(4:1:2)] were scraped off, eluted into *n*-hexane, back-extracted into the ceric sulfate–sulfuric acid solution, and examined by the oxidation-UV method. The resulting UV absorption spectra from both the unchanged methadone and the metabolite were identical to that of benzophenone. GLC examination of the heptane following reflux further substantiated that benzophenone was formed from both the parent drug and the metabolite.

DISCUSSION

The extended linearity of the spectrophotometric method permits the use of a single aliquot of the specimen to encompass most urine methadone concentrations encountered in patients receiving methadone maintenance. Analysis by the barium peroxide method required varying specimen volumes since linearity was achieved up to a concentration of 25 μg/ml.

A comparison of the procedures and results of the ceric sulfate and barium peroxide methods indicates several advantages of the former in addition to the extended linearity and elimination of the required alkaline wash. Oxidation of methadone by the method of this report gives a 84.6 ± 3.2% yield of benzophenone as opposed to a 77.8 ± 3.3% yield obtained with the barium peroxide method. In addition to the time saved through the elimination of an extraction step, the proposed spectrophotometric method requires a shorter reaction time; *e.g.*, 20 min reflux with barium peroxide provided only a 48% yield of benzophenone compared to an 82% yield obtained with ceric sulfate. Additionally, reflux conditions were less critical since no significant change in percent yield was observed as the amount of primary oxidant was varied from 200 to 300 mg, the molarity of the sulfuric acid was varied from 5 to 6, or the duration of reflux was varied from 25 to 50 min. Similar variations in the barium peroxide method resulted in significantly decreased yields; changes in the amount of acid, solvent, and heat of reflux produced similar effects in both methods.

The proposed methods provide rapid and sensitive quantitative methods for determining methadone at therapeutic levels in biological specimens. Since other diphenyl-substituted drugs are susceptible to cerium sulfate, utilization of the GLC technique in conjunction with the spectrophotometric method provides a highly specific analytical methodology for methadone in biological specimens. The methods are particularly useful as a quantitative test in suspected methadone overdose cases and as a mechanism to monitor pharmacokinetic studies. In methadone maintenance programs the techniques can be used in collaboration with TLC to confirm on a relative basis that patients are receiving, within broad limits, proper dosages of the drug.

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

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Abstract □ Thirty-one nitrogenous compounds with possible methionine activity were included as potential nitrogen sources in media for *in vitro* incubation of rumen microorganisms. Compounds not supporting cellulose digestion were included in studies of ammonia release and specific analyses designed to confirm resistance of the compounds to microbial deamination. *N*-Acetyl-DL-methionine (IV) and DL-homocysteine thiolactone hydrochloride (XIV) were highly stable when added individually and were selected for more intensive study. When added in combination with urea, XIV was more stable than IV as indicated by cellulose digestion, ammonia release data, and specific GLC analysis for the compounds. Compound XIV was also tested in aqueous buffers at pH 6.8 and 2.3. Little destruction was observed within 24 hr at either pH. These results indicate that dietary XIV has good potential for escaping rumen destruction in a form suitable for subsequent conversion to methionine.

Keyphrases □ Methionine substitutes—ruminant nutrition, stability of 31 nitrogen compounds compared to methionine, *in vitro* incubation with rumen microorganisms □ Nitrogen sources—methionine substitutes in ruminant nutrition, 31 compounds compared to methionine, *in vitro* incubation with rumen microorganisms □ Nutrition, ruminant—31 nitrogen compounds examined to circumvent rumen microbial amino acid deamination

Ruminant animals depend on mixtures of microbial and dietary proteins for their amino acid nutrition (1–5). Dietary proteins may vary greatly in both content and availability of amino acids, and limitations

have been observed in both the digestibility (6, 7) and biological value (8, 9) of microbial protein. This suggests that performance could be improved by providing additional amino acids to the postruminal digestive tract. This hypothesis is supported by responses in nitrogen balance or wool growth obtained during postruminal infusions of protein or amino acids (10–14) and parenteral administration of methionine (15). The possibilities for effecting such improvement through dietary additions of amino acids are severely limited by the instability of amino acids in the rumen (16, 17). This problem could be overcome if compounds with amino acid activity and resistance to deamination by rumen microorganisms could be discovered. Nitrogen-containing compounds related to methionine were tested for resistance to *in vitro* deamination by rumen microorganisms using procedures similar to those employed by Acord *et al.* (18) to evaluate a variety of individual nitrogen sources, including amino acids.

