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Methionine Substitutes in Ruminant Nutrition II: Stability of Nonnitrogenous Compounds Related to Methionine during *In Vitro* Incubation with Rumen Microorganisms

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Abstract □ Four new α-hydroxy derivatives related to methionine were prepared and tested for resistance to *in vitro* destruction by rumen microorganisms. 3-Hydroxydihydro-2(3*H*)-thiophenone was rapidly degraded by the microorganisms while its benzoate ester, 3-benzoyloxydihydro-2(3*H*)-thiophenone, escaped destruction as indicated by specific GLC analysis for the compounds. Two esters, methyl γ-methylmercapto-α-hydroxybutyrate and phenyl γ-methylmercapto-α-hydroxybutyrate, were found to be extensively destroyed upon incubation with rumen microorganisms.

Keyphrases □ Methionine substitutes—ruminant nutrition, stability of nonnitrogenous compounds compared to methionine, *in vitro* incubation with rumen microorganisms □ Nutrition, ruminant—stability of nonnitrogenous compounds compared to methionine, *in vitro* incubation with rumen microorganisms, effect of rumen microbial amino acid deamination □ Hydroxymethionine derivatives—synthesis, tested for resistance to *in vitro* destruction by rumen microorganisms

In ruminants, ingested feed passes immediately into the rumen and reticulum where it undergoes extensive degradation by a highly complex population of anaerobic bacteria and protozoa. The contents of these compartments are buffered at about pH 6.5–6.8; but since large amounts of organic acids are produced, it is not uncommon to observe pH values between 5.0 and 6.0. Proteins, amino acids, and other nitrogenous compounds are subjected to extensive deamination (1–3) to furnish ammonia, which is the preferred nitrogen source of much of the microbial population. Thus, much of the protein effectively available to the animal is that synthesized by the symbiotic microbial population (4). The mixture of microbial and feed proteins eventually passes from the rumen through the omasum to the abomasum where the pH is from 1 to 4. Here the proteins are subjected to enzymatic digestion by enzymes secreted by the animal. The next compartment of the digestive tract is the small intestine where conditions are slightly alkaline, protein digestion is completed, and most amino acid absorption takes place.

Normally, there is little absorption of amino acids from the rumen, and rumen microorganisms have been shown to degrade supplementary dietary amino acids (1–3). Furthermore, several studies have shown that nitrogen utilization has been improved by infusing amino acids postruminally (5–7). Thus, the development, through structural manipulation, of select amino acid preparations that resist microbial breakdown in the rumen could possibly prove useful for increasing nitrogen utilization in ruminants.

Thirty-one nitrogenous compounds related to methionine were previously examined (8) for their resistance to *in vitro* deamination by rumen microorganisms. DL-Homocysteine thiolactone hydrochloride (Ia) was found to be highly stable toward microbial destruction. Since an α-hydroxy analog of methionine (IIa) has been reported to produce a beneficial effect in ruminants (9), it was of interest to prepare α-hydroxyl derivatives of methionine and homocysteine thiolactone (Ia).

In the present study, four new nonnitrogenous compounds (α-hydroxy derivatives) related to methionine were tested for resistance to *in vitro* destruction by rumen microorganisms.

EXPERIMENTAL[1]

3-Hydroxydihydro-2(3*H*)-thiophenone (I*b*)—One hundred milliliters of concentrated hydrochloric acid was added to a solution of 50 g of homocysteine thiolactone (I*a*)[2] in 100 ml of distilled water, and the solution was cooled to ice bath temperature. A solution of 69 g of sodium nitrite in 300 ml of distilled water was then added dropwise with vigorous stirring. The resulting brownish-red oily liquid was separated from the aqueous layer. The water layer was then extracted with chloroform (3 × 150 ml). The chloroform fractions, combined with the oily liquid, were dried, and solvent was removed *in vacuo*. Distillation at 85° (0.05 mm) yielded 8.3 g (21%) of I*b*. Further distillation over a spinning band column afforded an analytical sample.

Anal.—Calc. for $C_4H_6O_2S$: C, 40.66; H, 5.12. Found: C, 40.56; H, 5.12.

3-Benzoyloxydihydro-2(3*H*)-thiophenone (Ic)—To a solution of 1.2 g of I*b*, 20 ml of methylene dichloride, and 1 ml of triethylamine was added dropwise 2 g of benzoyl chloride with stirring at room temperature. Stirring was continued for 30 min. To the reaction mixture was then added 50 ml of cold 10% NaOH solution. The resulting solution was extracted with chloroform (3 × 30 ml) and the combined extracts were dried. Removal of solvent *in vacuo* yielded Ic (1.9 g, 85%). Two recrystallizations from chloroform–methanol afforded white leaflets, mp 121–122°.

