Ruminal Bacterial Degradation of Benzo(b)thien-4-yl Methylcarbamate (Mobam) and Effect of Mobam on Ruminal Bacteria

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Mixtures of ruminal bacteria degraded benzo(b)thien-4-yl methylcarbamate (Mobam) to 4-hydroxybenzothiophene, CO₂, and polar products(s). The metabolite, 4-hydroxybenzothiophene, was identified (after acetylation) by comparative infrared and mass spectrometry with an authentic sample. Carbon dioxide and polar product(s) were produced by degradation of the methylcarbamate moiety. Ten previously characterized strains of ruminal bacteria with diverse physiological capabilities did not degrade Mobam. However, three tributyrin-hydrolyzing strains were isolated that did degrade Mobam. Mobam inhibited growth of two of ten strains isolated on Mobam-free glycerol-tributyrin enrichment medium. One of these strains was also sensitive to 2-carbomethoxy-propene-2yl dimethyl phosphate (Phosdrin). Mobam prevented some ruminal bacteria from producing zones of hydrolysis in tributyrin emulsion media and inhibited some ruminal bacteria from degrading 1-naphthyl acetate and fluorescein-3',6'-dicacetate.

Benzo(b)thien-4-yl methylcarbamate (Mobam) is an effective pesticide that exhibits broad spectrum insecticidal activity coupled with an apparent low mammalian toxicity (2, 7). Ruminants administered 14C-Mobam as single oral doses excreted 87 to 96% of the 14C dose via the urine in 24 hr (10). Cleavage of the methylcarbamate moiety of Mobam was evident in the production of two primary metabolites, 4-benzothiethyl sulfate and 4-benzothiethyl sulfate-1-oxide. The purpose of this investigation was to determine whether ruminal bacteria degrade Mobam. The effect of Mobam on ruminal bacteria was also considered.

MATERIALS AND METHODS

Chemicals. Mobam [benzo(b)thienyl-4-yl methylcarbamate-4, 7-¹⁴C] with a specific activity of 3.74 μCi/mg and unlabeled Mobam were supplied by Mobil Chemical Co., Metuchen, N.J. ¹⁴C-Mobam, labeled in the carbonyl position (5.0 μCi/mg), was synthesized by New England Nuclear Corp., Boston, Mass. ¹⁴C-methyl-labeled Mobam (165.9 μCi/mg) was supplied by G. L. Lamoureux, Plant Science Division, of this laboratory. Purity of the ¹⁴C-labeled Mobam preparations was greater than 99%, as determined by thin-layer chromatography (TLC).

Cultures. Characterized ruminal bacterial strains [Bacteroides amylophilus, H-18; B. ruminicola subspecies brevis, GA-33; B. succinogenes, S-85; Eubacterium ruminantium, B.C-23; Lachnospira multiformis, D-32; (Megasphaera Peptostreptococcus) elsdenii, B-159; E. limosum (Butyrribacterium rettgeri, Ramibacterium species), L-34; Selenomonas ruminantium, HD-1; Streptococcus bovis, FD-10; and Succinimonas amylolytica, B.4] were obtained from M. P. Bryant’s collection and were maintained on rumen fluid-Trypticase-yeast extract medium (RFTY, 3). Mixtures of bacteria in ruminal contents were obtained by stomach tube from calves (>70 days of age, free of ruminal ciliated protozoa) maintained on a pelleted ration of alfalfa, grain, and wheat bran (10:5:1) (13). Bacteria from the ruminal samples were concentrated by centrifuging the contents at 3,000 x g for 8 min and then centrifuging the resulting supernatant fluid at 13,000 x g for 20 min at 5 C. The sedimented bacterial cells were made up to one-fourth the original volume with basal medium (14) and incubated with ¹⁴C-Mobam (100,000 to 250,000 dpm/ml of medium with Mobam concentration adjusted to 72 μg/ml). Preparations of cell suspensions of pure cultures were obtained from each culture incubated in RFTY liquid medium for 48 hr at 39 C. The cultures were centrifuged, diluted in basal medium (14), and incubated with ¹⁴C-Mobam as above but for 18 hr. Cell densities for each culture were diluted so that a 1:10 dilution of
the incubating cell preparation had an absorbancy reading of 0.4 at 600 nm in 1.2-cm-diameter cuvettes.

