Transformation of Mercuric Chloride and Methylmercury by the Rumen Microflora

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The microflora in strained rumen fluid did not methylate or volatilize $^{203}$Hg$^{2+}$ at detectable rates. However, there was an exponential decay in the concentration of added CH$_3$Hg$^+$, which was attributed to demethylation. The major product of demethylation was metallic mercury (Hg$^0$), and it was released as a volatile product from the reaction mixture. Demethylation occurred under both anaerobic and aerobic conditions. The rate of demethylation was proportional to the concentration of added CH$_3$Hg$^+$-Hg from 0.02 to 100 $\mu$g of Hg per ml. The presence of HgCl$_2$ had almost no inhibitory effect on the rate of cleavage of the carbon-mercury bond of CH$_3$HgCl, but it completely inhibited volatilization of the Hg formed, when the concentration of HgCl$_2$-Hg reached 100 $\mu$g/ml. Three of 11 species of anaerobic rumen bacteria catalyzed demethylation. These were Desulfovibrio desulfuricans, Selenomonas ruminantium, and Megasphaera elsdenii. None of the 11 species caused detectable methylation, and only two caused limited volatilization of Hg$^{2+}$. Three species of bacteria out of 90 fresh aerobic isolates were demethylators: two were identified as Pseudomonas sp., and the third was a Micrococcus sp. Demethylation by the rumen microflora appeared to be carried out by both aerobic and anaerobic bacteria and, on the basis of Hg$^{2+}$ sensitivity, probably resulted from the activity of two enzymes, a CH$_3$Hg$^+$ hydrolase and a Hg$^{2+}$ reductase.

Toxicity of mercury compounds in ruminant animals has received considerable attention (20, 21). This concern stems from the widespread distribution of mercury compounds in our environment which have the potential for contaminating ruminant rations (1, 6, 10).

Studies using goats, calves, and milk cows as test animals have demonstrated that with single oral doses of CH$_3$HgCl, 59 to 80% is absorbed from the digestive tract (21, 27). In studies with single oral doses of HgCl$_2$ in goats, 86 to 99% of the total dose was excreted in the feces (15, 27). Those data clearly show that CH$_3$Hg$^+$ is readily absorbed from the digestive tract, whereas Hg$^{2+}$ is not. Consequently, any mechanism which results in the conversion of CH$_3$Hg$^+$ to Hg$^{2+}$ plays an important role in preventing the deposition of Hg in tissues.

Mercury compounds consumed by ruminant animals are immediately exposed to the rumen microflora. In a previous study, we found that fermentation by the rumen microflora was inhibited 50% by 20 and 23 $\mu$g of Hg per ml, added as HgCl$_2$ and CH$_3$HgCl, respectively (11). The individual species of rumen bacteria differed significantly from one another in their sensitivities to these compounds (11). From studies on other anaerobic habitats, we know that microorganisms are able to: (i) reduce Hg$^{2+}$ to metallic Hg (Hg$^0$); (ii) alkylate Hg$^{2+}$ to form CH$_3$Hg$^+$ in sediment (17), in soil (3), in human feces (8), and in gastrointestinal contents of rats (22); and (iii) demethylate CH$_3$Hg$^+$ in lake sediment (29) and in the intestinal contents of rats (24). However, no information is available on the influence of the rumen microflora on mercury compounds. Because of the potential capacity of the rumen microflora to modify the absorption characteristics of mercury compounds from the digestive tract of the ruminant by changing their chemical forms, a study was undertaken to determine the transformation of HgCl$_2$ and CH$_3$Hg$^+$ by the rumen microflora. A preliminary report of some of the present results has been published (S. Kozak and C. W. Forsberg, Proc. 28th Annual Meeting of the Canadian Society of Microbiologists, abstr. no. E11, 1978).

MATERIALS AND METHODS

Rumen microflora. Rumen fluid, unless stated otherwise, was obtained from either a fistulated 4-year-old Holstein cow fed a ration of first-cut Timothy hay and a Shur-Gain Dry Cow and Heifer Ration (Canada Packers Ltd.), or a group of three Western
Cross Breed (Suffolk and Border Lester) sheep fed alfalfa hay. Rumen fluid was collected from the animals via the fistula, 3 to 5 h after feeding, and strained through four layers of surgical gauze. The strained rumen fluid (SRF) was taken to the laboratory in a prewarmed Thermos flask and used within 1 h.

**Bacterial cultures, cultural conditions, and media.** The anaerobic rumen bacteria studied (Table 4) had, with one exception, been previously obtained from the culture collection of M. P. Bryant, University of Illinois. A rumen strain of *Desulfovibrio desulfuricans* was generously provided by B. H. Howard, Lincoln, Canterbury, New Zealand (14). For clarity, we have referred to it as strain BH.

The anaerobic technique used for culturing the bacteria was similar to that described by Bryant (4), except that we added the sodium carbonate and cysteine to the culture medium before sterilization, when preparing a semidefined medium based on the one described by Scott and Dehority (26; see Forsberg [12]) and modifications of it. The medium used for the growth of all anaerobic rumen bacteria, with the exception of the *Desulfovibrio* spp., was identical to that of Forsberg (12) except that the carbohydrate source consisted of 0.1% (wt/vol) each glucose, cellobiose, and soluble starch, and hemicellulose was included at a concentration of 0.01% (wt/vol).

