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Cobalamin-Mediated Mercury Methylation by *Desulfovibrio desulfuricans* LS†

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The prominence of sulfate reducers in mercury biomethylation prompted the examination of the methyl carrier and mercury methylation activity of *Desulfovibrio desulfuricans* LS. There was a low degree of mercury tolerance and a high degree of methylation during fermentative growth; the opposite was true during sulfate reduction. During 2 days of fermentative growth, up to 37% of $HgCl_2$ was methylated at 0.1 μg/ml, but only 1.5% was methylated at 10.0 μg/ml. Less than 1% of the added $HgCl_2$ was methylated under sulfate-reducing conditions. *D. desulfuricans* LS radioimmunoassay results were positive for cobalamin. The addition of $CoCl_2$ and benzimidazole to fermentative cultures increased methylation activity. From *D. desulfuricans* LS grown in the presence of $^{57}CoCl_2$, a corrinoid was extracted and purified. High-performance liquid chromatography analysis of the purified extract yielded a single peak with the retention time of cobalamin, and 97% of the ^{57}Co radioactivity was associated with this peak. Fast atom bombardment and UV and visible spectra of the isolated corrinoid matched those of cobalamin. When methylated with $^{14}CH_3I$, the isolated corrinoid methylated Hg^{2+} with a 93.9% preservation of ^{14}C specific activity. We conclude that *D. desulfuricans* LS methylates mercury via cobalamin (vitamin B_{12}). Under physiological conditions, the enzymatic catalysis of this reaction is likely.

Monomethylmercury is a highly potent and biomagnification-prone neurotoxin. In the Minamata incident, which involved a local discharge of methylmercury, tainted seafood caused 2,200 poisonings with 750 fatalities (20). Subsequently, methylmercury was recognized as a global health hazard due to the environmental conversion of less hazardous mercury pollutants to this very toxic form (12, 19). Even in pristine freshwater lakes of Wisconsin that receive atmospheric mercury inputs only, large fish accumulate methylmercury in excess of the 0.5-ppm Food and Drug Administration limit (10).

Recent work identified sulfate reducers of anoxic aquatic sediments as the principal environmental methylators of mercury (7, 8). Mercury-methylating strains of *Desulfovibrio desulfuricans* were isolated and described (7, 8), but much remains to be learned about the biochemistry of the mercury methylation process by these microorganisms. Methylcobalamin, which under appropriate conditions spontaneously methylates mercuric ions (6, 25), has long been suspected but never proven to mediate environmental mercury methylation. Paradoxically, methylcobalamin was identified in some acetogens and methanogens (22) which were ruled out as environmental methylators of mercury (7). Cobalt porphyrins were identified in *Desulfovibrio gigas* and *D. desulfuricans* Norway (11, 16), but these porphyrins do not appear to be methyl carriers. 5-Methylbenzimidazolyl cobamide, in its structure and function closely related to cobalamin, was identified in some sulfate-reducing bacteria (13). It is not known at this time whether this carrier can methylate mercuric ions.

Radioimmunoassays of a mercury-methylating strain of *D. desulfuricans*, strain LS, indicated the presence of tetrahydrofolate and cobalamin (3). The carbon flow in mercury biomethylation suggested that the methyl group donated to mercury originates as C-3 of pyruvate and is donated as C-3 of serine by serine hydroxymethyl transferase to tetrahydrofolate. From here, cobalamin or a closely related methyl carrier was postulated to transfer the methyl group to mercuric ions (3).

The aim of the study presented here was to positively identify the methyl carrier of *D. desulfuricans* LS and to document its involvement in the mercury methylation process.

MATERIALS AND METHODS

Cell growth and methylmercury analysis. The *D. desulfuricans* strain used in this study was previously isolated from an anoxic, low-salinity (0.4%) salt marsh sediment in Cheesequake State Park, N.J. (7). The strain designation of LS (for low salinity) was added to identify this isolate. When grown in lactate-sulfate medium, cells were motile and vibrio-shaped with the dimensions 0.5 by 1.0 μm. Media utilized in this study for supporting sulfate-reducing and fermentative growth were Postgate's lactate-sulfate medium C and pyruvate medium D, respectively (17). The purity of the strain was routinely monitored with ferrous sulfate-containing diagnostic medium, by tests for the presence of desulfoviridin, growth inhibition by molybdate, and methylation of mercury, and by microscopic observation (8). The maintenance of culture purity was a high priority since contaminants can easily take over under fermentative growth conditions. Cell growth was routinely determined by protein measurement with the dye-binding method of Bradford (4).

