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Carbon Flow in Mercury Biomethylation by *Desulfovibrio desulfuricans*†

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Radiocarbon incorporation from pyruvate and serine into monomethylmercury by *Desulfovibrio desulfuricans* was consistent with the proposal that the methyl group originates from C-3 of serine. Immunodiagnostic assays measured 4 to 35 μg of tetrahydrofolate and 58 to 161 ng of cobalamin or a closely related cobalt porphyrin per g of cell protein in *D. desulfuricans*. The light-reversible inhibition of mercury methylation by propyl iodide in *D. desulfuricans* indicates methyl transfer by a cobalt porphyrin.

The conversion of mercuric ions in anoxic sediments to the extremely neurotoxic and biomagnification-prone compound monomethylmercury greatly increases the environmental and human health impacts of mercury pollutants (7). The methylation process in anoxic sediments is predominantly microbial (1), and a variety of aerobic and anaerobic microorganisms were shown to be capable of mercury methylation in vitro (15). However, the recent use of selective inhibitors in anoxic sediments tied more than 95% of the mercury methylation to the activity of dissimilatory sulfate reducers (3, 4). This finding and the simultaneous isolation of mercury-methylating *Desulfovibrio desulfuricans* strains were not predicted by the previous in vitro studies, and it appears that earlier studies on the biochemistry of mercury methylation (15) were conducted with environmentally insignificant microorganisms. This insight prompted our preliminary investigation concerning the pathway of carbon and the nature of methyl donors in a mercury-methylating *D. desulfuricans* strain (3). Any attempt to control the environmentally undesirable mercury methylation process would benefit from a better understanding of the biochemical mechanism involved.

Anaerobic techniques. Media preparation, culture transfer, incubation, and cell harvest were performed anaerobically by Hungate techniques (5). N_2 or Ar gases, passed through a hot copper coil to remove traces of oxygen, were used to flush the headspaces of culture vessels or to blanket liquids during transfers or harvests.

Carbon flow experiments. The mercury-methylating sulfate reducer *D. desulfuricans* was isolated from low-salinity estuarine sediment (3). It was routinely maintained and pregrown for 48 h at 30°C in Postgate medium B (14) with 3.5 g of sodium pyruvate (substituted for lactate) per liter. A 2-ml inoculum from this culture was transferred to 18 ml of Postgate sulfate-free medium D (14) spiked with 7.5 μg of mercuric ion as $HgCl_2$ per ml. After 12 h at 30°C, radiolabeled pyruvate (2.75×10^7 dpm) or serine (2.54×10^7 dpm) was added along with an additional mercuric ion spike (7.5 μg/ml). After a 2-h incubation period at 30°C, methylmercury was extracted. Each extract was subdivided for gas chromatographic determination of methylmercury and liquid scintillation counting of radioactivity. Labeled [1-^{14}C]- and [3-^{14}C]sodium pyruvate (specific activities, 9.2 and 17.0 mCi/mmol; radiochemical purities, 99 and 98%, respectively) were obtained from New England Nuclear Corp., Boston, Mass.). L-[3-^{14}C]serine (56 mCi/mmol; radiochemical purity, 99%) was obtained from Amersham Corp., Arlington Heights, Ill.

Cobalamin and tetrahydrofolate assays. For these determinations, *D. desulfuricans* was grown either in medium C or medium G with 3.5 g of lactate per liter (14). Cells were harvested by a flow-through CEPA centrifuge (model CE; New Brunswick Scientific Co., Inc., Edison, N.J.) at 40,000 rpm, with cooling and with positive argon pressure. The SimulTRAC immunodiagnostic radiodilution assay kit for cobalamin (vitamin B_{12}) and tetrahydrofolate was used on the concentrated *D. desulfuricans* cell suspensions according to the enclosed instructions of the manufacturer (Becton Dickinson Immunodiagnostics, Orangeburg, N.Y.).

Propyl iodide inhibition of mercury methylation. Propyl iodide was obtained from Sigma Chemical Co., St. Louis, Mo. One-liter cultures of *D. desulfuricans* were grown in medium C (14) for 48 h at 30°C. In experiment I, the culture was divided into 250-ml aliquots and the cells were pelleted by centrifugation. Each aliquot was suspended in 20 ml of medium G (14) with no carbon source added. Two aliquots of the resting cells were treated with 5 mM propyl iodide, and two remained untreated. All samples were incubated for 30 min at 30°C in the dark, pelleted again, washed twice with Tris buffer (0.02 M, pH 7.6), and suspended in 20 ml of medium G (14) with 3.5 g of lactate per liter. All samples were spiked with 150 μg of mercuric ion per ml and were incubated at 30°C for 2.5 h. Unless specified otherwise, all samples were protected from light during preparation and incubation. The described preincubation procedure with the inhibitor was necessitated by the chemical reaction of propyl iodide with mercuric ions that would have rendered the ions unavailable for methylation.