Anaerobic dilution materials and culture methods used were essentially as reported by Bryant and Burkey (1). Serial dilutions of whole ruminal contents from $10^{-4}$ through $10^{-2}$ were each inoculated, in triplicate, into 0.25% glycerol-2% tributyrin-RFTY-agar medium (minus glucose, cellobiose, and starch) containing zero or 600 μg/ml levels of Mobam. The inoculated media were prepared as roll tubes and incubated at 39 C for 2 weeks. Isolates from colonies showing lytic zones (5, 13) were maintained in neoprene-stoppered tubes in Spirit Blue agar medium (Difco, Detroit, Mich.) modified by addition of 1% (v/v) tributyrin, 30% (v/v) clarified ruminal fluid, 0.4% Na₂CO₃, and cysteine·HCl and Na₂S·9H₂O (each 0.05%). The medium was adjusted to pH 6.85 under CO₂ in a medium-dispensing apparatus (11).

**Assays.** Thin-layer chromatograms were prepared in the laboratory with Silica Gel G (250 μm thick, Brinkmann) on glass plates (5 by 20 by 0.35 cm) and spotted with ¹⁴C-material. The TLC plates were developed ascendingly with hexane-ethyl acetate-acetic acid (70:30:2) and scanned for ¹⁴C distribution (14). Samples were dissolved in dioxane or toluene (12) scintillation solution. The dioxane formulation consisted of 7.0 g of POPP (2,5-diphenyloxazole), 100 g of naphthalene, 300 mg of POPP [1,4-bis(2-5-phenyloxazoyl)-benzene], and enough agent-grade dioxane to bring the volume to 1 liter. Counting of radioactivity was done with a liquid scintillation counter. Counting efficiencies were determined by internal standardization with toluene-¹⁴C or by channels ratio standardization. Counting times ranged from 2 to 30 min, depending on the level of radioactivity in the samples. Radioactive ¹⁴CO₂ produced in ¹⁴C-Mobam culture experiments was monitored as previously described (12). Gas production of ruminal bacteria in the presence of Mobam (100 to 500 μg/ml) was determined manometrically (14). Bacteria were assayed for carboxylesterase activity by incubating the preparations with 1-naphthyl acetate (NA) for 15 min at 38 C. The reaction was stopped by addition of 10% lauryl sulfate solution, and the 1-naphthol produced was coupled with fast garnet GBC (CI 37210, 4-amino-3,1'-dimethyl azobenzene) and read photometrically at 560 nm (9). Similarly, fluorescein-3',6'-diacetate (FDA) was used as a substrate for bacterial carboxylesterases, with fluorescein production determined with a spectrofluorometer (4). Protein content of sonically treated bacterial extracts was determined by the method of Lowry et al. (8), with bovine serum albumin (Sigma Chemical Co.) as the standard.

**Metabolite detection.** Bacterial cultures containing ¹⁴C-Mobam products were extracted with methylene chloride. The extract was evaporated to dryness, taken up in methanol, and applied to a column of Sephadex LH-20 (0.9 by 40 cm) in methanol. The column was eluted with methanol, and the radioactive fractions were analyzed by TLC and gas-liquid chromatographic (GLC) techniques. Metabolites were recovered from TLC plates by stripping ¹⁴C-containing bands and eluting with methylene chloride. The ¹⁴C material was taken to dryness under N₂ and derivatized with 1 μl of pyridine and 100 μl of acetic anhydride. The sample was then analyzed by GLC on an instrument equipped with a ¹⁴C monitor and effluent splitters to facilitate simultaneous flame ionization detection and trapping with glass capillary tubes (14). A 2% SE-30 Chromasorb W (60/80 mesh) column [6 ft (1.8 m) by 4 mm] programmed from 100 to 200 C at 5 C/min was used. Injection port and detector temperatures were set at 300 C. Helium (55 ml/min) was used as a carrier gas. Metabolites trapped were analyzed by infrared and mass spectrophotometric analysis as previously reported (14).