For the determination of monomethylmercury synthesis, either medium C or D received an appropriate concentration of $HgCl_2$ at the time of inoculation. After incubation at 27°C for 1 to 2 days, the cell suspensions were extracted by the method of Longbottom et al. (15), and monomethylmercury levels were measured with a Hewlett-Packard 5890 gas

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chromatograph equipped with a capillary column (0.53-mm inside diameter by 10 m long; Alltech AT-35, Deerfield, Ill.). Operating conditions were as follows: 95:5 Ar-CH_4 (vol/vol) carrier gas (Matheson Gas Products, East Rutherford, N.J.) at 40 ml/min, injector at 210°C, oven at 100°C, and electron capture detector at 250°C. Monomethylmercury peak (retention time of 1.25 min) areas were recorded by a Hewlett-Packard 3392A integrator.

Corrinoid extraction and purification. For determination of cobalamin in whole cells, a vitamin B_{12}-folate dual radioimmunoassay kit was used according to the instructions of the manufacturer (Amersham Inc., Arlington Heights, Ill.). To facilitate monitoring of the corrinoid during the purification, ^{57}Co label ($^{57}CoCl_2$, specific activity of 5.21 mCi/μg of Co; Amersham Inc.) was incorporated into the cells. Under strictly anaerobic conditions, 19 liters of lactate-sulfate medium in a 20-liter carboy received 0.2 mCi of $^{57}CoCl_2$ and was incubated at 27°C for 4 days. About 50 g (wet weight) of cells, including some inorganic sulfide precipitate, was harvested by centrifugation. Extraction with boiling KCN at a final concentration of 10 mM converted all of the corrinoid into the more stable cyano form. After removal of cell debris by centrifugation at 15,000 × *g* for 30 min, further purification was performed through a precleaned nonionic Amberlite XAD-4 (Supelco Inc., Bellefonte, Pa.) column (1.5 by 30 cm) and the same-sized aluminum oxide column on the basis of the procedure described by Stupperich et al. (24). A Rotavapor apparatus (RE 121; Büchi, Flawil, Switzerland) was used for the evaporation of methanol at a temperature not exceeding 40°C. The residue was dissolved in water. At each step, ^{57}Co radioactivity was measured with a gamma counter (model 1191; T. M. Analytic, Elk Grove Village, Ill.) with over 90% counting efficiency. Purification was carried out in the dark or under a dim red light to minimize any photodegradation of the corrinoid.

HPLC analysis. High-performance liquid chromatography (HPLC) was performed with a Partisphere 5-μm C_{18} column (0.46-cm inside diameter by 25 cm long; Whatman Inc., Clifton, N.J.), and a solvent gradient was generated with an Intelligent pump (model L-600; Hitachi). A UV-visible spectrophotometer (model L-4200; Hitachi) operating at 546 nm connected with a Chromato-integrator (model D-2000; Hitachi) was used for the detection of the corrinoid. The mobile phases were A, 0.1% acetic acid, and B, 100% methanol. The column was eluted at a flow rate of 1 ml/min with an initial 0- to 5-min isocratic elution (75% A–25% B) followed by a linear gradient to 35% A–65% B within 25 min. Cyanocobalamin standard was purchased from Sigma Chemical Co., St. Louis, Mo., and was, at times, injected together with the corrinoid extract. Eluted solvent was collected in 1.0-ml fractions, and the corresponding ^{57}Co radioactivity was measured with a gamma counter.