The *D. desulfuricans* strains were grown in a modification of the medium described by Howard and Hungate (14; Table 1).

For routine subculturing and the preparation of starter cultures for experiments, a 5% inoculum was used and cultures were incubated with shaking at 39°C for 24 h.

**Isolation and identification of aerobes.** The medium used for the isolation of aerobes was identical to that used for the anaerobes, except that air was the gas phase, the Na₂CO₃ buffer system was replaced by 0.05 M sodium phosphate (pH 7.0), and resazurin and cysteine were omitted. Agar was included when necessary at a concentration of 1.5%.

Aerobic bacteria were isolated from SRF by making dilutions in this liquid culture medium up to the 10⁻⁸ dilution. Volumes of 0.1 ml of the 10⁻⁸ to 10⁻⁴ dilutions were spread on the surfaces of prepared, dried agar plates in quadruplicate. The plates were incubated for 72 h at 39°C, and isolated colonies of different morphological types were picked from the highest dilutions and restreaked on the same medium. The isolates were tested for demethylating activity, and those with activity were identified to the level of genus.

**Methylation and demethylation by strained rumen fluid samples.** Twelve milliliters of the modified Scott and Dehority (26) medium, with 0.2% (wt/vol) cellulose powder (DF1, Whatman Ltd.) and 0.05% (wt/vol) soluble starch in the place of glucose and cellobiose, was transferred to each 125-ml Erlenmeyer flask aseptically and anaerobically, while flushing with CO₂. Each flask was sealed with a recessed butyl rubber stopper and equilibrated to 39°C. Fresh SRF (12 ml) was then added to each flask, and the stopper was fastened in place with wire to prevent it from coming out if the internal pressure increased during incubation. A 1-ml volume of a sterile solution of radioactive and nonradioactive mercury compound in water was injected with a syringe through the recessed stopper of each flask. The flasks were incubated at 39°C with shaking at 100 reciprocations per min (stroke length, 3.6 cm), and the first sample for processing was taken after 10 min to allow for complete mixing of the mercury compound with rumen contents.

**Sampling and counting total radioactivity in suspension and radioactivity in CH₃Hg⁺.** To determine the total ²⁰⁶Hg radioactivity present, a 1-ml sample was aseptically taken from each reaction mixture, and 0.5 ml was added to a gamma counting vial containing 0.5 ml of 5% (wt/vol) formaldehyde neutralized to pH 6.8 with 1 N NaOH. To determine the extractable radioactive CH₃Hg⁺, the remaining 0.5-ml portion was processed by a modification of the procedure described by Westoo (34) for the extraction of organomercury compounds (8). The sample was added to a conical centrifuge tube containing 0.5 ml of 5% (wt/vol) HgCl₂ and 0.5 ml of 6 N HCl containing 60 ng of nonradioactive CH₃Hg⁺ per ml. One milliliter of benzene was added, and the tube was mixed well on a Vortex mixer and centrifuged at 3,000 × g for 2 min to separate the phases. After centrifugation, the benzene layer was aspirated with a Pasteur pipette and placed in a gamma counting vial. The aqueous phase was extracted two more times with 1-ml volumes of benzene, and the benzene layers were added to the gamma counting vial.

In experiments where it was desirable to purify the CH₃Hg⁺ to a greater degree, the benzene extract was

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**Table 1. Composition of the medium used for the growth of *D. desulfuricans* cultures**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minerals A</td>
<td>60 ml</td>
</tr>
<tr>
<td>Minerals B</td>
<td>60 ml</td>
</tr>
<tr>
<td>Water</td>
<td>200 ml</td>
</tr>
<tr>
<td>Resazurin (0.1%, wt/vol)</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>Sodium lactate (30%, vol/vol)</td>
<td>4.5 ml</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.35 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1.75 g</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Thioglycolic acid</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>FeCl₃·4H₂O (2.5%, wt/vol)</td>
<td>1.1 ml</td>
</tr>
</tbody>
</table>

*The medium is a modification of that described by Howard and Hungate (14). Minerals A contained per liter: NaCl, 2.0 g; NH₄Cl, 6.0 g; KH₂PO₄, 3.0 g; CaCl₂·2H₂O, 0.6 g; MgCl₂·6H₂O, 0.5 g. Minerals B contained per liter: K₂HPO₄, 3.0 g. All ingredients, with the exception of thioglycolic acid and ferrous chloride, were added to a 500-ml round-bottom flask. The mixture was heated to boiling for 2 min, and then the thioglycolic acid and ferrous chloride were added and the medium was heated to boiling until it became reduced, while the surface was flushed with a gas mixture of CO₂-N₂ (20:80, vol/vol). After cooling, the gas mixture was bubbled through the medium for 5 min. The flask was sealed with a rubber stopper and sterilized by autoclaving at 121°C for 20 min. All subsequent manipulations were done under an atmosphere of CO₂-N₂.*
mixed with 1 ml of a cysteine acetate solution (8) using a Vortex mixer and then centrifuged to separate layers. The aqueous phase was removed with a Pasteur pipette and mixed thoroughly with 0.5 ml of 6 N HCl and 1 ml of benzene. This was centrifuged, and the upper benzene layer was removed and saved.