**Culture techniques.** Effect of Mobam on growth of mixed ruminal bacteria was determined as follows. Gauze-strained ruminal contents (0.1 ml) were inoculated, in duplicate, into 15-mm cuvettes each containing 4.9 ml of RFTY medium under CO₂ with either 0, 2, 10, 50, 100, or 200 μg of Mobam/ml of medium. The cuvettes were flushed with CO₂, sealed with neoprene stoppers, and incubated at 39 C for 24 hr. Absorbancy readings were taken at 600 nm. Readings (at 0, 4, 8, and 24 hr of incubation) were compared with uninoculated medium containing the appropriate concentrations of Mobam.

Mixtures of bacteria from ruminal fluid (300 ml) and newly isolated ruminal bacterial strains (see below; no. 29, 53, and 88, each grown 48 hr in 300 ml of RFTY liquid medium) were separately concentrated by centrifugation and suspended in 7.5-ml volumes of basal medium (14). These cells were then sonically treated at maximum intensity (Fisher probe model BP-5; generator model CW-5) for 3 min at 5 C and assayed for NA carboxylesterase activity (9). Agar well-diffusion experiments were run with the sonically treated preparations as follows. A medium of 1.5% agar in 0.2 m sodium phosphate buffer at pH 6.8 was poured into petri dishes containing Mobam dissolved in a minimum volume of ethanol. Final concentration of Mobam was 500 μg/ml of medium. Fifty to 75 μl of the sonically treated cell preparations was added to each 8-mm-diameter well cut in the solidified medium. The preparations were incubated at 39 C. At 0, 4, 8, and 24 hr of incubation, separate preparations were flooded with 10% ferric chloride solution and observed for development of dark blue-black zones due to the presence of 4-hydroxybenzoephene. These sonically treated cell preparations were compared to sonically treated mixed- and pure culture-cell preparations treated to temperatures of near 100 C for 5 min or treated with 15% ethanol. In some instances, FDA (100 μg/ml) was used as substrate. FDA degradation was indicated by development of a fluorescent zone surrounding the wells when viewed under ultraviolet light.

**RESULTS AND DISCUSSION**

**Metabolite identification.** Methylene chloride extracted 97 to 99.3% of the ¹⁴C from
mixed-cell suspensions incubated for 24 hr with \(^{14}C\)-ring-labeled (RL) Mobam. The radioactivity in the methylene chloride extracts separated into two fractions during column chromatography on Sephadex LH-20. Fraction 1 (41 to 43 ml) co-chromatographed (TLC) with 4-hydroxybenzothiophene, and fraction 2 (49 to 51 ml) co-chromatographed (TLC) with Mobam.

\(^{14}C\) radioactivity in fraction 1, when removed from TLC plates and acetylated, was released from the GLC column when the GLC column oven reached a temperature of 145 °C. The retention time of the compound compared favorably with an authentic sample of 4-hydroxybenzothiophene (which was acetylated according to the method used for the metabolite). Infrared and mass spectra of the metabolite were identical to the authentic sample. The metabolite was therefore identified as 4-hydroxybenzothiophene.

The \(^{14}C\)-compound in fraction 2 was degraded during GLC. It had an infrared spectrum identical with high-purity Mobam.