Anal.—Calc. for $C_{11}H_{10}O_3S$: C, 59.44; H, 4.54; S, 14.42. Found: C, 59.59; H, 4.61; S, 14.40.

Methyl γ-Methylmercapto-α-hydroxybutyrate (II*b*)—Fifteen grams of γ-methylmercapto-α-hydroxybutyric acid (II*a*)[3] was refluxed with 100 ml of 3% H_2SO_4 in methanol for 3 hr. The pH of the solution was then adjusted to 7.0 with 10% KOH in methanol, the solution was filtered, and the filtrate was evaporated to dryness under reduced pressure. Then 30 ml of water was added to the residue and the mixture was extracted with ether (3 × 50 ml). The combined ether extracts were washed with 10% aqueous KOH (3 × 20 ml) and then with water (2 × 20 ml). The dried ether extract was evaporated *in vacuo*. The oily residue was distilled, bp 85–90° (0.15 mm), to afford II*b* (8.5 g, 52%). Further distillation at 80° (0.1 mm) yielded an analytical sample.

Anal.—Calc. for $C_6H_{12}O_3S$: C, 43.88; H, 7.37. Found: C, 44.02; H, 7.31.

Phenyl γ-Methylmercapto-α-hydroxybutyrate (IIc)—For 2 hr, 7.5 g of γ-methylmercapto-α-hydroxybutyric acid (II*a*)[3], 5.5 g of phenol, and 0.5 g of *p*-toluenesulfonic acid in 100 ml of benzene were refluxed (Dean–Starke trap). The mixture was shaken with 20 ml of 0.5 *N* NaOH, and the benzene layer was subsequently dried and concentrated *in vacuo*. Distillation of the residue at 190–200° (0.15 mm) afforded IIc (6 g, 54%).

Anal.—Calc. for $C_{11}H_{14}O_3S$: C, 58.39; H, 6.24. Found: C, 58.06; H, 6.24.

***In Vitro* Fermentation with Rumen Microorganisms**—Rumen microorganisms, obtained from a fistulated steer according to a reported method (8), were washed and incubated under an atmosphere of carbon dioxide in 250 ml of a liquid medium developed by Cheng *et al.* (10). This medium was composed of inorganic salts, cellulose (14 mg/ml), and urea (0.5 mg/ml) as the sole nitrogen source. The concentrations of the test compounds in the fermentation medium were as follows (mg/ml): I*b*, 13.75; Ic, 5.95; II*b*, 8.2; and IIc, 11.3. The initial pH of the medium was adjusted to 6.86 with saturated sodium bicarbonate solution. Incubation experiments were conducted for 24 hr at 39° in 500-ml erlenmeyer flasks. Duplicate samples (2.0 ml), withdrawn from the fermentation mixture, were filtered through a filter[4], freeze dried, and subsequently analyzed by GLC. At the end of the fermentation period (24 hr), the samples were immediately frozen and their cellulose content was determined by the procedure of Crampton and Maynard (11) as modified by Donefer *et al.* (12).

GLC Analysis of I*b* and Ic—GLC analyses were conducted

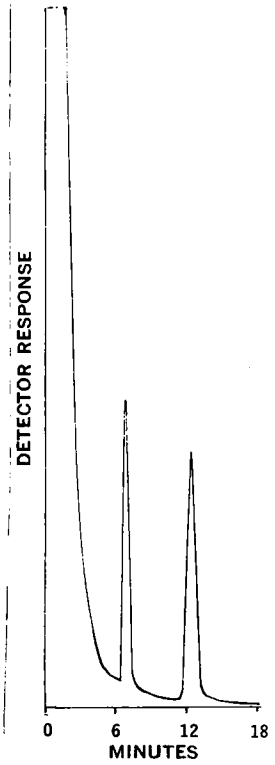


Figure 1—*GLC chromatogram obtained with a 2-μl sample from a solution of* I*b in fermentation fluid (10 mg/ml). Each sample was spiked with acetophenone (peak on the left), which exhibited a retention time of 6.9 min while that of* I*b (peak on the right) was 12.6 min. Chromatography of* I*b with a 1.52-m × 0.31-cm (5-ft × 0.125-in.) OV-17 column was performed under the following conditions: column temperature, 105°; injection block temperature, 200°; detector temperature, 200°; nitrogen flow rate, 30 ml/min; hydrogen flow rate, 30 ml/min; and air flow rate, 60 ml/min. The electrometer attenuation was at ×32, and the chart speed was kept at 0.17 in./min.*