In experiment II the same concentration of propyl iodide was present throughout the 48-h growth period, and in experiment III propyl iodide was present during growth and also during a 30-min preincubation of the concentrated resting cells. In experiments II and III, replicate samples were incubated either in the dark or in the light (20-W incandescent light bulb at a 20-cm distance).

Analytical. Cell protein was measured by the method of Lowry et al. (11), with bovine serum albumin as the standard. Methylmercury was extracted by the method of Long-

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TABLE 1. Comparison of specific activities of [1-^{14}C]pyruvate, [3-^{14}C]pyruvate and [3-^{14}C]serine with the specific activity of [^{14}C]methylmercury synthesized from them by *D. desulfuricans*

Labeled substrate	Sp act (μCi/μmol) of:		Ratio[a]
	Substrate	Methylmercury	
[1-^{14}C]pyruvate	9.2	1.0	0.11
[3-^{14}C]pyruvate	17.2	3.6	0.21
[3-^{14}C]serine	56.0	53.2	0.95

[a] Specific activity of methylmercury/specific activity of substrate.

bottom et al. (10) and determined by electron-capture gas chromatography (4); the detection limit was 2 ng/ml of culture solution. Radioactivity was measured by liquid scintillation counting with a Beta-Trac instrument (model 6895; TM Analytic, Elk Grove Village, Ill.) and a Scintiverse E (Fisher Scientific Co., Springfield, N.J.) scintillation cocktail. All counts were corrected for background and quenching by the external standard ratio method.

The report by Postgate on a minor formation of methane derived from C-3 of pyruvate (13) prompted our experiments with radiolabeled pyruvate. C-3 of ^{14}C-labeled pyruvate is twice as likely as C-1 of the pyruvate to be incorporated into methylmercury by cell suspensions of *D. desulfuricans* (Table 1). Considering the carbon flow from the pyruvate to the potential methylating systems (21) (Fig. 1), this result indicated that C-3 of serine, a principal methyl donor to tetrahydrofolate, was the most likely source of the methyl group in methylmercury. Under identical incubation conditions, the radiocarbon of C-3-labeled serine was incorporated into methylmercury by *D. desulfuricans* with 95% preservation of specific activity (Table 1). These results are consistent with the carbon flow scheme we propose in Fig. 1 for mercury methylation by *D. desulfuricans*. A less-efficient if significant incorporation of pyruvate C-1 into methylmercury may occur via decarboxylation, reduction of the CO_2 to formate by a formate dehydrogenase, and formation of formyltetrahydrofolate or a formyltetrahydropterin, as in the acetyl coenzyme A/CO dehydrogenase pathway in *Desulfovibrio baarsii* (6) and *Desulfobacterium autotrophicum* (16). The formyl group would be reduced to the methyl level and transferred to a corrinoid or a similar compound as the ultimate donor to the mercuric ion.

Of three major coenzymes (N^5-methyltetrahydrofolate, *S*-adenosylmethionine, and methylcobalamin) involved in methyl transfer, only methylcobalamin has been demonstrated to interact with mercuric ions in a spontaneous chemical reaction (18). *D. desulfuricans* has not been demonstrated to synthesize or require methylcobalamin, but a closely related cobalt-sirohydroporphyrin was identified in this bacterium (9) and might participate in the mercury methylation process. Using a SimulTRAC immunodiagnostic radiodilution kit, we measured in various culture batches of *D. desulfuricans* 58 to 161 ng of cobalamin and 4 to 35 μg of tetrahydrofolate per g of cell protein. It is possible, however, that the immunodiagnostic reagent measured the closely related cobalt sirohydroporphyrin (9) rather than cobalamin.

Additional evidence for the nature of the methyl donor in mercury methylation by *D. desulfuricans* was sought from inhibitor studies. The propylation of cobalamin by propyl iodide has been shown to inhibit transmethylation in the synthesis of both methionine and methane (2, 17, 20). Furthermore, this inhibition was shown to be reversible by exposure to light. If mercury methylation by *D. desulfuricans* was reversibly inhibited by propyl iodide, the involvement of cobalamin or a related cobalt porphyrin in the mercury methylation process would be indicated.