**4-C-Mobam degradation.** Degradation of \(^{14}C\)-Mobam by mixed-rumen bacterial preparations is shown in Table 1. Carboxyl-labeled Mobam was progressively degraded with release of 30.7% of the total \(^{14}C\) as \(^{14}CO_2\) in 18 hr. Mobam, at \(R_r\) 0.27 on TLC, accounted for 59.8% of the radioactivity, and 7.2% of the \(^{14}C\) remained at \(R_r\) 0 to 0.1. Total \(^{14}C\) recovered in \(^{14}CO_2\) and methylene chloride extractions was 97.7%. With \([^{14}C\text{-methyl}]\) Mobam, only a trace of \(^{14}CO_2\) (0.9%) was found at 18 hr. Mobam accounted for 62.0% of the \(^{14}C\), and the remaining radioactivity (15.1%) remained at the origin. Total \(^{14}C\) recovered was 78.0%. Attempts to account for the loss of the methyl \(^{14}C\) were not successful. With \([^{14}C\text{-RL}]\) Mobam, no \(^{14}CO_2\) was produced, and 33.8% of the \(^{14}C\) co-chromatographed with 4-hydroxybenzothiophene at \(R_r\) 0.49. Remaining \(^{14}C\) (65.1%) was at the same \(R_r\) as Mobam. Total \(^{14}C\) recovered was 98.9%. Since no \(^{14}C\) was found at the origin when \([^{14}C\text{-RL}]\) Mobam was incubated with mixed bacterial preparations but was found when incubated with Mobam labeled in the carbonyl or methyl positions of the methylene carbonate moiety, it indicated that only the methylene carbonate moiety was the source of carbon for production of polar product(s) by the bacteria. These products were not identified.

**Mobam-degrading bacteria.** A number of bacterial isolates from \(10^{-4}\) and \(10^{-7}\) dilutions of ruminal samples were effective in repeatedly showing zones of hydrolysis in tributyrin emulsion-Mobam medium. Three isolates from this medium were found to degrade Mobam. These obligately anaerobic bacterial strains (no. 29, 53, and 88) are gram-negative motile curved rods that appear similar to isolates of Hobson and Mann (5). These cultures on tributyrin emulsion-Spirit Blue agar medium showed colonies with clearing zones of hydrolysis surrounded by a dark blue coloration. Sonically treated cell preparations of these strains and mixtures of ruminal bacteria (2.1 to 4.2 mg of soluble protein/ml) in agar well experiments showed degradation of Mobam and FDA. Degradation was detectable as early as 4 hr after incubation. Zones of hydrolysis for both Mobam and FDA preparations with the strains and mixtures of bacteria ranged from 25 to 40 mm in diameter after 24 hr of incubation. Cell suspensions of strains 29, 53, and 88 incubated with \([^{14}C\text{-RL}]\) Mobam, degraded the \(^{14}C\)-substrate to 4-hydroxybenzothiophene at respective rates of 21.3, 23.3, and 18.4% in 18 hr. Characterized ruminal bacterial strains (L-94, GA-33, B.4, HD-1, B.7C-23, S-85, FD-10, B-

### Table 1. Mixed rumen bacterial degradation of \(^{14}C\)-Mobam

| Position of \(^{14}C\) label | Accumulative % \(^{14}C\) released as \(^{14}CO_2\) with time | Per cent \(^{14}C\) remaining in preparations at 18 hr according to TLC distribution* |
|-----------------------------|----------------------------------------------------------|----------------------------------------------------------------------------------|
|                             | 0 hr          | 4 hr          | 8 hr          | 18 hr         | \(R_r\) 0-0.1 | \(R_r\) 0.27 | \(R_r\) 0.49 |
| Carboxyl                    | 0             | 1.8           | 12.9          | 30.7          | 7.2           | 59.8        | 0            |
| Methyl                      | 0             | 0.1           | 0.4           | 0.9           | 15.1          | 62.0        | 0            |
| Ring-4, 7                   | 0             | 0             | 0             | 0             | 0             | 65.1        | 33.8         |

*Values in the tables are computed as means from three experiments for each \(^{14}C\)-Mobam preparation in basal medium (14). Controls (without ruminal bacteria) showed no degradation of \(^{14}C\)-Mobam. \(^{14}C\) levels used for Mobam preparations included: carbonyl (119,640 dpm/ml), methyl (236,794 dpm/ml), and ring (159,104 dpm/ml). Each preparation was adjusted with unlabeled Mobam to 72 μg/ml.