To determine the precision of the methods for measuring total radioactivity and extractable CH$_3$Hg$^+$ radioactivity, a mixture of radioactive and nonradioactive CH$_3$Hg$^+$ was added to sterile SRF to give a concentration of 1 µg of CH$_3$Hg$^+$-Hg per ml. The total count determined was 10,471 ± 83 cpm, and the radioactivity extracted as CH$_3$Hg$^+$ was 10,577 ± 21 cpm for the mean and standard error for six samples. In the experiments reported, all treatments were conducted in duplicate unless stated otherwise.

To count CH$_3$Hg$^+$ in SRF, 25-µl samples were mixed with 1-ml volumes of NCS tissue solubilizer (Nuclear Chicago Corp.) and incubated for 48 h prior to the addition of a xylene–Triton X-114 scintillation fluid (2). For counting CH$_3$Hg$^+$ in benzene, a toluene-base scintillation fluid was used which contained 0.4% 2,5-diphenyloxazole (PPO) and 0.01% p-bis-2-(5-phenyloxazolyl)benzene (POPPOP). A Beckman model LS3150T liquid scintillation counter was used.

Gamma counting was done using either a Nuclear Chicago Corp. 1185 series Automatic Gamma Counting System or a Nuclear Chicago Corp. model 4233 Automatic Gamma Counting System. When necessary, corrections were made to account for the decay of $^{203}$Hg.

Preparation of $^{203}$HgCl$_2$ and CH$_3$$^{203}$HgCl for experiments. $^{203}$HgCl$_2$ was obtained from Amersham Corp., Oakville, Ontario. Portions were diluted with nonradioactive HgCl$_2$ to give the desired specific activity and Hg concentration for a particular experiment. The solution was sealed in a glass tube and autoclaved at 121°C for 20 min.

The CH$_3$$^{203}$HgCl and CH$_3$HgCl$_2$ were obtained from New England Nuclear, Lachine, Quebec. The CH$_3$$^{203}$Hg$^+$ is subject to breakdown by primary external radiation. To ensure that the CH$_3$$^{203}$Hg$^+$ was radiochemically pure, the amount required for each experiment was purified by extraction just before use. The amounts of radioactive and nonradioactive CH$_3$Hg$^+$ required were added in a final volume of 0.5 ml to a conical centrifuge tube. HgCl$_2$, HCl, and benzene were added as described for the extraction of CH$_3$Hg$^+$, and the benzene phase was removed and added to 1 ml of distilled water in a test tube. The benzene was evaporated in a fume hood by using cool forced air. When nearly all of the benzene had evaporated, the aqueous phase was made to the desired volume and autoclaved in a sealed tube at 121°C for 20 min. The purified CH$_3$$^{203}$Hg$^+$ was radiochemically pure as demonstrated by thin-layer chromatography on Silica Gel G plates (Eastman Kodak, no. 6061) with 15-cm columns separated by etching to prevent horizontal spreading of spots. The solvent was a 90:10:10 (vol/vol/vol) solution of chloroform-methanol-formic acid (New England Nuclear Corp. data sheet). Immediately after drying, the plates were sprayed with 0.4% (wt/vol) diphenylthiocarbazone (dithizone) in chloroform solutions (8) to detect CH$_3$Hg$^+$. To locate radioactive compounds, the thin-layer chromatography plates were scanned with a Panax Thin-Layer Radiochromatogram Scanner System equipped with a PFR73 General Purpose Ratemeter.

Methane. Methane was determined with a Hewlett-Packard 5720 Gas Chromatograph fitted with a flame-ionization detector. The column, constructed of stainless steel, was 10 feet (ca. 3.05 m) long with a 0.124-inch (ca. 3.2-mm) external diameter. It was packed with 50/80-mesh Poropak N resin. The operating temperatures were 150°C for the injection port, 100°C for the column, and 200°C for the detector. The flow rate of the carrier gas, nitrogen, was 20 ml/min. The air and hydrogen flow rates to the detector were 240 and 20 ml/min, respectively. The retention time for methane was 50 s.

Characterization of the form of mercury volatilized. The trapping of $^{203}$Hg$^+$ vapor was carried out as described by Spangler et al. (28). The head gas from a culture in an all-glass vessel was flushed into two gas-washing bottles in series, each of which contained 50 ml of a mixture of 1.5% (wt/vol) HgBr$_2$ and 10% (wt/vol) KBr. At 0 and 48 h, 0.5-ml and 2-ml samples were removed from the culture and the bottles, respectively, for analysis of total radioactivity and CH$_3$Hg$^+$ radioactivity. The samples from the traps for determining CH$_3$Hg$^+$ were acidified with equal volumes of 10% KBr (wt/vol) in 1.5 N HCl, and this mixture was extracted with an equal volume of benzene for counting.

RESULTS

Methylation and volatilization of HgCl$_2$ by SRF. To determine whether the microflora in SRF had the capacity to methylate Hg$^{2+}$, ovine SRF was mixed in triplicate with equal volumes of liquid medium containing 0.2% (wt/vol) cellulose and 0.05% soluble starch as the sources of carbohydrate. The medium contained both $^{203}$HgCl$_2$ and nonradioactive HgCl$_2$ such that the final suspension was 1 µg of Hg$^{2+}$ per ml and 55,435 cpm/ml. Samples of 1 ml were removed at 0, 3, 6, 12, 18, 24, and 48 h and extracted to recover CH$_3$$^{203}$Hg$^+$ formed. No more than 0.09% of the $^{203}$Hg could be extracted at any of the sampling times from any sample. The amount extracted from control flasks containing autoclaved rumen fluid was 0.07%.

