Involvement of Vitamin B₆ in the Dethiomethylation of Methionine by Rumen Microorganisms

D. L. MERRICKS and R. L. SALSbury

Department of Animal Science and Agricultural Biochemistry, University of Delaware, Newark,
Delaware 19711

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The dethiomethylation of methionine by a dialyzed extract obtained from the protozoa-rich fraction of rumen fluid is stimulated 2.5-fold by pyridoxal phosphate and strongly inhibited by deoxypyridoxine, a pyridoxal phosphate antagonist. These effects are not seen with undialyzed extracts or with whole rumen fluid. It is suggested that the anaerobic dethiomethylation of methionine by rumen microorganisms requires pyridoxal phosphate as a cofactor.

It has been shown by Salsbury et al. (9, 10, 15) that methionine and several methionine analogues were extensively degraded by rumen microorganisms. Both S-methylcysteine and methionine were good substrates for dethiomethylation, with S-methylcysteine being the more reactive. In studies of dethiomethylation in this laboratory, several methionine analogues have been used to delineate a general substrate specificity for the dethiomethylation reaction. Results have indicated that substitution on the N, S, or C₂ atoms of methionine decreases or prevents activity (10).

Segal and Starkey (12) and Ruiz-Herrera and Starkey (8) have suggested that methionine is first deaminated and then dethiomethylated by aerobic bacteria. Bird (1) suggests that similar pathways may function in the rumen in spite of the anaerobic nature of the rumen environment. This assumption is not supported by the work of Salsbury and Merricks (10), who found that the methionine hydroxy analogue, oxidizable to the methionine keto analogue, was a poor substrate for dethiomethylation by rumen microorganisms, suggesting that in the rumen the oxidation of the amino group of methionine does not precede the dethiomethylation reaction.

Walker and Nader (6,14) have reported that when [³⁵S]methionine was added to a rumen fluid fermentation, 51% of the ³⁵S appeared in the H₂S pool. However, no attempt was made to distinguish between H₂³⁵S and CH₃⁴SH.

Ohigashi et al. (7) showed that an enzyme preparation isolated from Escherichia coli which was adapted to methionine degraded methionine to methanethiol and α-amino butyric acid. The E. coli enzyme preparation was stimulated both by anaerobic conditions and by pyridoxal plus adenosine 5'-triposphate (the latter constituting pyridoxal phosphate).

Pyridoxal phosphate is thought to be the cofactor most often involved in the metabolism of amino acids (2). Both homocysteine desulphydrase and cystathionase employ pyridoxal phosphate as the enzyme cofactor in a gamma-elimination reaction (13). The bonds between pyridoxal phosphate and the apoenzyme, once formed, are difficult to cleave. Phosphorylase can be resolved into pyridoxal phosphate and protein by dialysis against a deforming buffer (imidazole) and a specific carbonyl reagent (cysteine) but not by dialysis against either of these reagents alone (2).

This paper reports experiments done to determine whether pyridoxal phosphate is involved as a cofactor in the dethiomethylation of methionine by rumen microorganisms in vitro. A method is described for the preparation of an enzyme extract from a protozoa-rich fraction of rumen fluid that actively dethiomethylates methionine.

MATERIALS AND METHODS

Rumen fluid sample. Rumen fluid samples were taken from a mature, rumen-fistulated Guernsey cow at various stages of the lactation cycle. The cow was fed silage, hay, and pasture ad libitum, plus a concentrate during lactation. Samples were taken just after feeding grain but before feeding silage, strained through cheesecloth, and maintained at 39 C.

Mixed protozoal extract. Three liters of strained rumen fluid was allowed to stand at 39 C in a four-liter separatory funnel for 1 h, or until good separation of fractions occurred. When rumen fluid is
allowed to stand under these conditions, it separates into three recognizable layers: a thick, green top layer containing fine plant material, bacteria, and trapped protozoa; a middle layer consisting of a turbid, straw-colored or greenish liquid containing bacteria and a very few of the small, active protozoa; and a thick, cream-colored bottom layer composed mainly of protozoa. The protozoa-rich fraction was withdrawn from the bottom of the funnel several times as the protozoa settled. Successive portions were collected in 200-ml screw-cap centrifuge tubes and refrigerated at 8 C until all collections were completed. All collections were pooled and then centrifuged at 1,000 × g for 10 min at 0 C, giving a semisolid pellet. The supernatant was discarded, and the pellet was suspended in a mineral buffer at pH 7.0 (11) and recentrifuged at 1,000 × g for 10 min at 0 C. The resuspension-recentrifugation procedure was repeated until a clear supernatant was obtained. The pellet was once again suspended in the mineral buffer and sonicated for 4 min using a maximum setting (Bronson sonifier, LA 75). The sonified preparation was centrifuged at 10,000 × g for 60 min at 0 C, and the supernatant was designated as the mixed protozoal extract.