EXPERIMENTAL[1]

The compounds tested in this study are listed in Table I.

Compounds VIII, IX, XVIII, XXIX, and XXX were prepared according to literature procedures: VIII, mp 151–153° [lit. (19) mp 149°]; IX, mp 139–141° [lit. (20) mp 138–139°]; XVIII, mp 80–83° [lit. (21) mp 82–84°]; XXIX, mp 51–53° [lit. (22) mp 55–56°]; and XXX, bp 158° (10.8 mm), mp 49–50° [lit. (23) mp 59°]. Compounds XXIV and XXV are known (24) but testing samples contained octanoic and lauric acid impurities, respectively.

Compounds XXVI and XXVII were prepared by acylation of homocysteine thiolactone with the corresponding acid chloride in chloroform–sodium bicarbonate: XXVI, mp 84.5–85.5°, and XXVII, mp 148–149° [lit. (25) mp 134–136°]. Compound XXVIII was prepared by sulfuric acid-catalyzed esterification of XXV in methanol, mp 45–57°.

***In Vitro* Fermentation Test: Cellulose Digestion Experiment**—Rumen microorganisms were obtained 4–6 hr postprandial from a fistulated steer being maintained on 3 kg daily of a shelled corn–soybean meal concentrate mixture plus alfalfa hay *ad libitum*. The rumen ingestum (1–2 liters) was strained through four layers of cheesecloth into a Thermos bottle preheated to 39°. The microorganisms present in the liquid ingesta were centrifuged[2] at 1000×*g* for 2 min to remove any remaining feed particles. The supernate was decanted and diluted with an equal volume of carbon dioxide-saturated 39° water. The diluted material was then passed through a centrifuge[3] equipped with a continuous-flow apparatus to sediment the microbial cells. The flow rate was approximately 150 ml/min at a force of 20,000×*g*. The microorganisms were then washed from the centrifuge tubes and subsequently incubated under an atmosphere of carbon dioxide in 250 ml of a liquid medium developed by Cheng *et al.* (26). The chemical composition of this medium is shown in Table II. Urea, the only nitrogen source in the medium, was replaced by a test compound to provide 0.46 mg nitrogen/ml medium. The initial pH of the medium was adjusted to 6.86 with saturated bicarbonate. For the cellulose digestion experiments, incubations were conducted for 24 hr at 39° in 500-ml erlenmeyer flasks. At the end of the fermentation period (24 hr), the samples were immediately frozen and their cellulose content was determined by following the procedure of Cramptom and Maynard (27) as modified by Donefer *et al.* (28). For the GLC analyses of XIV and IV, the compounds were incubated with urea in 250 ml of the fermentation medium and without urea in another batch. Duplicate samples of 0.1 ml were withdrawn from each batch at various intervals and filtered through a filter[4], freeze dried, and subsequently analyzed by GLC.

***In Vitro* Ammonia-Nitrogen Release Studies**—Rumen fluid, obtained as outlined already, was centrifuged at 1000×*g* in a centrifuge[2] for 2–3 min. The supernate was decanted and diluted with an equal volume of the Cheng *et al.* (26) medium (Table II). To this incubation medium, 495.0 mg of XIV or 636.0 mg of IV/100 ml or the same quantities of the compounds plus 100 mg of urea/100 ml of medium was added. The pH of the medium was adjusted initially to 6.86 with a saturated solution of sodium carbonate and then the medium was incubated at 39°. Homocysteine or homocysteine and urea were used as control treatments (Table III). Samples were withdrawn at 1- and 2-hr intervals thereafter and the ammonia released by each sample was determined by the procedure of Conway (29).

GLC Analyses of XIV and IV in Buffers and Fermentation Fluid—A gas chromatograph[5] equipped with a flame-ionization detector and a recorder[6] was employed. Best results were obtained with a stainless steel chromatography column, 0.31-cm o.d. × 1.52 m (0.125-in. o.d. × 5 ft) packed with 3% OV-17[7] on 100–120-mesh Gas Chrom Q[7] as the solid support. Conditioning of a newly packed column was carried out at 275° with no carrier gas for the first 8 hr and then at 250° with carrier gas (nitrogen at a flow rate of 20 ml/min) for the next 24 hr. To maintain the column in good operating condition after extensive use, it was found that injection of 15 μl of a silylating agent[8] followed by heating at 200° for 30 min prior to use gave excellent results.