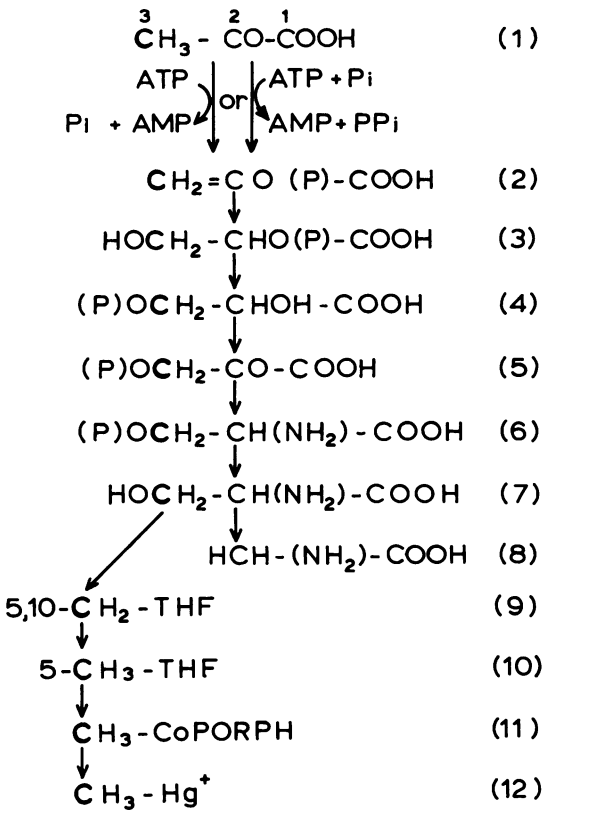

FIG. 1. Proposed pathway of carbon in *D. desulfuricans* during methylation of mercury. The carbon that becomes CH_3 in methylmercury is in boldface. Compounds: 1, pyruvate; 2, phosphoenolpyruvate; 3, 2-phosphoglycerate; 4, 3-phosphoglycerate; 5, phosphohydroxypyruvate; 6, phosphoserine; 7, serine; 8, glycine; 9, 5,10-methylene tetrahydrofolate; 10, 5-methyltetrahydrofolate; 11, methyl-cobalt porphyrin; 12, monomethylmercury. The enzyme proposed to transfer the C-3 group of serine to tetrahydrofolate is serine hydroxymethyl transferase (11).

Table 2 summarizes the results of several inhibitor experiments. In experiment I, with cell suspensions preincubated for 30 min with 5 mM propyl iodide, methylmercury synthesis was strongly inhibited compared with synthesis in propyl iodide-free controls. In experiment II, *D. desulfuricans* was grown in the presence of propyl iodide. Growth was normal,

TABLE 2. Light-reversible inhibition of mercury methylation by *D. desulfuricans* by preexposure to 5 mM propyl iodide

Expt	Preexposure to propyl iodide	Methylmercury formed (μg/g of protein per h)[a] in:	
		Darkness	Light
I	None (control)	5.2	
	30-min exposure	0.8	
II	Present during growth	0.8	5.5
III	Double exposure[b]	ND	0.4

[a] From 150 μg of mercuric ion per ml. ND, None detected.
[b] Propyl iodide was present during growth, and the resting-cell suspension was preexposed to propyl iodide for 30 min before the addition of mercury.

but the resulting cell suspension had low methylation activity. This activity was restored to control level by exposure of the organism to light. Since in experiments I and II propyl iodide-preexposed cells showed some residual mercury methylation activity, in experiment III the severity of treatment was increased by growing *D. desulfuricans* in the presence of propyl iodide and exposing the washed and concentrated resting-cell suspension to propyl iodide for an additional 30 min. *D. desulfuricans* preexposed in this manner failed to synthesize measurable amounts of methylmercury in the dark, but light exposure at least partially restored methylmercury synthesis. The failure of light to restore methylmercury synthesis fully in the latter case most likely reflects a partial enzyme inactivation due to the severity of the inhibitor treatment. The facts of the selective inhibition of mercury methylation activity by propyl iodide and the reversibility of this inhibition by light support the idea that transfer of the $-CH_3$ group to mercuric ions in *D. desulfuricans* is by a methyl donor that is either closely related or identical to methylcobalamin.

To date, no scientific consensus on the cause and mechanism of mercury methylation has emerged (15). Wood et al. (19) regarded mercury methylation as a reaction competing with methanogenesis, but the addition of spontaneously methylating methylcobalamin to their reaction mixtures seriously flawed these experiments. Subsequently, inhibitor experiments ruled out methanogens as significant environmental mercury biomethylators (3, 4). Landner (8) viewed mercury biomethylation as an incidental side reaction of methionine synthesis by *Neurospora crassa*. This fungus did not produce methylcobalamin, and an alternate methyl donor was not identified. On the other hand, the finding that auxotrophic mutants of *Clostridium cochlearium* that had lost their ability to synthesize methylcobalamin had also lost their ability to synthesize methylmercury (12) supported the idea that methylcobalamin was the direct methyl donor to mercury. Mercuric ions were more toxic to the auxotrophic mutant than to the parent strain. The fragmentary evidence suggests to us that there may be more than one way to biomethylate mercury. The reaction may be incidental or may serve to reduce the toxicity of mercuric ions to the methylating microorganism.