Gas chromatographic system. A dual-column, hydrogen flame ionization gas chromatograph (F and M Scientific, model 810) was used with stainless-steel columns (0.25 inch by 12 feet; ca. 0.635 by 365.76 cm), packed with 10% silicone oil DC-200, on a 60- to 80-mesh diatoport S support (Hewlett Packard, Avondale, Pa.). Hydrogen and air were maintained at 20 lb/in² (20 × 703.1 kg/m²) and 33 lb/in² (33 × 703.1 kg/m²), respectively. The carrier gas, helium, was maintained at 40 lb/in² (35 × 703.1 kg/m²) with rotometers set at 2.0 for a flow rate of 47.2 ml/min. The injection port and the ionization detector were maintained at 300 ± 10 C, and the column oven was kept at 60 ± 2 C. All analyses were carried out isothermally.

Portions (10 ml) of the mixed protozoal extract were incubated in 20-ml vials in the presence of 134 μmol of methionine. The vials were fitted with channel rubber stoppers to facilitate sampling by syringe and were maintained at 39 C in a water bath. Samples of the gas in the headspace above the mixed protozoal extract were obtained with a gas-tight syringe, and 250 μl was injected directly into the injection port. Headspace gas samples were taken periodically over a 4-h period, and results were quantitated on a disk chart integrator.

Fermentation system, gas scrubber. Portions (30 ml) of the mixed protozoal extract were incubated in 60-ml serum vials in the presence of 1 μCi (17.86 nmoi of [methyl-14C]) of methionine obtained from Amersham/Searle, plus 4.08 μmol of pyridoxal phosphate or deoxyriboflavin, or with no vitamin B12 analogue. The serum vials were fitted with channel rubber stoppers and two 17-gauge syringe needles. Tygon tubing was used to connect one needle to a nitrogen carrier gas source and the other needle to a gas scrubber train. The train consisted of three 10-ml portions of 1 N NaOH in test tubes (22 by 175 mm) connected in series by Tygon tubing. All treatments were in duplicate, and values reported are mean values. Incubation was for 4 h at 39 C. At the end of the incubation period, 10-ml portions of 6 N HCl were injected into the serum vials, the the mixtures were maintained at 39 C for another 15 min to permit the collection of residual methionine dethiomethylation by the acid.

Liquid scintillation counting procedure. The three 10-ml portions of 1 N NaOH were pooled after each experiment, and five 500-μlter volumes were placed in 7-ml plastic vials that contained 5 ml of scintillation cocktail (Phase combining system, Amersham/Searle). Each 7-ml vial was fitted with a plastic sleeve to permit counting in a liquid scintillation counter requiring 20-ml vials (Nuclear-Chicago, ISOCAP 300). Counts were taken at a discriminator setting of 4.5 to 150.0 keV (program 2B) for 2 min at 0.25 sigma error and repeated three times. Blanks were handled identically and subtracted from experimental values. Quench correction was accomplished by using an internal standard because of high chemical and color quench from rumen samples, protein, and strong NaOH solutions. The disintegrations per minute determined for the 5 volumes were averaged disintegrations per minute and the mean was multiplied by 60 to determine the total disintegrations per minute in all the traps of each scrubber train. This gave a figure for total [14C]methanethiol production for each fermentation.

RESULTS

Evaluation of dethiomethylase activity. An experiment was carried out to determine the rate of methanethiol production by whole rumen fluid, the top layer from rumen fluid which had been incubated for about 1 h, and the bottom layer obtained under the same conditions (Fig. 1). When the amount of methanethiol which had accumulated after incubation for 160 min was taken to represent the relative activities of these three fractions, the bottom layer had an activity per unit volume (160 × 10⁹ integrator units) that was 10% of the corresponding activity of whole rumen fluid (1,600 × 10⁹ integrator units).