Silylation of XIV—The sample[9] to be silylated was filtered[4], and a 0.1-ml aliquot from the resulting clear solution was taken. The aliquot was then freeze dried in a half-dram vial and immediately covered with a rubber septum to avoid unnecessary exposure to moisture[10]. To the lyophilized sample, 0.2 ml of silylating solution[11], consisting of 0.05 ml of *N,O*-bis(trimethylsilyl)trifluoroacetamide and 0.15 ml of pyridine, was added and the resulting clear solution was shaken for 30 min at room temperature. A 1-μl sample was then analyzed by GLC according to the conditions described in Fig. 1.

Silylation of IV—The general procedure for the silylation of IV was similar to that used for XIV. Both *N,O*-bis(trimethylsilyl)trifluoroacetamide and *N,O*-bis(trimethylsilyl)acetamide[12] were found to silylate IV. However, best results were obtained when a 0.1-ml aliquot from the sample of IV was treated for 30 min at room temperature with 0.2 ml of a silylation solution[11] containing 0.05 ml *N,O*-bis(trimethylsilyl)acetamide and 0.15 ml of pyridine. A 1-μl sample was then analyzed by GLC according to the conditions described in Fig. 2.

Stability Studies with XIV—Stability studies were performed in 10.0-ml volumetric flasks by dissolving 0.10 g of XIV in 10.0 ml of the appropriate buffer and adjusting the pH. A phthalate buffer was used for pH 2.3, and phosphate buffer was used for pH 6.9. The flasks were shaken on a water bath shaker at 39°. Then 0.1-ml samples, withdrawn at various intervals, were immediately lyophilized and analyzed for their thiolactone content by GLC.

RESULTS AND DISCUSSION

The results reported here represent the first step in the evaluation of compounds that might be of special value for the improvement of methionine nutrition of ruminants. The nature of the ruminant digestive tract poses special problems to those interested in altering the amino acids available to the tissues (17, 39–41). Ingested feed passes immediately into the rumen and reticulum. These stomach compartments constitute a large fermentation vat

[1] Elemental analytical results were within ±0.3% of the theoretical values; the following compounds were analyzed for the elements indicated by the symbols: Compound XXX ($C_9H_{17}NO_3S$)—C, H, N, S; Compound XXVI ($C_{16}H_{29}NO_2S$)—C, H, N; Compound XXVII ($C_{11}H_{11}NO_2S$)—C, H, N; and Compound XXVIII ($C_{18}H_{35}NO_3S$)—C, H, N. Melting points were taken on a Thomas-Hoover apparatus and are uncorrected.

[2] International model V2 centrifuge.

[3] Sorvall model SS-1.

[4] Millipore.

[5] Varian Aerograph model 1700.

[6] Varian Aerograph model 20.

[7] Applied Science Laboratories, State College, PA 16801

[8] Silyl 8, Pierce Chemical Co., Rockford, Ill.

[9] Samples from aqueous buffer solutions or fermentation media were treated similarly.

[10] The silylation reaction was found to be sensitive to moisture.

[11] The silylation solution was freshly prepared and stored in a half-dram vial covered with a rubber septum. The transfer of 0.2 ml from that solution to the lyophilized sample of XIV or IV was performed by means of a glass syringe and stainless steel hypodermic needle.

[12] Pierce Chemical Co., Rockford, Ill.

Table I—*In Vitro* Cellulose Digestion by Rumen Microorganisms Incubated with DL-Methionine Derivatives or Analogs