The experiment was repeated using bovine SRF with HgCl$_2$-Hg concentrations of 0.5 and 5 µg/ml in the absence and presence of 0.1% of cysteine in the medium. Again no extractable organic mercury compounds were detected. Similarly, no methylation of $^{203}$Hg$^{2+}$ was detected when the cells were washed with medium, under anaerobic conditions, to remove any residual sulfide before the incubation. In these and other experiments, samples were taken for measurement of the total counts per minute of $^{203}$Hg in the medium at times 0 and 48 h. No detectable loss of $^{203}$Hg was observed.

Demethylation of CH$_3$Hg$^+$ by SRF. The
data in Fig. 1 show the effects of time on the loss of extractable \(^{14}\text{CH}_3\text{Hg}^+\) from ovine SRF. There appeared to be an initial short lag followed by a loss of \(^{14}\text{CH}_3\text{Hg}^+\). The correlation coefficient for the relationship between the logarithm of the percent \(^{14}\text{CH}_3\text{Hg}^+\) remaining and time was 0.9940, suggesting that there was an exponential loss of \(^{14}\text{CH}_3\text{Hg}^+\). A similar loss of \(^{14}\text{C}\) from solution was observed for \(^{14}\text{CH}_3\text{Hg}^+\) added to bovine SRF when samples of the reaction mixture were treated with a tissue solubilizer and the total radioactivity in solution was determined.

The results presented in Fig. 2 show the effect of incubation time on the loss of CH\(_3\)\(^{203}\text{Hg}^+\) and total \(^{203}\text{Hg}\) from bovine SRF. There was no appreciable loss of \(^{203}\text{Hg}\) when the SRF was sterilized by either autoclaving or gamma irradiation. In samples containing undiluted SRF, the loss of extractable CH\(_3\)\(^{203}\text{Hg}^+\) closely paralleled the loss of total \(^{203}\text{Hg}\) from solution during the first 24 h and more slowly thereafter. By 48 h, between 85 to 96% of the total CH\(_3\)\(^{203}\text{Hg}^+\) was lost from solution. Dilution of SRF with an equal volume of culture medium caused a slight decrease in the rate of loss of CH\(_3\)\(^{203}\text{Hg}^+\), but since this method proved to be more reproducible, it was adopted for subsequent experiments.

It was evident that after 16 to 24 h there was a decrease in the rate of loss of CH\(_3\)\(^{203}\text{Hg}^+\) (Fig. 2). This was not due to a depletion of nutrients or other related effects, since an identical loss profile was observed when CH\(_3\)\(^{203}\text{Hg}^+\) was added to SRF that had been preincubated for 24 h.

To determine the effect of oxygen on the demethylation process, the loss of CH\(_3\)\(^{203}\text{Hg}^+\) from SRF was assayed in the presence and absence of aeration of the culture fluid. Control flasks containing autoclaved SRF were set up for each series to correct for any changes in radioactivity due to volume changes that could occur during the incubation (not illustrated). The data in Fig. 3 show that there were similar losses of total \(^{203}\text{Hg}\) and CH\(_3\)\(^{203}\text{Hg}^+\) in the absence and presence of aeration, indicating either that the demethylation process was insensitive to oxygen or that demethylation was occurring at anaerobic microsites in the flasks sparged with air. There was no loss in CH\(_3\)\(^{203}\text{Hg}^+\) from the sterile control flasks. The presence of anaerobic microsites was tested for by incubating flasks aerobically for 48 h, then adding 10 ml of culture medium to each, gassing each flask with CO\(_2\) and sealing them with stoppers, followed by incubation for 48 h and testing for methane. Methane was detected in these flasks, demonstrating that at least some of the oxygen-sensitive methanogenic bacteria survived the aeration treatment. It would, therefore, appear that some anaerobic microsites were present at which demethylation could occur anaerobically.

**Form of Hg volatilized.** To determine the form of the Hg volatilized from SRF, the culture flask and traps containing HgBr\(_2\)-KBr as the Hg trapping agent were sealed with butyl rubber stoppers and connected by Tygon tubing. Carbon dioxide was flushed through the system at 50 ml/min. In one experiment there was a 70% loss in CH\(_3\)\(^{203}\text{Hg}^+\) from the SRF in 48 h. Of the CH\(_3\)\(^{203}\text{Hg}^+\) added to the flask, 70.3% of the ra-
radioactivity was accounted for, of which 42.1% remained in solution, 55.7% was bound to the rubber stopper in the flask, 2.1% was bound to the tubing connecting the flask with the trap, and only 0.1% reached the trapping solution. The radioactivity bound to the rubber stopper was not extracted into an acid-benzene mixture.

