Effect on Rumen Bacteria of Methionine Hydroxy Analog and Sulfur-Containing Amino Acids, *in Vitro*[1] (37091)

L. A. Gil, R. L. Shirley, J. E. Moore, and J. F. Easley

Department of Animal Science, University of Florida, Gainesville, Florida 32601

When DL-methionine was substituted for an isonitrogenous amount of urea in a semi-purified diet for lambs, Barth *et al.* (1), and McLaren, Anderson and Barth (2) showed that nitrogen retention was significantly increased. In the study by Polan, Chandler and Miller (3) with lactating cows fed high urea diets, methionine hydroxy analog (MHA) not only improved nitrogen retention but also the digestibility of crude fiber. Salsbury *et al.* (4) demonstrated that MHA acted like methionine in preventing inhibition of cellulose digestion by ethionine. Gil and Shirley (5, 6) showed that MHA increased the rate of glucose utilization and the synthesis of bacterial N (crude protein) and dry matter (DM).

The present studies were conducted to help clarify: (a) The effect of MHA on the logarithmic rate of growth and the lag phase, or both; (b) the extent that MHA could have a reducing effect making anaerobiosis of the media more complete; (c) the molecular components of MHA responsible for rumen bacterial growth stimulation; (d) similarities of other organic and inorganic sources of sulfur in their effect on growth of rumen bacteria, and (e) extent that other amino acids stimulate bacterial protein synthesis and growth.

Materials and Methods. In vitro culture of rumen bacteria in the absence of protozoa were carried out using 1 vol of 160 ml of culture consisting of 128.6 ml of artificial saliva (7), 7 ml of valeric acid (5 μg/ml), 0.7 of PABA (50 μg/ml), 2.8 ml of biotin (10 μg/ml), 1 ml of urea solution (126 mg/ml), and 20 ml of inoculum in each fermentation bottle. The medium was saturated with CO_2 and the pH adjusted to 6.9 with Na_2CO_3. One gram of glucose was placed into each bottle as the energy providing substrate. Carbon dioxide was bubbled through the media continuously. Once the temperature, pH and CO_2 saturation were attained, each bottle was inoculated with 20 ml of centrifuged (250 RCF for 10 min) supernatant rumen fluid. The rumen fluid was collected prior to the morning feeding from a fistulated steer fed moderate quality grass hay, 1.0 kg soybean meal and trace mineralized salt, plus vitamins A, D and E. The rumen fluid was immediately strained through four layers of cheese cloth, collected under carbon dioxide in a bottle, tightly capped and centrifuged within the following 5 min.

Duplicate aliquots of 20 ml were withdrawn at scheduled times from the bottles during fermentation and transferred to weighed centrifuge tubes. Proteins were precipitated with tungstic acid and centrifugation at 2000 RCF for 20 min (8) and dried in a vacuum at 60° prior to gravimetric bacterial DM and bacterial N determination by the micro-Kjeldahl (9) or the phenol–nitroprusside (10) methods. Samples of supernatant solutions were kept refrigerated until glucose (11) and ammonia (10) determinations were made.

Results. Logarithmic growth rate and lag phase. The treatment group consisted of four fermentation bottles containing 0.025 mmoles of MHA/160 ml of medium. These and an equal number of control bottles had aliquots removed every 0.5 hr for 8 hr.

Values for bacterial DM indicates that the MHA acted by increasing the logarithmic growth rate and had very little effect on the lag phase (Fig. 1). However, the values for

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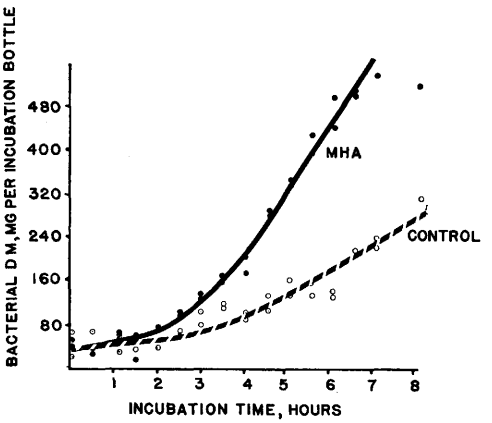

FIG. 1. Rumen bacterial dry matter production rate in medium containing glucose and urea, with and without methionine hydroxy analog (MHA).

bacterial N (Fig. 2) indicate that the MHA was a stimulant during the lag phase as there was almost a doubling of the bacterial N during the first 2 hr of fermentation.