Compound Number	Name	Percent Cellulose Fermented
	Trial 1	
I[a]	Methionine sulfoxide	46
II[b]	DL-Methionine amide hydrochloride	95
III[a]	DL-Methionine sulfone	30
IV[a]	*N*-Acetyl-DL-methionine	0
V[a]	DL-Methionine methyl ester hydrochloride	70
VI[a]	DL-Methionine	28
	Trial 2	
VII[c]	Glycyl-DL-methionine	8
VIII	Benzoyl-L-methionine	3
IX	DL-2-Ureido-4-methylthiobutyric acid	63
X[d]	*N*-Hydroxymethyl-DL-methionine calcium salt	0
XI[d]	*N*-Acetyl-DL-homocysteine thiolactone	45
XII[d]	*N*-Phthalyl-DL-methionine	0
XIII[b]	*N*-*tert*-Butyloxycarbonyl-L-methionine dicyclohexyl ammonium salt	0
XIV[c]	DL-Homocysteine thiolactone hydrochloride	0
XV[d]	*N*-Formyl-DL-methionine	40
VI	DL-Methionine	46
XVI[a]	Urea	67
	Trial 3	
XVII[e]	*N*-Propionyl-DL-methionine	9
XVIII	*N*-Benzoyl-DL-methionine amide	45
XIX[b]	*N*-Benzoyl-DL-methionine methyl ester	45
XX[a]	*N*-*tert*-Butyloxycarbonyl-L-methionine-*p*-nitrophenyl ester	0
XXI[a]	DL-Methionine methyl sulfonium chloride	19
XXII[a]	*N*-Carbobenzoxy-DL-methionine	0
XXIII[a]	DL-Homocysteine	49
VI	DL-Methionine	21
XVI	Urea	62
	Trial 4	
XXIV	*N*-Octanoyl-DL-methionine	30
XXV	*N*-Lauryl-DL-methionine	19
XXVI	*N*-Lauryl-DL-homocysteine thiolactone	30
XXVII	*N*-Benzoyl-DL-homocysteine thiolactone	44
XXVIII	*N*-Lauryl-DL-methionine methyl ester	28
XXIX	*N*-Lauryl-DL-methionine ethyl ester	30
XXX	*N*-Acetyl-DL-methionine ethyl ester	50
XXXI	Poly-L-methionine	45
XVI	Urea	65

[a] Sigma Chemical Co. [b] Cyclo Chemical. [c] Aldrich Chemical Co. [d] Mann Research Labs. [e] Smith Kline & French. [f] Miles Laboratories.

where the feed undergoes extensive degradation by a highly complex population of anaerobic bacteria and protozoa. The contents are heavily buffered at about pH 6.5–6.8, but large amounts of organic acids are produced and pH values from 5.5 to 7.0 are not considered abnormal or unusual. Proteins, amino acids, and other nitrogenous compounds are subject to extensive, although variable, deamination to furnish ammonia, the preferred nitrogen source of much of the microbial population. Consequently, much of the protein effectively available to the animal is synthesized by the symbiotic microbial population. There is normally little absorption of amino acids at this point, and most of the free amino acids furnished in the diet or resulting from microbial digestion of protein serve mainly as nonspecific nitrogen sources for microorganisms. The mixture of microbial and feed proteins resulting from these processes eventually passes through the omasum to the abomasum, where the pH ranges from 1 to 4, and is there subjected to the first stages of protein 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. Thus, an effective way of increasing the amount of an amino acid available to the animal tissues might involve providing it in a form that is stable to the fermentive activity in the rumen but that can be absorbed from the lower digestive tract and is capable of conversion to the desired amino acid either before or after absorption.

The data reported here are the results of a screening procedure involving subjecting test compounds to *in vitro* fermentation by microbial populations taken from the rumen. The procedures do not provide satisfactory conditions for protozoal growth and probably result in some alteration of the bacterial population. Compounds surviving this test would logically be tested next for stability to the microbial population in the animal. Determining absorbability, establishing methionine potency in monogastric laboratory animals, and demonstrating improved methionine status in ruminants under laboratory and practical conditions would follow.

In the present study, a multidisciplinary effort was made to develop an *in vitro* test that would permit the screening of several compounds as methionine substitutes in ruminant nutrition. Since microbial destruction of methionine in the rumen is a major problem, an attempt was made to select methionine analogs that would permit correlation between structural characteristics and their resistance to microbial attack.

Table II—Composition of Nutrient Medium

Constituent	Amount, g/liter
Cellulose[a]	14.0
Urea	2.0
KH_2PO_4	0.6
Na_2HPO_4	0.64
$NaHCO_3$	3.5
KCl	4.0
NaCl	4.0
$CaCl_2$	0.55
$MgSO_4 \cdot 7H_2O$	0.29
$CuSO_4 \cdot 5H_2O$	0.002
$MnSO_4 \cdot H_2O$	0.00046
$ZnSO_4 \cdot 7H_2O$	0.00008
$FeSO_4 \cdot 7H_2O$	0.075
$CoCl_2 \cdot 6H_2O$	0.002

[a] SolKa-floc BW-200, a purified wood cellulose, Brown Co., Berlin, N.H.