To improve the recovery of radioactivity in the traps, a modified all-glass 125-ml gas-washing bottle was used as the culture vessel with direct attachment to the traps. The results from these experiments are summarized in Table 2. The extent of demethylation was 13.5 and 12.5% after 48 h as compared to the 85 to 90% range usually observed. Whether this was due to the shape of the flask, less mixing, or some other less evident factor is not known. However, the flasks were anaerobic, the medium was satisfactorily reduced, and flushing CO2 over the surface of the medium was found in separate experiments to have no effect on the course of demethylation. The radioactivity trapped in the HgBr2-KBr accounted for 41 and 25% of that evolved for trials 1 and 2, respectively, and of that trapped,

**TABLE 2. Estimation of radioactivity released as volatile 203Hg from SRF containing added CH3203Hg*, by trapping in HgBr2-KBr solution**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Trial 1</th>
<th>Trial 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Total cpm in the culture at time zero</td>
<td>1.13 x 10⁷</td>
<td>1.39 x 10⁶</td>
</tr>
<tr>
<td>2. cpm lost from the mixture in 48 h</td>
<td>1.52 x 10⁶</td>
<td>1.74 x 10⁵</td>
</tr>
<tr>
<td>3. cpm in trap 1 (HgBr2-KBr)</td>
<td>6.16 x 10⁴</td>
<td>4.42 x 10⁴</td>
</tr>
<tr>
<td>4. cpm in trap 2 (HgBr2-KBr)</td>
<td>2.5 x 10⁴</td>
<td>2.68 x 10³</td>
</tr>
<tr>
<td>5. cpm extracted into benzene from trap 1</td>
<td>6.13 x 10³</td>
<td>7.5 x 10²</td>
</tr>
<tr>
<td>6. cpm extracted into benzene from trap 2</td>
<td>7 x 10²</td>
<td>1 x 10²</td>
</tr>
<tr>
<td>7. Counts on Teflon tape and sections of glass tubing</td>
<td>2.67 x 10⁵</td>
<td>5.57 x 10⁴</td>
</tr>
</tbody>
</table>

Percent recovery of counts volatilized: 59% in Trial 1, 58% in Trial 2.

*The SRF inoculum was diluted with an equal volume of culture medium. The concentration of CH3Hg* was 2 μg/ml. Radioactivity is expressed as counts per minute (cpm).*
only 1 and 1.8%, respectively, was extracted into benzene. These results indicate that the volatile $^{203}\text{Hg}$ was metallic mercury.

The culture flask outlet was fastened to the gas-washing bottle with a glass junction and sealed with Teflon tape. Volatile radioactivity, presumably Hg$^0$, was absorbed onto both the glass junction and tape. Thus the lower recovery of Hg$^2+$ in the trapping solution was probably due to binding on glass surfaces and the Teflon tape.

Distribution of added mercury compounds between the supernatant and particulate fractions of SRF. The distribution of added CH$_3$$^{203}$Hg$^+-$ in the supernatant and particulate fractions of autoclaved and unautoclaved SRF during a 48-h incubation period is shown in Fig. 4. In the mixed autoclaved sample, there was a slight decrease in the total counts per minute over time, which was a departure from the usual results observed for samples containing autoclaved SRF. The $^{203}$Hg in the supernatant fraction decreased by approximately 60% in 48 h, while there was a corresponding increase in that bound to the particulate fraction, both apparently reaching an equilibrium. In contrast to these results, in the unautoclaved samples there was the usual decrease in CH$_3$Hg$^+$ concentration in the mixed suspension. There was a more rapid decrease of CH$_3$Hg$^+$ in the supernatant fraction, with a nearly complete loss of CH$_3$Hg$^+$.$^+$-$^+$. There was a transient increase in CH$_3$Hg$^+$ in the particulate fraction up to 8 h of incubation, followed by a decrease.

The distribution of added $^{203}$HgCl$_2$ between the supernatant and particulate fractions of SRF is shown in Fig. 5. There was no detectable loss of Hg$^2+$ from either the autoclaved or the untreated SRF suspension during the 48-h incubation period. In both cases, Hg$^2+$ in the supernatant decreased during the incubation and increased in the particulate fraction. These trends were much greater in the untreated than in the sterilized samples. The decreased binding of Hg$^2+$ by the particulate fraction of the autoclaved cell suspension may have been due to a lack of active uptake of Hg$^{2+}$, but more likely was a result of extensive solubilization of cellular material which bound Hg$^{2+}$ but remained in suspension after centrifugation.

Influence of CH$_3$HgCl concentration and HgCl$_2$ concentration on demethylation by SRF. The effect of CH$_3$Hg$^+$ concentration on the course of demethylation is shown in Fig. 6 and Table 3. At all concentrations of CH$_3$Hg$^+$ tested, the decrease in extractable CH$_3$Hg$^+$ and loss of total Hg from solution were closely correlated. Only the slowest and fastest courses of loss of CH$_3$Hg$^+$ are shown in Fig. 6. The amount of CH$_3$Hg$^+$ remaining in suspension after 24 h for the various treatments is shown in Table 3. There was a small decrease in the proportion of CH$_3$Hg$^+$ lost as the CH$_3$Hg-Hg concentration was increased from 0.02 µg/ml up to 100 µg/ml.

There was an exponential decay in the concentration of $^{203}$Hg in all samples ($r = 0.9800$). The initial rates of demethylation were estimated for all treatments using the concentrations of CH$_3$Hg$^+$ remaining at 3 and 6 h. When these were plotted against substrate concentration a linear relationship was observed ($r = 0.9916$). Since the initial velocity of demethyla-
Fig. 5. Effect of time on the distribution of added $^{203}\text{HgCl}_2$ between the supernatant and particulate fractions of SRF. The concentration of HgCl$_2$ was 2.0 $\mu$g/ml and 300,000 cpm/ml. Fractions were prepared as described in Fig. 5. Symbols: Autoclaved SRF, open symbols (---); untreated SRF, closed symbols (---); counts in the mixed suspension (O, ●), the supernatant fraction (Δ, △), and the particulate fraction (□, ■).