*Remaining \(^{14}C\) in the preparations was methylene chloride extracted and quantitated by liquid scintillation methods. The percentage of \(^{14}C\) recovered was then assigned to the \(R_r\) values according to thin-layer chromatography (TLC) scanning information for each preparation.
159, H-18, and D-32) did not degrade Mobam. Whether these strains could be adapted over a long period of time to degrade Mobam is unknown. With soil bacterial species, adaptation to chlorophenyl-carbamates is necessary before degradation of these substrates occurs (6).

**Mobam inhibition of bacteria.** Manometric experiments with mixtures of ruminal bacteria showed no suppression of endogenous gasses produced in 80 min of incubation in the presence of Mobam concentrations of up to 500 \( \mu g/ml \). Values obtained at the 500-\( \mu g \) level were 2.86 \( \mu l \) of gas produced per min per 38 mg of bacteria (dry weight) from the experimental preparations and 2.65 \( \mu l \) of gas produced per min per 38 mg of bacteria (dry weight) from the control preparations (without Mobam). Gas production by the 10 characterized ruminal strains was also not inhibited by Mobam. Growth of mixtures of ruminal bacteria in RFTY liquid medium containing Mobam up to 200 \( \mu g/ml \) suggested that the actively growing members of the mixed population apparently were not suppressed.

Evidence that growth could be inhibited by Mobam was demonstrated with a gram-negative, tributyrin-degrading bacillus which was isolated and maintained in Mobam-free medium. This obligately anaerobic ruminal bacterial isolate was inhibited by Mobam and an organophosphate, Phosdrin, as shown in Table 2. Both pesticides at \( 10^{-4} M \) concentrations and above were inhibitory at 72 hr of incubation. This strain (no. 102) was one of only two strains out of ten isolated on Mobam-free medium that was inhibited by Mobam. Strain 53, which was isolated on a medium containing Mobam, as expected, showed a resistance to Mobam at the levels tested in Table 2. Also, strains L-34 and HD-1 showed resistance to these Mobam concentrations. No other strains have been tested at this time.

Suppression of ruminal bacterial populations showing zones of tributyrin hydrolysis was demonstrated when numbers of colonies growing in the presence of Mobam (600 \( \mu g/ml \)) were compared to counts growing in the absence of Mobam. At 2 weeks of incubation, \( 1.2 \times 10^{7} \) colonies/g of ruminal contents showed zones in the presence of Mobam. In comparison, \( 4.6 \times 10^{4} \) colonies/g of ruminal contents showed zones in the absence of Mobam. Total culturable counts on 0.25% glycerol-2% tributyrin-RFTY-agar medium with and without Mobam, respectively, were \( 1.4 \times 10^{7} \) and \( 1.8 \times 10^{3} \) g of ruminal contents. (On RFTY-agar medium containing glucose, cellobiose, and starch, culturable counts ranged from 7.5 to 14 \( \times 10^{6} \) g ruminal contents (13)).

When Mobam (500 \( \mu g/ml \)) was incorporated in the agar well preparations with sonically treated preparations of ruminal bacteria, fluorescein production was suppressed. To determine the extent of Mobam inhibition, suspensions of ruminal bacteria were incubated with NA and FDA as shown in Table 3. Mobam, at a concentration of \( 6.5 \times 10^{-4} M \), inhibited 65.5% of the production of 1-naphthol. However, only 5% inhibition was observed with FDA in combination with Mobam at a concentration of \( 9.7 \times 10^{-4} M \). With isolate no. 53, 69.5% inhibition of FDA hydrolysis was observed with Mobam at a concentration of \( 13.4 \times 10^{-4} M \). With isolate no. 29, 35.7% inhibition of FDA hydrolysis with Mobam at \( 9.7 \times 10^{-3} M \) was obtained.