Table III—Ammonia–Nitrogen Released during Fermentation (Milligrams per Milliliter)

Treatment	Hours							
	0	1	2	4	6	8	10	12
XIV	0.074	—	—	—	—	0.002	—	—
XIV and VI	0.221	0.353	0.290	0.647	0.440	0.618	0.601	0.597
IV	—	0.031	—	0.118	0.059	—	0.059	0.031
IV and VI	—	0.619	0.542	0.706	0.901	0.901	1.190	1.000
XXIII	—	—	—	—	0.357	0.266	0.308	0.294
XXIII and VI	0.147	0.588	0.470	0.470	0.706	0.266	0.322	0.294

Table II shows the composition of the nutrient medium developed by Cheng *et al.* (26) used for the *in vitro* fermentation experiment. In this medium, rumen microorganisms, obtained from fistulated steers, were incubated. The only nitrogen source in the fermentation fluid was urea or one of the methionine analogs being tested. In the fermentation test, the extent of cellulose digestion was utilized as an index of the susceptibility of the test compounds to microbial destruction (30). Thus, compounds supporting a high level of cellulose digestion (*i.e.*, an indication of good microbial growth) were considered easily degradable by rumen microorganisms; compounds not supporting *in vitro* cellulose destruction were considered as *potentially* stable to microbial destruction.

Thirty-one known methionine derivatives were subjected to the test, and the results of four different trials are shown in Table I. DL-Methionine and/or urea was used in each trial as a control. Of the compounds tested, *N*-acetyl-DL-methionine (IV), glycyl-DL-methionine (VII), benzoyl-DL-methionine (VIII), *N*-hydroxymethyl-DL-methionine calcium salt (X), *N*-phthalyl-DL-methionine (XII), *N*-*tert*-butyloxycarbonyl-L-methionine dicyclohexyl ammonium salt (XIII), DL-homocysteine thiolactone hydrochloride (XIV), *N*-propionyl-DL-methionine (XVII), *N*-*tert*-butyloxycarbonyl-L-methionine *p*-nitrophenyl ester (XX), and *N*-carbobenzoxy-DL-methionine (XXII), did not support *in vitro* cellulose digestion. The rest of the methionine analogs exhibited a wide range in ability to support *in vitro* cellulose fermentation (Table I).

Examination of the structural characteristics of the 31 methionine derivatives tested for resistance to rumen microbial degradation, evidenced by increased cellulose digestion *in vitro*, revealed the following:

1. Various acyl groups, by blockade of the amino function, may or may not have the ability to impart desirable resistance.
2. Esterification of the methionine carboxyl function does not appear to give products with enhanced resistance.
3. Conversion of the thioether linkage of methionine to sulfoxide, sulfone, or a methylsulfonium salt does not seem to increase resistance.

Belasco (30) showed that excellent bacterial growth resulted in the *in vitro* artificial rumen fermentation test, as indicated by high levels of cellulose digestion, when the medium was provided with certain nitrogenous compounds. More recently, Acord *et al.* (18, 31) found that arginine, glutamic acid, lysine, methionine, serine, and valine were poorly utilized as single nitrogen sources for *in vitro* starch digestion by mixed rumen bacteria. Aspartic acid was found to be inferior to urea as a nitrogen source for the microorganisms. However, three-way and four-way combinations of urea, valine, aspartic acid, and lysine significantly increased starch digestion. These results show that enrichment of the fermentation medium with a combination of various nitrogenous sources results in enhanced microbial growth with subsequently increased cellulose or starch digestion. It, therefore, follows that compounds that appear to be degraded under artificial rumen conditions, especially in the absence of other nitrogenous sources, would almost certainly be degraded in the rumen, in which a great variety of nitrogenous substances, enzymes, and protozoa is present. Thus, potential methionine substitutes supporting cellulose digestion were discarded (Table I). Conversely, a compound's apparent resistance to *in vitro* degradation, as indicated by the extent of cellulolytic activity of the rumen microorganisms, should not be considered as an indicator of resistance to rumen degradation. Furthermore, lack of ability of a compound to support cellulose digestion is not unequivocable proof of resistance to microbial attack. Thus, further evaluation of the stability of