FAB-MS and UV-visible spectral analysis. Fast atom bombardment-mass spectrometry (FAB-MS) analysis was performed with a ZAB-T high-resolution mass spectrometer (model 8058; Vacuum Generators, Manchester, England) with a technique known as liquid secondary ionization mass spectrometry. The sample was dissolved in "magnetic bullet" matrix, which is a 3:1 mixture of dithiothreitol and dithioerythritol dissolved in methanol. Experimental conditions were 10 kV of accelerating potential and 35 kV of cesium ion gun. Data were acquired in the continuum mode, and the signal was averaged over the entire analysis (100 scans).

UV and visible absorption spectra were obtained in water with a Shimadzu spectrophotometer UV-265.

Transmethylation. By using the previously described spectrophotometer, spontaneous transmethylation by methylcobalamin was recorded as a time lapse (cycle time of 10 min, 15 cycles) spectral change to hydroxycobalamin in a wavelength range of 280 to 400 nm. The reaction mixture contained 30 μM methylcobalamin and 60 μM $HgCl_2$ in 0.1 M Na-acetate buffer, pH 4.5. The reaction was initiated by a direct addition of concentrated $HgCl_2$ solution into a quartz cuvette.

For the demonstration of mercury-methylating activity with the purified corrinoid, 50 μCi of $^{14}CH_3I$ (specific activity of 8 mCi/mmol; New England Nuclear Corp., Boston, Mass.) diluted in 100 mg of unlabeled CH_3I was added to 20 ml of corrinoid extract, obtained from a cell mass equivalent to 40 mg of protein. The reaction was allowed to proceed at room temperature under reducing conditions generated by a gentle stream of N_2 gas (Matheson Gas Products) and the addition of 20 mg of sodium borohydride. The cleanup included the extraction of the methylcobalamin into the phenol phase and back-extraction from the phenol. Details for the exchange of the $-CN$ to the $-CH_3$ group were the same as described for the chemical synthesis of methylcobalamin from cyanocobalamin (9). The methylated corrinoid extract in 0.1 M Na-acetate buffer (pH 4.5) received 1 mg of $HgCl_2$ and was allowed to stand at room temperature and in the dark for 6 h. After extraction of monomethylmercury, the final benzene extract was analyzed by gas chromatography and the transfer of the ^{14}C label was measured by a liquid-scintillation counter (BetaTrac 6895; T. M. Analytic). Counting efficiency was determined by the external standard ratio method. A control experiment was performed by using the same procedure, except the purified corrinoid was omitted.

RESULTS AND DISCUSSION

Culture conditions and methylmercury synthesis. The absolute amounts of methylmercury produced and the percentage of the mercuric ion methylated by *D. desulfuricans* LS strongly depended on the culture conditions and on the levels of the mercury added. It was reported earlier that more methylmercury was produced under fermentative than under sulfate-reducing conditions (7). When in the present study three serial transfers on pyruvate medium excluded sulfide carryover, this effect was much more pronounced (Fig. 1). From the data presented, we calculated that on the basis of micrograms of protein, up to 17.1 and 2.9 ng of methylmercury were produced during 2 days of incubation under fermentative and under sulfate-reducing conditions, respectively. Under fermentative conditions, 37.0, 12.6, and 1.5% of 0.1, 1.0, and 10.0 μg of $HgCl_2$ per ml, respectively, were transformed to methylmercury. Under sulfate-reducing conditions, 0.7, 0.5, and 0.2% of 10, 25, and 50 μg of $HgCl_2$ per ml were methylated, respectively. In the fermentative culture, $HgCl_2$ levels above 10 μg/ml strongly inhibited both cell growth and methylation activity. In the sulfate-reducing culture, however, the absolute amounts of methylmercury produced increased up to 25 μg of $HgCl_2$ added per ml and declined above this $HgCl_2$ level only gradually. Cell yields were still substantial at 50 μg of $HgCl_2$ per ml. Of course, under sulfate-reducing conditions, the numbers reflect only the added rather than the available $HgCl_2$ concentrations because most of the mercuric ions precipitated as insoluble HgS. When added within the tolerance range, methylmercury synthesis generally paralleled the increase in cell protein, as illustrated in Fig. 2.