Because of differences in numbers of protozoa in the rumen fluid samples and in losses during preparation and storage of extracts, there were wide differences in activity between the individual mixed protozoal extracts used in this study. Consequently, comparisons have generally been limited to treatments within the same experiment. Nevertheless, to obtain an indication of the activity of the mixed protozoal extracts relative to whole rumen fluid, the results shown in Fig. 2 for a protozoal extract were compared to those shown in Fig. 1. Comparison of the very low activity of the washed-cell suspension with the much higher activity of the sonically treated extract suggests that the activity measured was
Effect of vitamin B₆ on dethiomethylation by the mixed protozoal extract. Incubation of undialyzed mixed protozoal extract with methionine plus pyridoxal phosphate or methionine plus deoxypyridoxal did not yield good evidence for stimulation of the dethiomethylation reaction by pyridoxal phosphate or inhibition by deoxypyridoxine (Fig. 5). After dialysis of 80 ml of extract for 60 h against 5 liters of distilled water at 10°C, the activity of the extract was reduced, but stimulation by pyridoxal phosphate and inhibition by deoxypyridoxine could be demonstrated (Fig. 6).

Effect of vitamin B₆ on dethiomethylation by whole rumen fluid. Previous experimentation (unpublished work from this laboratory) had shown that pyridoxal phosphate (Fig. 3) had little effect on dethiomethylation by rumen microorganisms in experiments with whole rumen fluid. To test the possibility that this lack of stimulation reflected a decreased uptake of the vitamin by rumen microorganisms when supplied as pyridoxal phosphate, an experiment was conducted with pyridoxal (Fig. 3) as a vitamin B₆ analogue addition to the reaction mixture. The experiment also included addition of the vitamin B₆ antagonist deoxypyridoxine (Fig. 3) as one of the treatments. It can be seen from Fig. 4 that neither deoxypyridoxine nor pyridoxal had any effect on the dethiomethylation reaction when whole rumen fluid was used.

Fig. 1. Methanethiol production from fractions of rumen fluid. Whole rumen fluid, ——; top layer, -----; bottom layer, ——.

Fig. 2. Methanethiol production. Mixed protozoal extract, ——; washed protozoal cells, -----.

Fig. 3. Vitamin B₆ analogues used. Pyridoxal; R¹=CHO, R²=OH. Deoxypyridoxine; R¹=CH₃, R²=OH. Pyridoxal phosphate; R¹=CHO, R²=OPO₃.
To obtain a more precise measure of the involvement of pyridoxal phosphate in dethiomethylation, [methyl-\textsuperscript{14}C]methionine was used as the substrate (1 \textmu Ci/30 ml of the mixed protozoal extract) and pyridoxal phosphate or deoxypyridoxine was added to the reaction mixture. In these experiments, measurements of \textsuperscript{[14}C]methanethiol production were used to determine enzyme activity. Table 1 shows the relative amounts of \textsuperscript{[14}C]methanethiol produced by various 4-h experiments. A different mixed protozoal extract was used for each experiment, and each was dialyzed for 36 h against distilled water (80 ml of extract to 5 liters of water at 10 °C) prior to incubation. The mixed protozoal extract used for experiment 1 was much more concentrated than those used for the other two experiments, and this is reflected in the values shown in the last column of Table 1. However, almost twice the amount of \textsuperscript{[14}C]methanethiol was produced by the pyridoxal phosphate-treated preparation as by the untreated preparation in all three experiments. These findings give substantial support to the
TABLE 1. Effect of pyridoxal phosphate or deoxypyridoxine on [14C]methanethiol production from [methyl-14C]methionine by the mixed protozoal extract

| Expt | Treatmenta | dpm recoveredb | t-tests | Conversion (%)c |
|------|------------|----------------|---------|-----------------|
| 1    | 1          | 772 ± 39       |         | 21.05           |
|      | 2          | 1,468 ± 22     | 31.106* | 39.87           |
| 2    | 1          | 1,105 ± 29     |         | 3.36            |
|      | 2          | 2,117 ± 47     | 4.529*  | 6.49            |
| 3    | 1          | 1,030 ± 38     | (1 vs. 2) 26.761* | 2.81 |
|      | 2          | 1,718 ± 35     | (1 vs. 3) 47.125* | 4.69 |
|      | 3          | 137 ± 3        |         | 0.37            |