Focusing on the sulfate reducers, known to be responsible for over 95% of mercury methylation in anoxic aquatic sediments (3, 4), our note establishes a carbon flow to mercury through serine. It also strongly suggests a sequential participation of tetrahydrofolate and cobalamin (or a closely related corrinoid) in the transfer of the methyl group to mercuric ions. Sulfate-limiting conditions favor the mercury methylation process both in pure cultures and in anoxic aquatic sediments (3, 4).

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LITERATURE CITED

1. **Berman, M., and R. Bartha.** 1986. Levels of chemical versus biological methylation of mercury in sediments. Bull. Environ. Contam. Toxicol. **36**:401–404.
2. **Brot, N., and H. Weissbach.** 1965. Enzymatic synthesis of methionine: chemical alkylation of the enzyme-bound cobamide. J. Biol. Chem. **240**:3064–3070.
3. **Compeau, G. C., and R. Bartha.** 1985. Sulfate-reducing bacteria: principal methylators of mercury in anoxic estuarine sediment. Appl. Environ. Microbiol. **50**:498–502.
4. **Compeau, G. C., and R. Bartha.** 1987. Effect of salinity on mercury-methylating activity of sulfate-reducing bacteria in estuarine sediments. Appl. Environ. Microbiol. **53**:261–265.
5. **Hungate, R. E.** 1969. A roll-tube method for cultivation of strict anaerobes, p. 117–132. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 3. Academic Press, Inc., New York.
6. **Jansen, K., R. K. Thauer, F. Widdel, and G. Fuchs.** 1984. Carbon assimilation pathways in sulfate reducing bacteria. Formate, carbon dioxide, carbon monoxide and acetate assimilation by *Desulfovibrio baarsii*. Arch. Microbiol. **138**:257–262.
7. **Jeffries, T. W.** 1982. The microbiology of mercury. Progr. Ind. Microbiol. **16**:23–75.
8. **Landner, L.** 1971. Biochemical model for the biological methylation of mercury suggested from methylation studies *in vivo* with *Neurospora crassa*. Nature (London) **230**:452–454.
9. **LeGall, J., and G. Fauque.** 1988. Dissimilatory reduction of sulfur compounds, p. 587–639. *In* A. J. B. Zehnder (ed.), Biology of anaerobic microorganisms. John Wiley & Sons, Inc., New York.
10. **Longbottom, J., R. Dressman, and J. Lichtenberg.** 1973. Metals and other elements: gas chromatographic determination of methylmercury in fish, sediment and water. J. Assoc. Off. Agric. Chem. **56**:1297–1303.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**:265–275.
12. **Pan-Hou, H. S., and N. Imura.** 1982. Involvement of mercury methylation in microbial mercury detoxication. Arch. Microbiol. **131**:176–177.
13. **Postgate, J. R.** 1969. Methane as a minor product of pyruvate metabolism by sulfate-reducing and other bacteria. J. Gen. Microbiol. **57**:293–302.
14. **Postgate, J. R.** 1984. The sulfate-reducing bacteria, 2nd ed., p. 32–33. Cambridge University Press, Cambridge.
15. **Robinson, J. B., and O. H. Tuovinen.** 1984. Mechanism of microbial resistance and detoxification of mercury and organomercury compounds: physiological, biochemical, and genetic analyses. Microbiol. Rev. **48**:95–124.
16. **Schauder, R., A. Preuss, M. Jetten, and G. Fuchs.** 1989. Oxidative and reductive acetyl CoA/carbon monoxide dehydrogenase pathway in *Desulfobacterium autotrophicum*. 2. Demonstration of the enzymes of the pathway and comparison of CO dehydrogenase. Arch. Microbiol. **151**:84–89.
17. **Weissbach, H., B. G. Redfield, H. Dickerman, and N. Brot.** 1965. Studies on methionine synthesis: effect of alkyl-cobamide derivatives on the formation of holoenzyme. J. Biol. Chem. **240**:856–862.
18. **Wood, J. M.** 1984. Alkylation of metals and the activity of metal alkyls. Toxicol. Environ. Chem. **7**:229–240.
19. **Wood, J. M., F. S. Kennedy, and C. G. Rosen.** 1968. Synthesis of methyl-mercury compounds by extract of a methanogenic bacterium. Nature (London) **220**:173–174.
20. **Wood, J. M., and R. S. Wolfe.** 1966. Propylation and purification of a B_{12} enzyme involved in methane formation. Biochemistry **5**:3598–3603.
21. **Zubay, G.** 1988. Biochemistry, 2nd ed., p. 374, 454–457, 764–765. Macmillan Publishing Co., Inc., New York.