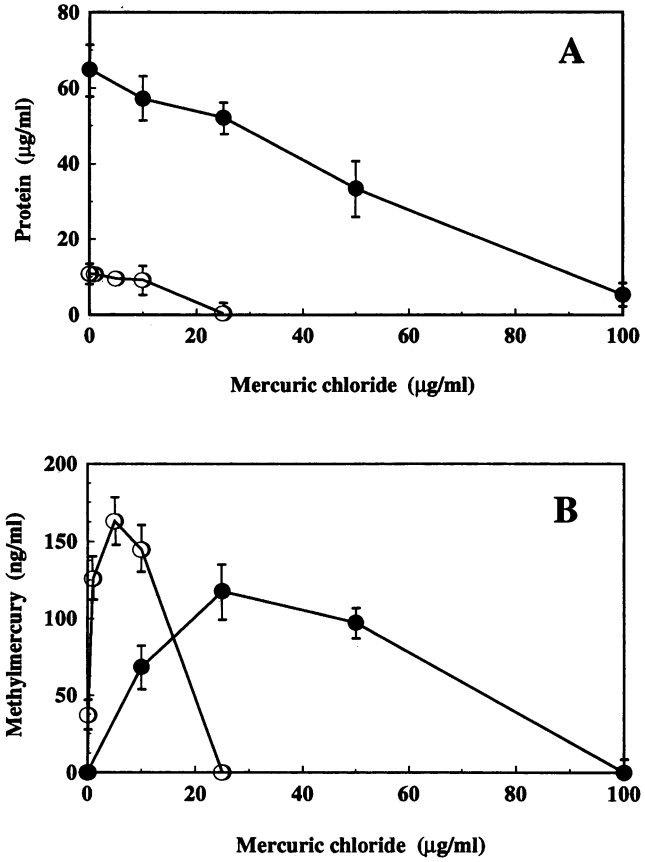

FIG. 1. Cell protein synthesis (A) and methylmercury production (B) during 2 days of incubation of *D. desulfuricans* LS with varying concentrations of $HgCl_2$. ●, sulfate-reducing conditions; ○, fermentative conditions. Data points are averages of triplicate samples; error bars represent standard deviations.

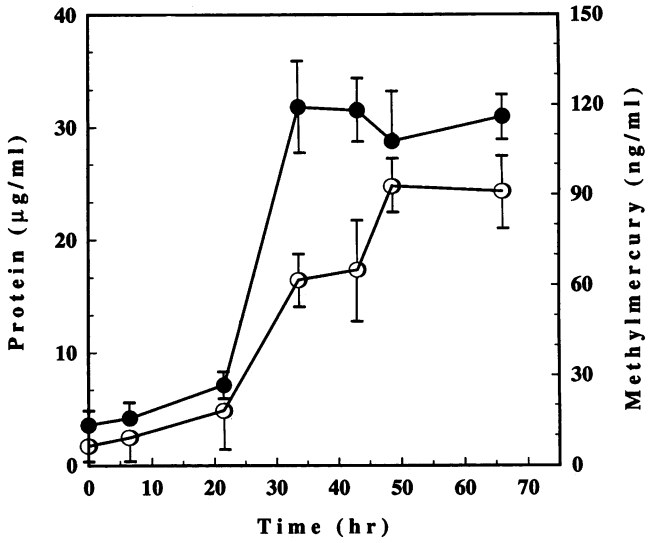

FIG. 2. Cell protein synthesis and methylmercury production with time by *D. desulfuricans* LS under sulfate-reducing conditions and in the presence of 50 μg of $HgCl_2$ per ml. ●, protein; ○, methylmercury. Data points are averages of triplicate measurements; error bars represent standard deviations.

When cells were grown fermentatively, the combined addition of 5 mg of $CoCl_2$ and 20 mg of benzimidazole per liter increased methylmercury production 2.5-fold compared with that of the control with no additions (data not shown). A radioimmunoassay for vitamin B_{12} detected 265.2 ± 14.6 pg of cobalamin per mg of protein. Both results prompted us to extract and analyze *D. desulfuricans* LS for corrinoids as the suspected methyl transfer agents in the mercury methylation process. However, the limited specificity of the radioimmunoassay did not assure us that we had in fact identified cobalamin in *D. desulfuricans* LS.