Considerable disappearance of glucose (Fig. 3) was apparent during the 2-hr lag phase of bacterial growth in both the MHA and control tubes. However, the rate of disappearance was greatly increased during the accelerated growth phase in case of the MHA supplemented tubes. All the glucose was exhausted in the tubes with MHA after 6.5 hr of fermentation when approximately half the glucose was still present in the control tubes.

Ammonia N in both MHA and control media (Fig. 4) increased linearly immediately from the start of fermentation and increased at the same rate during the lag phase (first 2 hr). Ammonia in the medium with MHA reached a maximum by 4 hr, after which time a decrease began. The decrease was concurrent with the higher bacterial growth rate taking place in the MHA media. The ammonia concentration in the control medium continued to increase after 4 hr of fermentation but at a slower rate. This was a likely consequence of the slower bacterial growth in the control medium.

Redox potential of MHA in relation to anaerobic rumen bacterial medium. The possibility that MHA would increase the growth rate of the anaerobic rumen bacteria by acting as a reducing agent was tested. Comparisons of relative redox potentials were made using cysteine–cystine, $E^0 = -0.140$ V at pH 7.0 and 30° as the reference system and methylene blue, $E^0 = -0.011$ V at pH 7 and at 30° as redox indicator. The dye is colored at redox potentials above its E^0 and colorless when at lower (more negative) potentials.

Twenty milliliters of fresh centrifuged (250 RCF for 10 min) rumen fluid and equal molar concentrations of cysteine, MHA, or DL-methionine were added to the culture medium; and their redox power measured with a Beckman potentiometer fitted with a pair of platinum–glass electrodes. Culture medium inoculated with bacteria caused the most negative shift of the needle; cysteine when added to the medium without bacteria resulted in a less negative shift, and MHA and methionine gave an almost imperceptible shift. This indicates that bacteria in culture media develop a lower redox potential than added cysteine, MHA or methionine.

A second approach was made by adding a few drops of 0.4% methylene blue solution to culture medium (no bacteria) equilibrated with carbon dioxide, with and without MHA, at pH 6.9 and 39°. No color change occurred in any of the bottles after 0.5 hr of incubation. Then 20 ml of inoculum were added to 140 ml of medium in each bottle and fermentation was continued. The blue color dis-

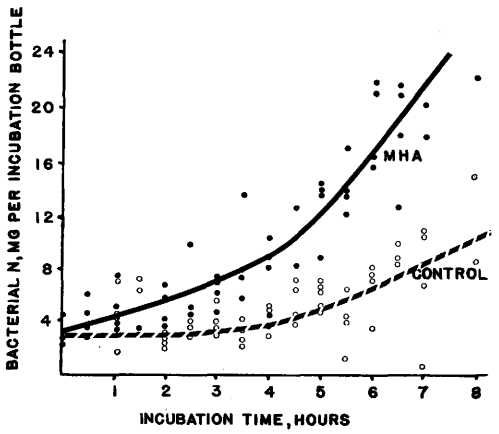

FIG. 2. Rumen bacterial nitrogen production rate in medium containing glucose and urea, with and without methionine hydroxy analog (MHA).

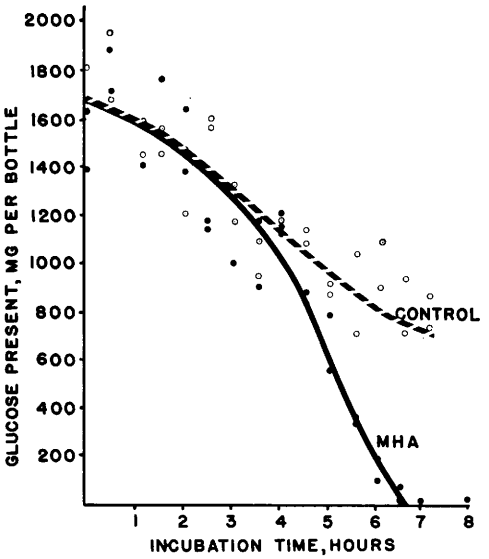

FIG. 3. Glucose utilization by rumen bacteria in media with urea, with and without methionine hydroxy analog (MHA).

appeared from both the MHA supplemented and unsupplemented media in an average time of 4.5 min after inoculation with the bacteria. This indicated that the bacterial activity generates a redox potential lower (more negative) than $E^0 = -0.011$ V. It may be concluded that MHA probably does not exert a reductive action in the media inoculated with rumen bacteria since the activity of the microbes generate a lower E^0 than the redox potential of MHA.