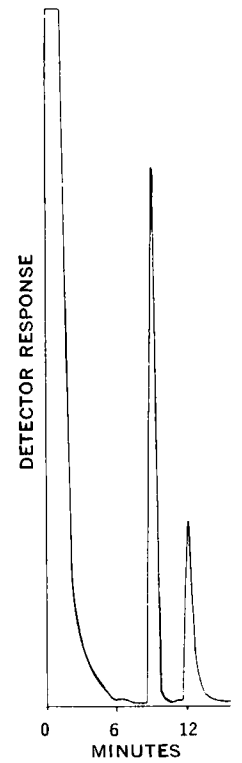


Figure 2—*GLC chromatogram obtained with 1-μl sample from a solution of* Ic *in fermentation fluid (5 mg/ml). Each sample was spiked with phenobarbital (peak on the right), which exhibited a retention time of 12.0 min while that of* Ic *(peak on the left) was 9.6 min. Chromatography of* Ic *with a 1.52-m × 0.31-cm (5-ft × 0.125-in.) OV-17 column was performed under the following conditions: column temperature, 225°; injection block temperature, 250°; detector temperature, 260°; nitrogen flow rate, 30 ml/min; hydrogen flow rate, 30 ml/min; and air flow rate, 300 ml/min. The electrometer attenuation was at ×32, and the chart speed was kept at 0.17 in./min.*

[1] Melting points were determined on a Thomas-Hoover melting-point apparatus and are uncorrected. Elemental microanalyses were performed by Microanalysis, Inc., Wilmington, DE 19808. The structures of all compounds were supported by IR and NMR spectra determined on Beckman IR-8 and Varian A-60 spectrometers, respectively. UV spectra were determined on a Cary 15 spectrophotometer in methanol.

[2] Compound I*a* was obtained from Aldrich Chemical Co., Milwaukee, Wis.

[3] Compound II*a* was obtained from its calcium salt preparation (Hydan, Nutritional Biochemical Corp., Cleveland, Ohio) by removal of calcium with 10% H_2SO_4 in methanol, extraction of II*a* with 10% aqueous KOH, and subsequent acidification and extraction with chloroform. The obtained II*a* was distilled, bp 135–140° (0.25 mm).

[4] Millipore.

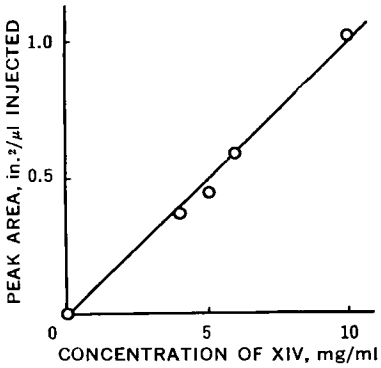


Figure 3—*Calibration curve for the GLC assay of XIV in fermentation fluid.*

with a gas chromatograph[5] equipped with a flame-ionization detector, using a 1.52-m × 0.31-cm (5-ft × 0.125-in.) stainless steel column, packed with 3% OV-17[6] on 100–120-mesh Gas Chrom Q[6] as the solid support. Conditioning of newly packed columns was carried out as previously described (8). Solutions of varying concentrations in fermentation medium were utilized for the preparation of calibration curves (Figs. 3 and 4). The concentrations ranged from 1.0 to 20.0 mg/ml for I*b* and from 0.4 to 7.0 mg/ml for Ic. Samples of 2.0 ml each were extracted twice with 5-ml increments of ether. The combined ether extracts were dried over 0.2 g of anhydrous sodium sulfate and subsequently evaporated to dryness. Then 1 ml of an internal standard was added to each sample. Acetophenone in chloroform (1 mg/ml) was used as the internal standard for I*b*, while phenobarbital in ethanol (1 mg/ml) was utilized for Ic. Samples of I*b* and Ic were then analyzed by GLC according to the conditions described in Figs. 1 and 2, respectively.

GLC Analysis of II*b* and IIc—Samples (2.0 ml) from fermentation medium containing II*b* and IIc were extracted four times with 2-ml increments of ether. The extracts were reduced to approximately 1.5 ml under reduced pressure and their volume was adjusted to 2.0 ml. A 5-μl sample was then analyzed on a 1.52-m × 0.31-cm (5-ft × 0.125-in.) stainless steel column, packed with 3% SE-30 on 100–120-mesh Varaport[7] 30. A gas chromatograph[5], equipped with a flame-ionization detector and a linear temperature programmer, was utilized. The GLC analyses of II*b* and IIc were performed under the following conditions, respectively: initial column temperature, 110°, 107°; final column temperature, 140°, 140° at a rate of 12°/min; injection block temperature, 220°, 215°; detector temperature, 220° (II*b* and IIc); nitrogen flow rate, 14 ml/min (II*b* and IIc); hydrogen flow rate, 30 ml/min (II*b* and IIc); air flow rate, 300 ml/min (II*b* and IIc). The retention times were 6 min for II*b* and 8 min for IIc. The electrometer attenuation was at ×32, and the chart speed was kept at 0.17 in./min.