The levels of Mobam used in these experiments are higher than one might expect in a contaminant of ruminant rations. The rate of Mobam degradation by ruminal bacteria appears to be adequate to detoxify the insecticide. The biological activity of the hydrolysis product (4-hydroxybenzothiophene) is unknown. Data presented on the effect of Mobam

| Table 2. Mobam and Phosdrin inhibition of growth of a rumen bacterial isolate (strain 102)* |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Pesticide and incubation time (hr) | Pesticide molarities and absorbancy readings (600 nm) | \( 10^{-1} M \) | \( 10^{-2} M \) | \( 10^{-3} M \) | \( 10^{-4} M \) | \( 10^{-5} M \) | \( 0 M \) |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Mobam                           |                 |                 |                 |                 |                 |                 |
| 27                              | 0               | 0.05            | 0.02            | 0.09            | 0.13            | 0.14            | 0.21            |
| 49                              | 0               | 0.07            | 0.02            | 0.20            | 0.47            | 0.52            | 0.83            |
| 72                              | 0               | 0.05            | 0.04            | 0.34            | 0.75            | 0.75            | 0.75            |
| Phosdrin                         |                 |                 |                 |                 |                 |                 |
| 27                              | 0.02            | 0.11            | 0.11            | 0.12            | 0.21            | 0.22            | 0.21            |
| 49                              | 0.03            | 0.11            | 0.15            | 0.33            | 0.81            | 0.83            | 0.83            |
| 72                              | 0.01            | 0.13            | 0.20            | 0.54            | 0.89            | 0.80            | 0.75            |

*Phosdrin, 2-carbomethoxy-propene-2yl dimethyl phosphate. Bacteria inoculated in glycerol-rumen fluid. Trypticase-yeast extract medium minus glucose, cellobiose, and soluble starch.
on ruminal bacterial endogenous gas production, growth in RFTY liquid medium, and total culturable counts suggested that ruminal bacteria were not inhibited by Mobam. However, colony counts of bacteria showing zones of hydrolysis on tributyrin differential media were reduced in the presence of Mobam, and Mobam inhibited hydrolysis of aromatic ester compounds by pure cultures of ruminal bacteria. The relative importance to rumen metabolism of the bacterial reactions inhibited by Mobam presently cannot be assessed. Further basic knowledge on ruminal bacterial carboxyl-esterases in relation to water-insoluble aromatic ester compounds and rumen metabolism may be helpful in evaluating Mobam inhibition of microbial reactions.

**Table 3. Mobam inhibition of ruminal bacterial suspensions degrading 1-naphthyl acetate and fluorescein-3',6'-diacetate**

| Bacteria   | Mobam molarity* | Assay | Per cent inhibition |
|------------|-----------------|-------|---------------------|
| Mixture    | 6.5 x 10^{-4} M | NA    | 65.5                |
|            | 6.5 x 10^{-3} M | NA    | 8.3                 |
| Mixture    | 9.7 x 10^{-2} M | FDA   | 5.0                 |
| Strain 53  | 13.5 x 10^{-4} M| FDA   | 69.5                |
|            | 29.5 x 10^{-6} M| FDA   | 6.3                 |

*Mobam was incubated for 10 min at 39 C with the bacterial suspensions (1:10 dilutions of the incubated mixed and strain 53 cell preparations in basal medium had respective absorbancy readings of 0.56 and 0.39 at 600 nm) in basal medium (14) before adding substrate (NA or FDA) and subsequent incubation for 15 min. Noninhibited control mixtures of bacteria produced 45.5 x 10^{-4} m moles of 1-naphthol/15 min, and strain 53 cells produced 9.8 x 10^{-7} m fluorescein/15 min.

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