Table 3. Effect of CH$_3$HgCl concentration on the loss of total $^{203}$Hg and CH$_3$$^{203}$Hg$^+$ from bovine SRF

<table>
<thead>
<tr>
<th>CH$_3$HgCl-Hg (μg/ml)</th>
<th>% Remaining after 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>0.02</td>
<td>25.2</td>
</tr>
<tr>
<td>0.2</td>
<td>24.8</td>
</tr>
<tr>
<td>2.0</td>
<td>25.2</td>
</tr>
<tr>
<td>10.0</td>
<td>29.6</td>
</tr>
<tr>
<td>50.0</td>
<td>38.3</td>
</tr>
<tr>
<td>100.0</td>
<td>36.6</td>
</tr>
</tbody>
</table>

*The results illustrated are the total and extractable $^{203}$Hg in suspension at 24 h for the experiment illustrated in Fig. 6.

Figure 6. Effect of CH$_3$HgCl concentration on the loss of $^{203}$Hg and CH$_3$$^{203}$Hg$^+$ from bovine SRF. The concentrations of CH$_3$Hg$^+$-Hg tested were 0.02, 0.2, 2.0, 10, 50, and 100 $\mu$g/ml. The $^{203}$Hg radioactivity at time 0 was 78,000 cpm/ml. Symbols of data illustrated are those for the slowest and fastest rates of demethylation: 50 $\mu$g of Hg per ml, total $^{203}$Hg (O---□) and extractable CH$_3$Hg$^+$ (O---●); 0.2 $\mu$g of Hg per ml, total $^{203}$Hg (O---□) and extractable CH$_3$Hg$^+$ (O---●).

The results illustrated are the total and extractable $^{203}$Hg in suspension at 24 h for the experiment illustrated in Fig. 6. The concentration increased in direct relation to the substrate concentration, this suggested that the demethylating enzymes were functioning below saturation.

Clark et al. (5) reported that the mercury volatilization system of some plasmid-bearing bacteria can be induced by exposure to Hg$^{2+}$ or organomercurials. To see whether the presence of Hg$^{2+}$ would have any effect on demethylation and volatilization of CH$_3$Hg$^+$, increasing concentrations of HgCl$_2$ were added to flasks containing SRF and a constant concentration of CH$_3$Hg$^+$ (Fig. 7). Mercuric chloride at all concentrations tested from 1.0 to 100 $\mu$g of HgCl$_2$-Hg per ml appeared to have little or no effect on the course of demethylation as indicated by the losses of extractable CH$_3$Hg$^+$. However, as the concentra-
tion of HgCl₂ was increased, volatilization of Hg⁰ was decreased to the extent that at 100 µg of HgCl₂-Hg per ml there was complete inhibition of volatilization of Hg⁰. Indeed, as little as 1 µg of HgCl₂-Hg per ml decreased the radioactivity lost from solution at 48 h by 9.9% as compared to samples to which no HgCl₂ was added.

**Demethylation and methylation activity of anaerobic and aerobic rumen bacteria.** Cultures representing the major groups of anaerobic bacteria present in the rumen were tested for CH₃Hg⁺-demethylating activity (Table 4). Of the 16 cultures tested, 3 exhibited demethylating activity. These included: *Desulfovibrio desulfuricans*, *Selenomonas ruminantium* D, and *Megasphaera elsdenii*. *M. elsdenii* showed demethylating activity on only one of the two occasions that it was tested.

The anaerobic rumen bacteria listed in Table 4 were also tested for Hg²⁺-volatilizing activity during growth in the presence of 0.5 µg of HgCl₂-Hg per ml for 5 days. There was a 25% loss in Hg⁺ from uninoculated controls, and only two cultures had greater losses of Hg²⁺ than this. These were *S. ruminantium* D and *Butyrivibrio fibrisolvens*, with 32 and 37% losses in Hg²⁺, respectively. No cultures showed greater than 1% apparent methylation of ²⁰⁶HgCl₂ as indicated by extraction of CH₃²⁰⁶Hg⁺. When the concentrated benzene extracts from cultures were chromatographed, all radioactivity re-

