1998) Mercury methylation and demethylation in anoxic lake sediments and by strictly anaerobic bacteria. Appl Environ Microbiol 64:1013-1017

Postgate JR (1984) The sulfate-reducing bacteria. 2nd. Ed. Cambridge University Press, Cambridge, UK

Robinson JB, Tuovinen OH (1984) Mechanism of microbial resistance and detoxification of mercury and organo-mercury compounds: Physiological, biochemical and genetic analyses. Microbial Rev 48:95-124

Thayer JS (1988) The Metal-Carbon Bond: Methods of Synthesis. pp. 12-14. In Organometallic chemistry-An overview. VCH Publishers Inc New York