*a Incubated with mixed protozoal extract for 4 h at 39 C.
bTreatment 1, dialyzed mixed protozoal extract; treatment 2, dialyzed mixed protozoal extract plus pyridoxal phosphate; treatment 3, dialyzed mixed protozoal extract plus deoxypyridoxine.
c dpm, Disintegrations per minute. Given as mean of two fermentations, five portions each, ± s.d. dpm.
*Given as [dpm in traps]/(dpm in traps plus dpm in supernatant) x 100.
* P < 0.01.

gas chromatographic data presented in Fig. 6, which suggest the involvement of pyridoxal phosphate in the dethiomethylation reaction. Further, the results obtained in experiment 3 (Table 1) indicate that deoxypyridoxine did indeed inhibit dethiomethylation relative to the control.

DISCUSSION

The crude enzyme preparation used in this study was prepared from the protozoa-rich fraction of rumen fluid and represented only a small part of the total methionine dethiomethylase activity of the whole rumen fluid. The decision to use this fraction was based on the assumption that the mixed protozoal extract would present a less complex array of demethylases than would an extract prepared from whole rumen fluid. The very low dethiomethylase activity of the washed-cell suspension, when compared with the sonic extract prepared from it (Fig. 2) and with the fractions of rumen fluid (Fig. 1), suggests that this was indeed the case and that the activity was endogenous to the protozoa. It remains to be determined whether the activity lost during dialysis was similar to that studied or indicated the presence of different, more labile enzymes. Some of our more recent work (unpublished data) indicates that the dialyzed extract may contain more than one dethiomethylase.

The absence of an appreciable treatment effect from the addition of pyridoxal phosphate or deoxypyridoxine when whole rumen fluid was used (Fig. 4) suggests that dethiomethylation by whole rumen fluid may be a different reaction than that of the mixed protozoal extract. However, the undialyzed mixed protozoal extract also failed to show inhibition by deoxypyridoxine or stimulation by pyridoxal phosphate (Fig. 5), and it was not until the preparation had been dialyzed that a treatment effect from added analogue could be demonstrated (Fig. 6). We interpret this to indicate that pyridoxal phosphate is tightly bound to the enzyme and cannot be displaced by deoxypyridoxine or added pyridoxal phosphate. Dialysis apparently releases some of the bound pyridoxal and permits the demonstration of the inhibitory or stimulatory effects of the analogues. It is possible that this interpretation, as well as the assumption that pyridoxal is not required for the bulk of methionine dethiomethylase activity shown by whole rumen fluid, could account for the lack of analogue effect with whole rumen fluid.

The dethiomethylation reaction described in this report appears to resemble the one described by Ohigashi et al. (7), who suggested that a phosphorylated derivative of pyridoxal was required as a cofactor. Mitsuhashi (3), Mitsuhashi and Matsuo (4), Miwatani et al. (5), and Ohigashi et al. (7) reported that their enzyme preparations dethiomethylated methionine much more actively under anaerobic than under aerobic conditions. This may indicate that the mechanism(s) of dethiomethylation by these enzymes was such that they functioned more effectively at low oxygen tensions or that aerobic conditions favored destruction of the enzymes. Segal and Starkey (12) proposed a mechanism in which an initial oxidative deamination of methionine was followed by dethiomethylation of the α-keto-γ-mercaptobutyric acid formed. This would exclude the possibility of involvement of pyridoxal phosphate as a cofactor in their system.

Ruiz-Herrara and Starkey (8) have shown that a purified methionine dethiomethylase from Aspergillus utilized both methionine keto analogues and methionine hydroxy analogues as substrates. This would be expected if the dethiomethylation were preceded by deamination. We have found that methionine hydroxy analogues can be dethiomethylated by whole rumen fluid (9), although much less effectively than is methionine, but not by the mixed protozoal extract. The failure of the methionine hydroxy analogue to act as a substrate for the mixed protozoal extract is readily explained by the
requirement for the amino group in order to form a Schiff base with the pyridoxal moiety. The dethiomethylation of the analogue by whole rumen fluid may indicate that a portion of the dethiomethylase activity of the rumen fluid is attributable to an enzyme(s) not requiring pyridoxal as a cofactor, or it may reflect an initial amination of the methionine hydroxy analogue to form methionine, which then serves as a substrate for the pyridoxal-requiring dethiomethylase.

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