**TABLE 4. Demethylation of CH₃²⁰⁶HgCl by pure cultures of rumen bacteria**

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>% ²⁰⁶Hg remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Desulfovibrio desulfuricans</em> BH</td>
<td>48</td>
</tr>
<tr>
<td><em>D. desulfuricans</em> ATCC 27774</td>
<td>42</td>
</tr>
<tr>
<td><em>Ruminococcus albus</em> 7</td>
<td>102</td>
</tr>
<tr>
<td><em>R. albus</em> B37</td>
<td>104</td>
</tr>
<tr>
<td><em>R. albus</em> B199</td>
<td>98</td>
</tr>
<tr>
<td><em>R. flavifaciens</em> C-94</td>
<td>96</td>
</tr>
<tr>
<td><em>Bacteroides ruminicola</em> 23</td>
<td>95</td>
</tr>
<tr>
<td><em>B. ruminicola</em> B4</td>
<td>93</td>
</tr>
<tr>
<td><em>B. succinogenes</em> S85</td>
<td>100</td>
</tr>
<tr>
<td><em>B. amylophilus</em> 70</td>
<td>100</td>
</tr>
<tr>
<td><em>Eubacterium ruminantium</em> GA-195</td>
<td>107</td>
</tr>
<tr>
<td><em>Selenomonas ruminantium</em> D</td>
<td>76</td>
</tr>
<tr>
<td><em>S. ruminantium</em> HD4</td>
<td>94</td>
</tr>
<tr>
<td><em>Megasphaera elsdenii</em> B159</td>
<td>67</td>
</tr>
<tr>
<td><em>Butyrivibrio fibrisolvens</em> D1</td>
<td>102</td>
</tr>
<tr>
<td><em>Streptococcus bovis</em> S-b-3</td>
<td>102</td>
</tr>
<tr>
<td>Control (uninoculated)</td>
<td>104</td>
</tr>
</tbody>
</table>

* Each culture was 5 ml in final volume and contained 0.5 µg of CH₃Hg⁺-Hg per ml. The total count at time 0 varied slightly from one tube to another but was approximately 10,400 cpm/ml. At time 0 and 5 days, two 0.5-ml samples were taken: one was for a total count, and the other was for extraction of remaining CH₃Hg⁺. Control tubes consisted of medium with added CH₃²⁰⁶HgCl but were uninoculated.

mained at the origin, indicating a lack of mercury-methylating activity.

Aerobic bacteria present in SRF were enu-
merated using a modification of the medium used for the cultivation of anaerobes, with bicarbonate buffer replaced by a phosphate buffer, and cysteine and resazurin omitted. After 3 days of incubation at 39°C, between 10⁶ and 10⁷ colony-forming units per ml were observed. Ninety colonies from the highest dilutions were picked, purified, and tested for aerobic demethylation of CH₃Hg⁺. Of those tested, only three were found to be positive for demethylation. There was a 94% loss of extractable CH₃Hg⁺ and an 80% loss in total ²⁰⁳Hg for culture no. 46 after 5 days of incubation. There was a 98% loss in extractable CH₃Hg⁺ and a 77% loss of total ²⁰³Hg from culture 75A after 5 days of incubation. Culture 45 showed some demethylation activity with a 27% loss in extractable CH₃Hg⁺ and a 9% loss of total ²⁰³Hg after 5 days.

Tests were performed to identify cultures 45, 46, and 75A. Culture 45 was a gram-negative, short rod, oxidase negative, with glucose catabolism occurring oxidatively. With an API 20E test strip, the organism was tentatively identified as a Pseudomonas species. Culture 75A was a large gram-negative rod that was oxidase negative. It lacked the ability to catabolize glucose. The bacterium was keyed out to a Pseudomonas species. Culture 46 was a gram-positive coccus that grew in tetrads. It produced a yellow pigment, was oxidase negative, and catabolized glucose oxidatively. On the basis of this information, the bacterium appeared to be a Microcococcus species.

**DISCUSSION**

This is the first report in the scientific literature on the transformation of Hg²⁺ and CH₃Hg⁺ by the rumen microflora. There was no detectable methylation or volatilization of added Hg²⁺ by the rumen microflora under the conditions tested. From a screening of 11 species representing some of the predominant types of rumen bacteria, none was found to be capable of methylation and only two caused limited volatilization of Hg²⁺. Mercury-methylating activity was expected because it has been observed in other anaerobic habitats including aquatic sediment (17), soil (3), human fecal material (8), and gastrointestinal contents of rats (22).

Edwards and McBride (8) reported that the extent of Hg²⁺ methylation in human feces increased as the concentration of Hg²⁺ added was increased up to 1.4 μg/ml of fluid. The maximum amount of CH₃Hg⁺ was formed after 2 days; it then decreased rapidly. The decrease observed by Edwards and McBride was attributed to a loss of CH₃Hg⁺, which had a half-life of greater than 7 days. In contrast, the half-life of CH₃Hg⁺ in SRF was approximately 15 to 18 h. Thus the inability to detect CH₃Hg⁺ resulting from the methylation of Hg²⁺ by SRF presumably could be accounted for by a rapid loss of CH₃Hg⁺ as a result of demethylation. However, degradation of CH₃Hg⁺ formed would have resulted in volatilization of ²⁰⁷Hg. The fact that no loss of ²⁰³Hg²⁺ was found strengthens the conclusion that methylation of Hg²⁺ was not occurring at a detectable rate in SRF.

The presence of sulfide in rumen contents (19) could cause the precipitation of added Hg²⁺ as mercuric sulfide, thereby inhibiting methylation, as has been demonstrated in sediment with pure mercuric sulfide (9). The influence of sulfide may not be that severe, however, since Edwards and McBride (8) observed methylation of Hg²⁺ in the presence of added sodium sulfide.

Methanogenic bacteria have, in the past, been considered important in the methylation of Hg²⁺ in anaerobic environments because cell-free extracts catalyzed methylation of Hg²⁺ (35). Recently, McBride and Edwards (18) reported that whole cells of these bacteria lacked methylating capacity and therefore probably would play only a minor role in the rumen. Screening pure cultures, representing eight of the major genera of rumen bacteria, did not reveal any with methylating capacity. Rowland et al. (25) reported that some strains of Bacteroides and Bifidobacteria isolated from human fecal material were able to methylate Hg²⁺, but the amounts produced were much less than by other bacterial types.