MHA functional groups effective in microbial growth stimulation. The free acid form of MHA (MHA-H) was prepared by precipitating the Ca in MHA as Ca-oxalate from an acid solution. The identity of the resulting substance was established by nuclear magnetic resonance spectroscopy of an aqueous solution of MHA-H. If –OH were essential, MHA and MHA-H, but not methionine would increase growth rate. If the Ca form were essential, only MHA would enhance growth. If none of the above were essential, all three substances would likely give the same response.

The initial concentration of MHA, MHA-H and methionine were 0.1% of the total media. Results are presented for 0, 3, 4.5, 7 and 10 hr of fermentation in Table I. These data indicated that the –OH group or the Ca in MHA were not essential to increase rumen bacterial growth rate. Increase in DM and crude protein were quite linear with time for all four treatments up to 7 hr of fermentation. At this time, apparently, all the glucose was exhausted except in the control group where fermentation continued. The rates of production of bacterial DM and crude protein during the logarithmic phase with MHA, MHA-H, and methionine in the media were approximately double that of the control ($p < .01$). At 3, 4.5 and 7 hr, the three supplemented groups were not significantly different from one another; which indicated that the hydroxyl group or calcium in the MHA were not factors in its growth stimulation.

Rumen bacterial growth stimulation by various sources of sulfur in addition to MHA. A study was made to determine if the increased growth rate of rumen bacteria utilizing glucose in the presence of MHA was due to the extra sulfur added to the media as MHA by testing if other sources of sulfur could produce the same effect. MHA, methionine, cysteine–HCl, $MgSO_4$, Na_2SO_4 and Na_2SO_3 were added in amounts equivalent to 10 μg of sulfur/incubation bottle.

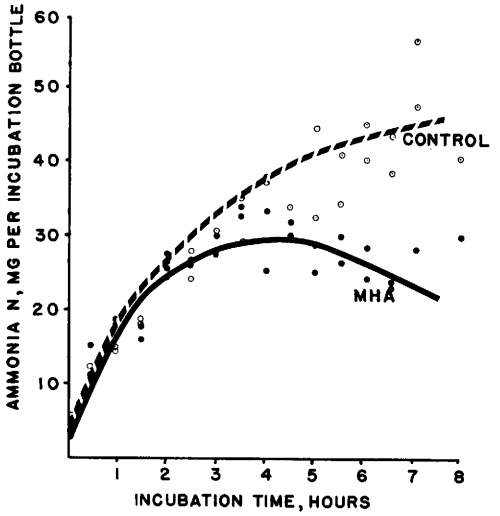

FIG. 4. Ammonia from urea as function of incubation time with rumen bacteria in medium that also contained glucose, with and without methionine hydroxy analog (MHA).

TABLE I. Effect of $(MHA)_2$-Ca, MHA-H, and DL-Methionine on *in Vitro* Synthesis of Rumen Bacterial Dry Matter (DM) and Nitrogen (N) and Glucose Disappearance at Increasing Fermentation Times.[a,b]

Incubation time (hr)	Control			$(MHA)_2$-Ca			MHA-H			DL-Methionine		
	DM	N	%Gl[c]	DM	N	%Gl[c]	DM	N	%Gl[c]	DM	N	%Gl[c]
0	228	8	0	226	8	0	222	8	0	218	8	0
3	287	—	11	502	14	23	447	14	24	483	5	28
4.5	506	13	18	829	26	69	901	25	78	987	30	85
7	739	21	42	915	30	96	1002	32	97	1002	30	98
10	1080	34	96	889	30	98	968	28	98	960	25	99

[a] Each value is an average of three determinations; DM and N expressed as milligrams per 160 ml medium.

[b] Significance: Controls $<$ $(MHA)_2Ca$ = MHA-H = Methionine ($p < .01$) at 3, 4.5 and 7 hr of incubation for DM, N and glucose.

[c] %Gl, percentage glucose disappearance.

Aliquots were taken at 0 and 4.5 hr of fermentation. The data obtained are summarized in Table II.