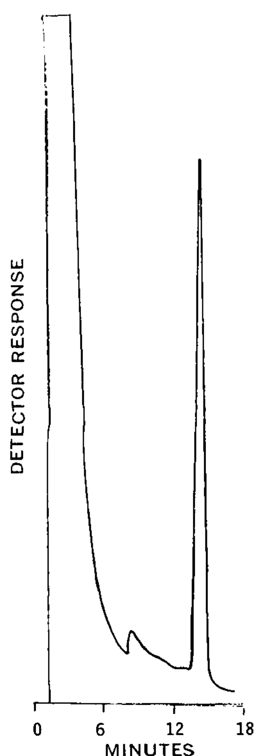


Figure 1—*GLC chromatogram obtained with a 1-μl sample from a solution of XIV in fermentation fluid (5 mg/ml). Compound XIV was silylated with* N,O-*bis(trimethylsilyl)trifluoroacetamide and its chromatography with a 1.52-m × 0.31-cm (5-ft × 0.125-in.) OV-17 column was performed under the following conditions: column temperature, 125°; injection block temperature, 225°; detector temperature, 250°; nitrogen flow rate, 20 ml/min; hydrogen flow rate, 30 ml/min; and air flow rate, 300 ml/min. The retention time of XIV was 15 min. The electrometer attenuation was at ×32, and the chart speed was kept at 0.17 in./min.*

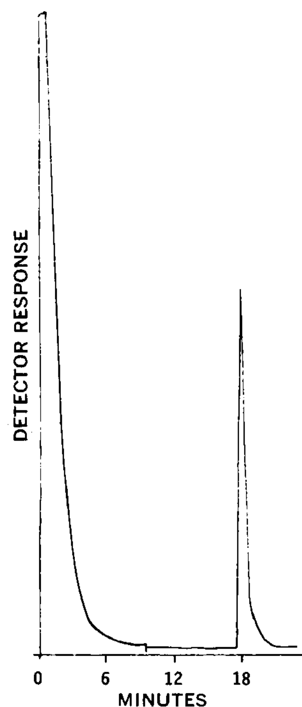


Figure 2—*GLC chromatogram obtained with a 1-μl sample from a solution of IV in fermentation fluid (0.5 mg/ml). Compound IV was silylated with* N,O-*bis(trimethylsilyl)acetamide and its chromatography with a 1.52-m × 0.31-cm (5-ft × 0.125-in.) OV-17 column was performed under the following conditions: column temperature, 155°; injection block temperature, 250°; detector temperature, 275°; nitrogen flow rate, 20 ml/min; hydrogen flow rate, 30 ml/min; and air flow rate, 300 ml/min. The retention time of IV was 19.0 min. The electrometer attenuation was at ×64, and the chart speed was kept at 0.17 in./min.*

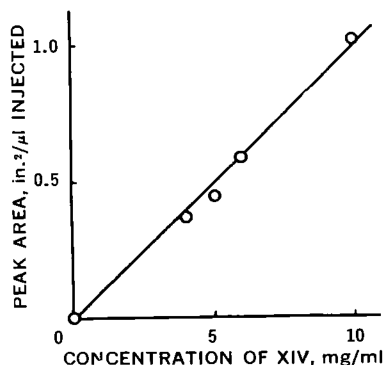


Figure 3—*Calibration curve for the GLC assay of XIV in fermentation fluid.*

these compounds is necessary before animal experimentation begins.

Of the 10 compounds that appeared to exhibit a considerable degree of resistance to microbial attack (Table I), IV and XIV were chosen for further testing. These choices were made because the compounds fulfilled the following criteria: (*a*) both compounds are relatively easily synthesized, (*b*) they are commercially available and relatively inexpensive, an important criterion in view of the large quantities involved in cattle feeds; and (*c*) IV has been shown to replace methionine in the diet of growing chicks (32). Compound XIV is well known. Its synthesis has long been reported (33), and the compound has been disclosed as a pharmaceutical. Compound XIV exhibits protection against irradiation and nitrogen mustards (34) and detoxification against tetanus toxin (35). The compound supports growth of methionine-dependent mutants of *Pseudomonas tabaci* (36). Homogenized, lyophilized, or sliced rat liver methylates XIV to methionine (37). *In vivo* experiments on young rats fed a diet of highly purified amino acids showed synthesis of methionine from XIV if the diet is properly constituted otherwise (38). This fact has been verified in these laboratories, and the details of this study will be reported.