Purification and identification of the corrinoid in *D. desulfuricans* LS. The purification of the corrinoid was greatly facilitated by the ^{57}Co label incorporated by *D. desulfuricans* LS cultures. When cultures were grown under sulfate-reducing conditions, a high proportion of the added $^{57}CoCl_2$ was precipitated as ^{57}CoS. This precipitate sedimented with the cells and prevented an exact calculation of label incorporation efficiency. However, inorganic cobalt solubilized by the KCN treatment was not retained on the XAD column. The retained radioactivity on the column, calculated from activity loss of the solution passing through, was our closest measure of the actual amount of ^{57}Co incorporated by the cells and served as the starting value (100%) in the subsequent purification sequence (Table 1). Of the radioactivity, 80.7% was retained in the course of purification. During HPLC of the purified sample, 97.0% of the radioactivity was associated with a single peak (Fig. 3). On the basis of an HPLC retention time that failed to match our known standards, we first reported preliminary evidence for a novel corrinoid (5). Follow-up work showed this report to be an error due to instrument problems. When a well-functioning instrument was used, the retention time of the isolated corrinoid matched perfectly the retention time of authentic cyanocobalamin (12.91 min). A 1:1 mixture of the unknown sample and the analytical standard chromatographed as a single symmetrical peak, with no additional corrinoids detected in the cell extract (data not shown). This fact and the 97% association of the ^{57}Co radioactivity with the described cobalamin peak during HPLC (Fig. 3) strongly indicated that

TABLE 1. Purification of ^{57}Co-labeled corrinoid from *D. desulfuricans* LS

Sample characteristic(s)	Vol (ml)	Total counts[a] (cpm)[b]	Recovery[c] (%)
Harvested cells	50.0	ND[d]	
Extraction with hot KCN	450.0	1.298×10^8	
XAD-4 column attached fraction	450.0	5.740×10^7	100
XAD-4 column eluate with 80% methanol	292.0	5.416×10^7	94.4
Evaporated and redissolved in water	2.0	5.150×10^7	89.7
Applied to neutral Al_2O_3 and eluted	150.0	4.645×10^7	80.9
Concentrated for HPLC analysis	1.6	4.633×10^7	80.7

[a] Averages of duplicate measurements. Deviations from the mean were less than 1.8%.
[b] Counting efficiency over 90%.
[c] Deviations from the mean were less than 3.5%.
[d] ND, not determined.

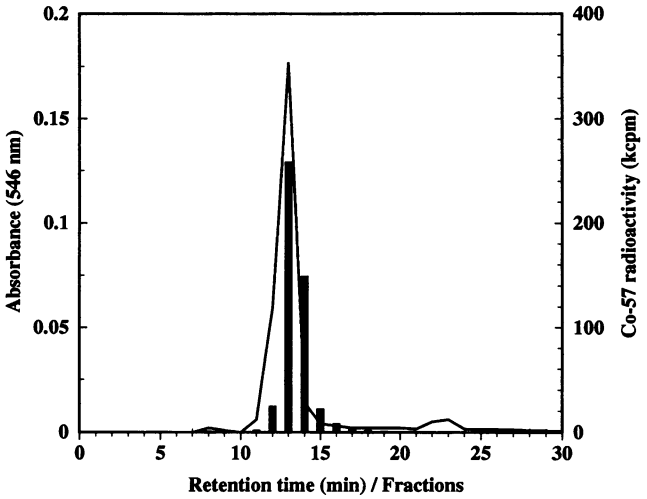

FIG. 3. HPLC separation of the *D. desulfuricans* LS corrinoid and the association of the ^{57}Co radioactivity (bars) with the collected fractions. The slight offset is due to volume holdup between detector and collection tubes.

cobalamin was the only corrinoid present in *D. desulfuricans* LS.