The process of demethylation in the rumen is very important, because it has the potential to decrease the absorption of CH₃Hg⁺ consumed by converting it to Hg²⁺ and Hg. In ruminants, almost complete absorption of CH₃Hg⁺ (95%) has been reported (13). In ruminants, absorption is reduced and ranges from 59 to 80% (21, 27), suggesting a real involvement of demethylating activity. The turnover time for the liquid fraction within the rumen is 12 to 14 h (16), and that for the particulate fraction is somewhat longer. Thus, with a half-life for CH₃Hg⁺ of 15 to 18 h (estimated from our in vitro data) in the rumen, and with some absorption of CH₃Hg⁺ through the rumen wall, probably not more than 40% of CH₃Hg⁺ consumed would be degraded. Therefore, the in vivo animal data and our in vitro experimental results appear to be complementary.

Characterization of the products of demethylation was not entirely satisfactory. When the volatile products of CH₃²⁰³Hg⁺ were trapped in HgBr₂-KBr, nearly all was unextractable into benzene, suggesting that it was metallic mercury and not dimethylmercury (28). However, the
recovery of Hg²⁺ volatilized from solution was only 59%. Radioactivity was detected on a glass-Teflon junction between the culture vessel and gas-washing bottle, suggesting that the unrecovered radioactivity was bound to the culture vessel and the exposed surfaces of the gas-washing bottle. Spangler et al. (28) achieved nearly complete recovery when they took into account radioactivity bound to glass. In separate experiments, we observed that ^14CO₂ and ^14CH₄ are produced from the hydrolysis of ^14CH₃HgCl by rumen fluid (Forsberg and Kozak, unpublished data). Methane has been found to be the usual product of demethylation (28). The formation of CO₂ from the methyl group of CH₃HgCl may have been the result of metabolism of methane by the rumen microflora, since anaerobic degradation of methane has been reported (32, 38).

The rate of demethylation of CH₃Hg⁺ increased as the concentration of CH₃Hg-Hg was increased from 0.02 µg/ml up to the highest concentration tested (100 µg of CH₃Hg⁺-Hg per ml). Thus the enzymes involved in the demethylation process apparently were not saturated, even by the highest concentration of CH₃Hg⁺ tested. Spangler et al. (28) noted a similar phenomenon with pure cultures of aerobic demethylators.

The degradation of organomercurial compounds by Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus is the result of two enzymes acting in sequence. The first is a hydrolase that cleaves the carbon-mercury bond, and the second is a reductase that reduces divalent mercury to volatile metallic mercury (30, 31, 33). Our data on the effect of HgCl₂ on the course of demethylation of CH₃Hg⁺ revealed that up to 100 µg of HgCl₂-Hg per ml had nearly no effect on cleavage of the carbon-mercury bond of CH₃Hg⁺, but inhibited the volatilization step, thus demonstrating that both a hydrolase and a reductase were likely involved. The degree of volatilization was dependent upon the concentration of HgCl₂ present, but it is not known whether this was due to competitive or noncompetitive inhibition. There was no loss of added Hg²⁺ from SRF, even though the presence of mercuric ion reductase in the rumen microflora would lead one to expect this reaction to occur. The outstanding question, therefore, is why added HgCl₂ can inhibit volatilization of Hg²⁺ resulting from cleavage of the CH₃Hg⁺, and yet HgCl₂ added by itself is not volatilized. It would be interesting to test the influence of HgCl₂ on the CH₃Hg⁺ hydrolysis and volatilization of Hg²⁺ in pure cultures of bacteria with characterized demethylating activity to determine whether they would respond in a similar manner.

Demethylation of CH₃Hg⁺ by the rumen microflora was observed to proceed at similar rates under both aerobic and anaerobic conditions, suggesting that microbes other than anaerobes might be responsible for demethylation. The isolation of aerobic bacteria including two Pseudomonas sp. and a Micrococcus sp. helps to explain the observed aerobic demethylation by rumen fluid. However, since the methanogenic bacteria were not killed by aeration for 48 h, one could argue that demethylation by anaerobes could proceed under aerobic conditions due to the presence of anaerobic microsits. Anaerobes causing a loss in CH₃Hg⁺ were D. desulfuricans, S. ruminantium, and M. elsdenii. Thus the loss of CH₃Hg⁺ from rumen fluid under aerobic conditions would appear to be due to the activities of the combination of aerobic, facultatively anaerobic, and anaerobic bacteria. However, under anaerobic conditions it is likely that the anaerobes are primarily responsible for demethylation since they are present in larger numbers and are more active metabolically.

Methylmercury can also be lost from solution in the form of a volatile sulfur complex. Rowland et al. (23) reported that H₂S could react with CH₃Hg⁺ to produce a volatile sulfur derivative of CH₃Hg⁺ which, according to Craig and Bartlett (7), is dimethylmercury. This mechanism would not appear to be active in rumen fluid since metallic mercury (Hg⁰) is the primary product of demethylation, although we have not excluded it from being the mechanism of CH₃Hg⁺ loss catalyzed by D. desulfuricans.

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LITERATURE CITED