More information on the stability of IV and XIV under the *in vitro* fermentation conditions was obtained from the ammonia release experiment. The data in Table III show net increases in ammonia concentrations over control fermentations with no nitrogen added. Such values represent a balance between the release of ammonia from nitrogenous compounds and the use of ammonia by active microorganisms. Total release of ammonia from the experimental compounds used alone without any uptake by microorganisms would have yielded an ammonia concentration of 0.46 mg/ml. With urea added, the potential concentration became 0.92 mg/ml (0.46 mg/ml from the experimental compound plus 0.46 mg/ml from urea). Decomposition of proteins from dead microorganisms would provide additional potential for ammonia release. Ammonia uptake varies greatly with activity of the microbial population. Consequently, instantaneous ammonia concentrations may provide indications of compound stability but are subject to considerable variation and are by nature likely to be somewhat inconclusive. The data for single additions of XIV and IV provide little indication of ammonia release from either compound. This observation is consistent with the lack of support of cellulose digestion by these compounds in previous experiments. Assuming no ammonia uptake and no other source of ammonia, it can be calculated that 0.0, 7.8, and 62.0% of XIV, IV, and homocysteine, respectively, were degraded. When urea was added, there was considerable evidence of compound degradation (levels above the 0.46-mg/ml potential contribution of urea), and a suggestion of ammonia release from bacterial protein (levels above the 0.92-mg/ml potential contribution of the added nitrogen source) in the *N*-acetyl methionine treatment. Similar degradation might have occurred on other treatments without being detected due to the masking effect of factors such as use of ammonia by the microorganisms.

Because of the above-mentioned difficulties with the ammonia release experiment, more definitive data, on the stability of IV and XIV toward microbial attack, were sought. Thus, GLC assays were developed for both in aqueous solutions and fermenta-

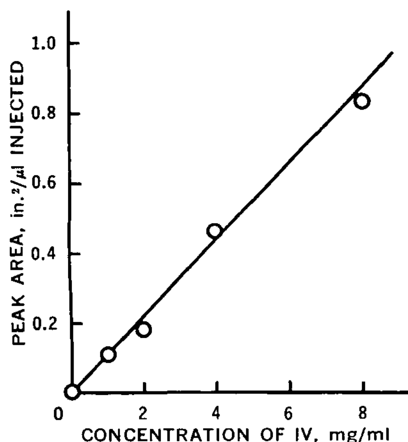


Figure 4—*Calibration curve for GLC assay of IV in fermentation fluid.*

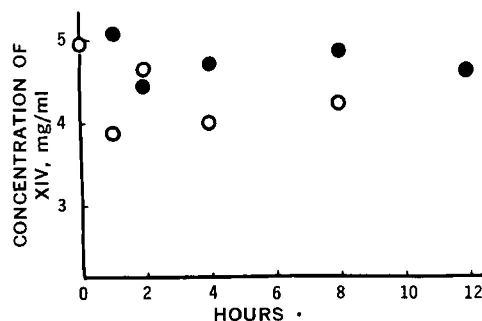


Figure 5—In vitro *microbial stability of XIV. Key:* ○, *4.95 mg XIV/ml fermentation fluid; and* ●, *4.05 mg XIV and 0.99 mg urea/ml fermentation medium.*

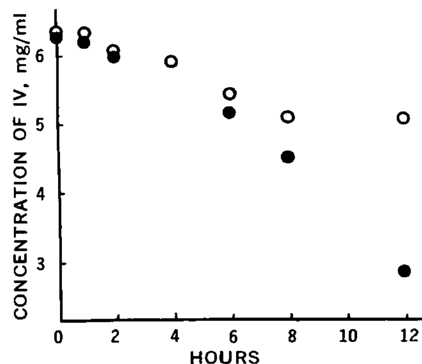


Figure 6—In vitro *microbial destruction of IV. Key:* ○, *6.36 mg IV/ml fermentation fluid; and* ●, *6.36 mg IV and 0.99 mg urea/ml fermentation medium.*