The identity of the isolated corrinoid was confirmed by FAB-MS. The positive ion spectrum of the unknown corrinoid indicated a molecular weight of 1,354, identical to that of cyanocobalamin (Fig. 4). Loss of the β axial ligand (cyano group) resulted in an intense peak at *m/z* 1329. An interpretation of the fragmentation is as follows: *m/z* 1286 [1329 − CONH]$^+$, *m/z* 1270 [1329 − CH_3CONH_2]$^+$ or [1329 − Co]$^+$, *m/z* 1257 [corrin-dimethylbenzimidazole-sugar-$OPO_2OCH(CH_3)CH_2$]$^+$, *m/z* 1199 [corrin-dimethylbenzimidazole-sugar-OPO_2]$^+$, *m/z* 1183 [1329 − dimethylbenzimidazole]$^+$, *m/z* 1069 [corrin-$CH_2CH_2CONHCH_2CH(CH_3)OPO_2OH$ + H]$^+$, *m/z* 989 [corrin-$CH_2CH_2CONHCH_2CH(CH_3)OH$]$^+$, and *m/z* 971 (989 − H_2O)$^+$. These data are in good agreement with the ones published by Barber et al. (1).

The UV and visible absorption spectra showed a series of spectral bands at wavelengths of 275, 360, 480, and 540 nm, with a relative peak height ratio of 1:0.46:0.06:0.09. Again, this spectrum is in good agreement with published data for cyanocobalamin (21).

The described characteristics confirm that the corrinoid isolated from *D. desulfuricans* LS is identical to cobalamin.

TABLE 2. Conversion of *D. desulfuricans* LS corrinoid to its ^{14}C-methyl form and spontaneous methylation of Hg^{2+} by this methylcorrinoid

Exptl step	Radioactivity (dpm)	
	Control[a]	Corrinoid extract
Corrinoid methylation and removal of $^{14}CH_3I$		
Addition of $^{14}CH_3I$	1.11×10^8	1.11×10^8
Water extract of organic layer	2.03×10^5	1.20×10^6
Mercury methylation and methylmercury extraction		
Starting sample for Hg^{2+} methylation	3.00×10^4	9.31×10^5
Toluene extract	3.40×10^2	6.18×10^5
Sodium thiosulfate extract	0	5.04×10^4
Benzene extract	0	4.25×10^4

[a] Identical treatment, except that the *D. desulfuricans* LS corrinoid was omitted.

Role of cobalamin in the methylation of mercury by *D. desulfuricans* LS. Cyanocobalamin isolated from *D. desulfuricans* LS was methylated by $^{14}CH_3I$, and any residual $^{14}CH_3I$ was carefully removed. When the prepared $^{14}CH_3$-cobalamin was allowed to react with mercuric ions in a pH 4.5 acetate buffer, 0.038% of the original $^{14}CH_3I$ label partitioned into benzene and was determined by gas chromatography as 99 μg of methylmercury. No radioactivity remained in the negative control (Table 2). The corresponding specific activity ratio (specific activity of the methylmercury produced/specific activity of the added $^{14}CH_3I$) was 93.9%, indicating that the methylated *D. desulfuricans* LS corrinoid spontaneously transferred its methyl group to mercuric ion. Thus, in addition to spectral evidence, the functional identity of the *D. desulfuricans* LS corrinoid to methylcobalamin was also established.

Under the appropriate conditions, such as low-pH acetate buffer, the transfer of the methyl group from methylcobalamin to mercuric ions occurred spontaneously and quite

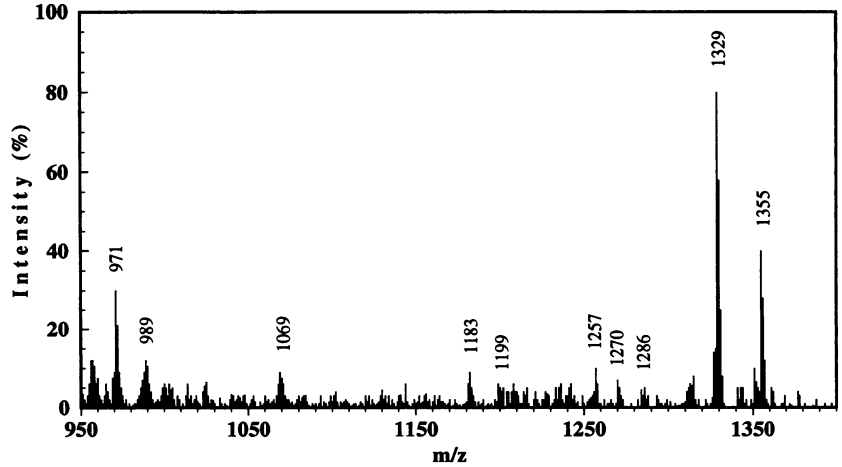

FIG. 4. The positive ion FAB mass spectrum of the purified corrinoid extract from *D. desulfuricans* LS. Numbers represent the positions of peaks of interest (see text).