RESULTS AND DISCUSSION

In a previous publication (8) and in the introductory remarks,

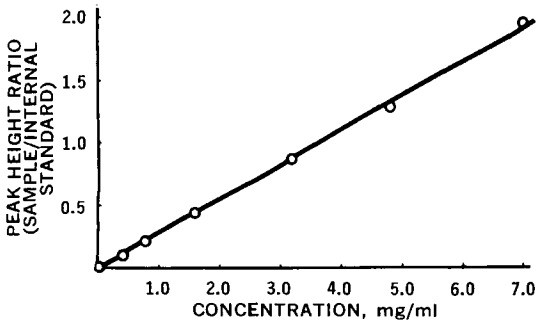


Figure 4—*Calibration curve for the GLC assay of Ic in fermentation fluid.*

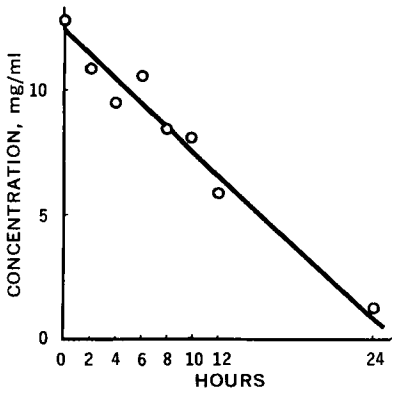


Figure 5—In vitro *microbial destruction of* Ib (*13.75 mg of* Ib*/ml of fermentation fluid*).

it was mentioned that proteins, amino acids, and other nitrogenous compounds are subjected in ruminants to extensive chemical degradation by rumen microorganisms. It appears that a large portion of the protein effectively available to these animals is that synthesized by the symbiotic microbial population. Recent research indicates that rumen microbial protein may be deficient in methionine, lysine, and threonine for supporting maximum protein utilization by growing lambs (13). Supplementary dietary amino acids are also degraded by rumen microorganisms (1–3). However, postruminal infusion of amino acids appears to improve nitrogen utilization (5–7). Thus, it is clear that resistance of an amino acid preparation toward microbial breakdown in the rumen could possibly find utility for increasing nitrogen utilization in the ruminant.

Recently it was reported (9) that an α-hydroxy analog of methionine[8] (II*a*) improved milk production in dairy cows. DL-Homocysteine thiolactone hydrochloride (I*a*) was found (8) to resist rumen microbial destruction *in vitro* and was stable in aqueous buffers at pH 2.3 and 6.8, typical pH values for the abomasum and small intestine, respectively. Furthermore, I*a* has been shown to support growth of methionine-dependent mutants of *Pseudomonas tabaci* (14), and sliced rat livers have been shown to convert the thiolactone to methionine (15). *In vivo* experiments on young rats fed a diet of highly purified amino acids showed synthesis of methionine from DL-homocysteine thiolactone (I*a*) (16). This dietary property of I*a* was also observed in growing rats[9]. In addition, evidence has been obtained that the compound is absorbed more readily than methionine by using an *in situ* rat gut absorption technique described by Doluisio *et al.* (17).

These findings suggested that α-hydroxy analogs of DL-homocysteine thiolactone (I*a*) would have good potential for escaping rumen destruction and act as good methionine precursors. Thus, 3-hydroxydihydro-2(3*H*)-thiophenone (I*b*) was synthesized by diazotization of I*a*. Subsequent esterification of I*b* with benzoyl chloride led to the synthesis of Ic.

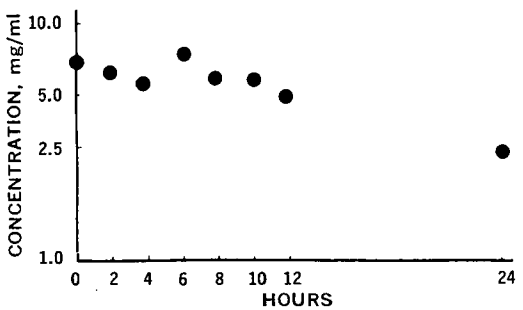
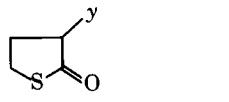


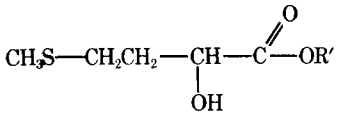
Figure 6—In vitro *stability of* Ic (*5.95 mg of* Ic*/ml of fermentation fluid*).