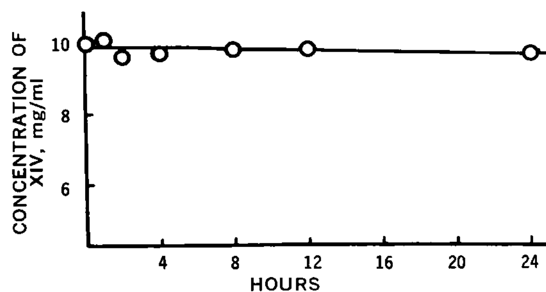


Figure 7—In vitro *stability of XIV at pH 2.3 (phthalate buffer) at 50°.*

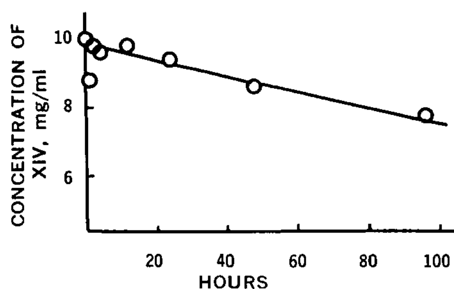


Figure 8—*In* vitro *stability of XIV at pH 6.8 (phosphate buffer) at 39°.*

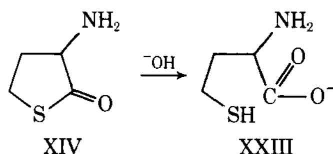
tion media. The compounds exhibited sharp, symmetrical peaks in their GLC chromatograms after silylation in pyridine (Figs. 1 and 2). Figure 1 shows a GLC chromatogram obtained from a 1-μl aliquot of a solution containing 5 mg XIV/ml fermentation fluid.

Calibration curves were obtained with various solutions of IV and XIV in aqueous solutions and fermentation media. These were found to be linear over the range of 0.5–10 mg/ml of solution for both compounds (Figs. 3 and 4). No attempt was made to determine the lowest possible sensitivity limits of the GLC assays since the concentrations of XIV and IV in the fermentation fluid were 4.95 and 6.36 mg/ml, respectively, at the start of the experiment and since good linearity was obtained with their calibration curves around these concentrations.

The extent of microbial destruction of XIV was followed by analyzing for the compound in the fermentation fluid by GLC. Compound XIV was highly stable in either the presence or the absence of urea (Fig. 5). Likewise, IV was fairly stable in the fermentation medium that contained no urea. However, when 0.99 mg of urea was added per milliliter of the medium as a nitrogen source, a considerable destruction of IV was observed (Fig. 6). This is not too surprising in view of what has been discussed earlier on the effect of nitrogen-containing substances on bacterial growth.

The potential of XIV as a methionine substitute for ruminants was further investigated by studying its *in vitro* chemical stability at pH 2.3 and 6.8. These studies were conducted by analyzing for XIV using the GLC assay. Figure 6 shows that XIV is stable at pH 2.3, even at 50°, suggesting that the compound would survive degradation due to pH in the abomasum. The stability of XIV at pH 6.8 and 39° is shown in Fig. 8. These data (Figs. 7 and 8) suggest that XIV is fairly stable at pH 2.3 and 6.8. However, the stability of the compound appears to decrease as the pH of the solution is increased, and this finding is in agreement with what is known about the stability of thiolactones. These are cyclic thioesters that are known to be hydrolyzed in basic media (42–44). Thus, XIV is expected to yield XXIII in an alkaline medium (Scheme I). The conversion of XIV to XXIII is probably catalyzed by esterolytic enzymes. This is substantiated by the fact that homogenized rat liver converts XIV to VI (37). This is probably achieved by conversion of XIV to XXIII, with subsequent methylation to produce VI.

The chemical characteristics of XIV suggest that it could be considered as a potential precursor "prodrug" of methionine in ruminants (45, 46). This means that XIV and its analogs could perhaps pass through the rumen and abomasum unchanged and subsequently be absorbed from the intestines where most absorption occurs. After absorption, the compound could be biotransformed to methionine as previously mentioned. Encouraging preliminary results on the *in situ* absorption of XIV in rats were recently obtained. By using the *in situ* rat gut absorption studies described by Doluisio *et al.* (47), it was possible to show that the compound is absorbed more readily than VI, exhibiting a half-life of absorption of about 20 min. Details on these findings will be reported.



Scheme I

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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 (I*a*) was found to be highly stable toward microbial destruction. Since an α-hydroxy analog of methionine (II*a*) 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 (I*a*).