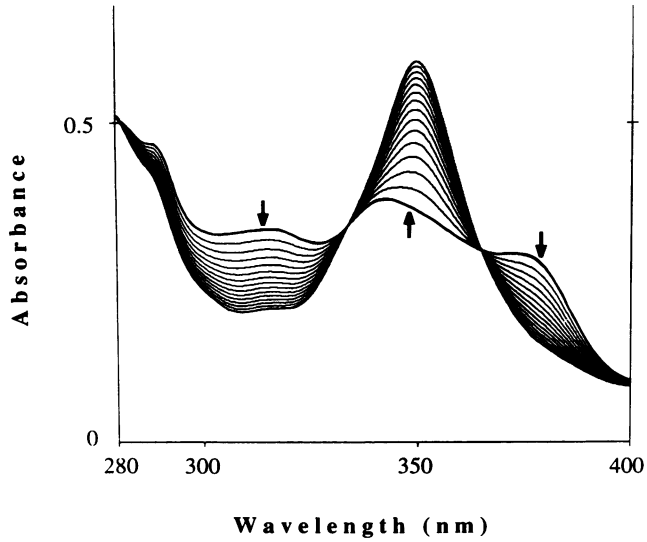

FIG. 5. Spontaneous methylation of $HgCl_2$ by methylcobalamin. A time lapse (cycle time of 10 min, 15 cycles) spectral change from methylcobalamin to hydroxycobalamin was recorded. Arrows point at the zero-time spectrum of methylcobalamin. The reaction mixture contained 30 μM methylcobalamin and 60 μM $HgCl_2$ in 0.1 M Na-acetate buffer, pH 4.5. The methylcobalamin spectrum remained unchanged without the addition of $HgCl_2$ during the reaction time period (150 min).

rapidly (Fig. 5). At a more physiological pH of 7.0, transmethylation occurred three times more slowly (data not shown). In addition, various anions (Cl^-, HCO_3^-) were shown to interfere substantially with the spontaneous transmethylation process (6). Therefore, there is reason to believe that under physiological conditions, mercury methylation may be an enzymatically catalyzed process rather than a spontaneous chemical reaction.

Until recently, sulfate-reducing bacteria were believed neither to require nor to synthesize vitamin B_{12} or closely related corrinoids (14). The identification of 5-methylbenzimidazolyl cobamide in several sulfate reducers (13) and of cobalamin in *D. desulfuricans* LS changed the situation but did not elucidate the physiological function of these compounds in sulfate reducers. Mercury methylation is most likely an incidental rather than a primary function of cobalamin. Comparative data on methylcorrinoid occurrence and mercury methylation ability in sulfate reducers are currently not available. In a sediment-water microcosm, no significant correlation between the concentration of vitamin B_{12} and methylmercury production was found (18). This is not surprising because anoxic sediments contain, in addition to sulfate reducers, numerous methanogens which are well known to contain corrinoids because of their C_1 metabolism (22, 23). The presence of methylcorrinoids in these microorganisms does not appear to be correlated with significant mercury methylation activity (7). In a very limited study, Berman (2) tested the ability of *D. desulfuricans* ATCC 2774, *D. desulfuricans* Norway 4, *D. gigas* ATCC 29494, *Desulfobulbus propionicus* FP, and *Desulfotomaculum orientis* ATCC 19365 to methylate mercury in lactate-sulfate medium. Only *D. desulfuricans* Norway 4 had any detectable ability to methylate mercury in this test, suggesting that mercury methylation is not an attribute of all sulfate-reducing bacteria.

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