[5] Varian Aerograph model 1700.
[6] Applied Science Laboratories, State College, PA 16801
[7] Varian Instrument Division, Palo Alto, CA 94303

[8] Hydan.
[9] H. E. Amos, G. T. Schelling, G. A. Digenis, J. V. Swintosky, C. O. Little, and G. E. Mitchell, Jr., to be published.



Ia: $y = NH_2 \cdot HCl$
Ib: $y = OH$
Ic: $y = OCOC_6H_5$



IIa: $R' = H$
IIb: $R' = CH_3$
IIc: $R' = C_6H_5$

To investigate the resistance of I*b* and I*c* toward microbial degradation, a specific GLC analysis was developed for the compounds. Samples from the fermentation experiments were extracted with ether, and an internal standard was added to the residues obtained from the extracts. Acetophenone was used as a marker for I*b*, and phenobarbital was used for I*c* (Figs. 1 and 2). Figures 3 and 4 illustrate that an excellent linear relationship could be obtained for the calibration curves by measuring the peak height ratios of the unknown compounds with their respective markers.

When I*b* and I*c* were incubated *in vitro* with rumen microorganisms, according to the previously described technique (8), it was found that I*b* was quickly degraded by the microorganisms (Fig. 5). In contrast, however, the ester I*c* appeared to be considerably resistant to microbial destruction (Fig. 6). The results obtained with I*b* are somewhat surprising in view of the fact that I*a* was previously shown to escape microbial destruction under identical conditions (8).

As shown in Figs. 7 and 8, both II*b* and II*c* were extensively destroyed during *in vitro* incubation. The rapid rates of destruction observed during the first few hours declined considerably and permitted 10–20% of the added materials to remain after 24 hr. If a similar pattern applies to *in vivo* degradation, a sufficient quantity of these compounds might escape destruction in the rumen to permit significant passage to the lower digestive tract.

Experiments are now underway to assess the value of these compounds as dietary methionine substitutes in ruminants. The results of these studies will be reported elsewhere.

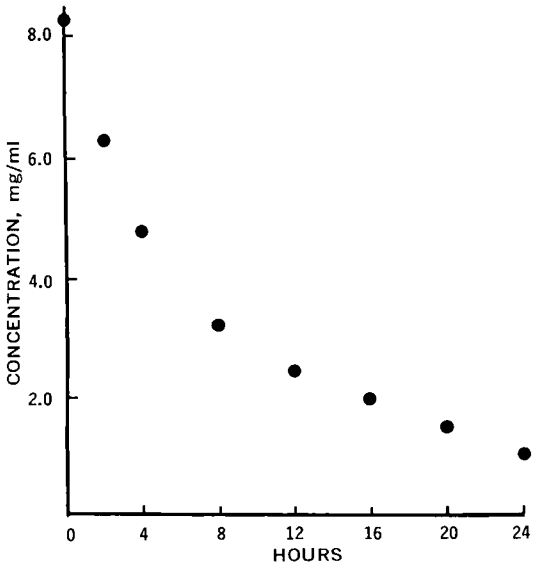


Figure 7—In vitro *microbial destruction of II*b (*8.2 mg of II*b*/ml of fermentation fluid*).

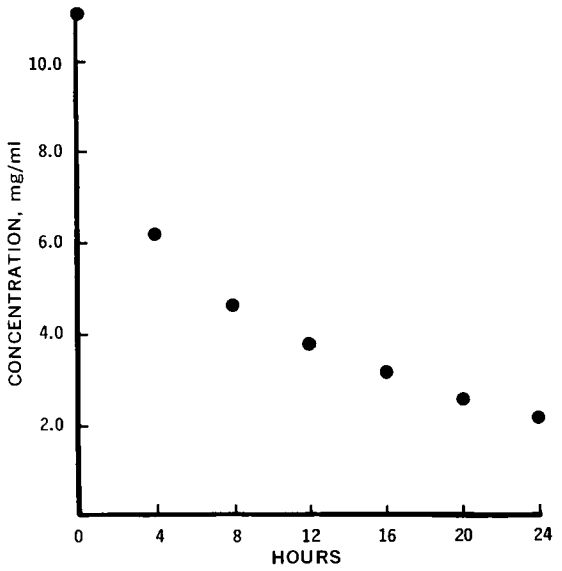


Figure 8—In vitro *microbial destruction of IIc* (*11.3 mg of IIc/ml of fermentation fluid*).

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