MHA and the two sulfur containing amino acids resulted in bacterial DM and bacterial N production two or three times greater than occurred in the bottles that had equal amounts of S as inorganic salts. Glucose was utilized in amounts relative to the DM and crude protein synthesized. The three inorganic sources of sulfur did not differ significantly ($p < .05$) from the control values in any of the responses measured.

Comparison of 18 amino acids and MHA on growth of rumen bacteria. Eighteen amino acids and MHA were tested for their effect on bacterial DM and bacterial N production as well as glucose consumption during 4.5 hr of fermentation. Each amino acid was tested separately at a concentration of 0.050 mmoles/bottle. MHA was added at the level of 0.025 mmoles of the Ca salt.

TABLE II. Effect of Added Organic and Inorganic Sources of Sulfur[a] on Bacterial Dry Matter (DM) and Bacterial N Production and Glucose Utilization, at 4.5 hr of Fermentation.[b]

	mg/160 ml medium		
	DM[a]	N[c]	Glucose[c]
Control	178	8.2	1000
$Na_2S_2O_3$	171	8.3	1120
Na_2SO_4	169	7.9	924
$MgSO_4$	151	8.2	1156
DL-Met	465	21.6	392
Cysteine	570	26.3	216
MHA	362	15.5	528

[a] Concentration of treatment substances are equivalent to 10 μg of S/bottle.

[b] Each value is the average of four determinations.

[c] Initial concentrations of DM, N and glucose were 85 $\pm$ 6, 4.1 $\pm$ 1 and 1002 $\pm$ 180 mg/incubation bottle, respectively.

Means of two determinations for each amino acid and four determinations for the control are presented in Table III. The sulfur-containing amino acids and MHA increased bacterial DM and bacterial N to approximately 1.7 times more the control. The other amino acids resulted generally in a slightly higher production of bacterial DM and crude protein. This increase of metabolic activity was also indicated by an increased glucose utilization.

Discussion. Most of the nonsulfur-containing amino acids had a slight stimulation on growth of cells utilizing glucose. Probably, the energy spared in the synthesis of an amino acid can be diverted to other metabolic activities of growth. Only the sulfur-containing amino acids caused growth to proceed at approximately 1.7 times the rate of unsupplemented bacteria. The growth stimulation indicated that the conversion of inorganic forms of sulfur to sulfur-containing amino acids was a slow process that limits the potential of protein synthesis and, therefore, rumen bacterial growth. It may be that MHA can be rapidly aminated to methionine by rumen bacteria and incorporated into protein. MHA and methionine had a similar effect on growth

TABLE III. Effect of Amino Acids and MHA (25 mmole/160 ml) on Rumen Bacterial DM and Bacterial N Production and Glucose Utilization at 4.5 hr of Fermentation.[a]

Item	mg/160 ml medium		
	DM	N	Glucose
Control	435	18.4	792
Val	467	19.6	499
Phe	445	9.0	738
Ala	447	20.3	577
Ser	470	20.3	558
Gly	500	21.2	574
Pro	416	20.6	617
Leu	469	21.6	535
Thr	455	20.5	596
Ileu	434	19.3	802
Arg	492	20.8	574
Asn	501	20.9	717
Asp	482	21.0	689
Lys	481	20.5	613
Tyr	487	21.9	638
His	498	21.0	796
Cys	708	29.0	210
Met	746	31.7	66
Cystine	738	34.2	120
MHA	725	33.0	138

[a] The initial DM, N and glucose concentrations were 216 ± 9, 9.4 ± 2 and 1068 ± 146 mg/160 ml medium, respectively.

in the short fermentation periods with glucose reported in the present study. Rumen bacteria seem to have the capacity to convert any of the S-containing amino acids into the others very rapidly as demonstrated by the growth stimulation caused by any of the S-amino acids and MHA.

Summary. Fermentation of glucose by rumen bacteria utilizing urea as the only source of nitrogen was carried out with or without MHA. The logarithmic growth rate was observed to increase about 2.5 times due to MHA. The lag phase was markedly stimulated in regard to bacterial N synthesis but not to total bacterial dry matter production. Bacterial activity generated a more negative redox potential than MHA making it improbable that MHA functioned as a reducing agent aiding in the anaerobiosis of the medium. The free hydroxyl group or the Ca salt of MHA were not essential for the stimulatory effect of MHA on bacterial growth. Inorganic sources of sulfur or amino acids other than the sulfur-containing amino acids did not have a growth-stimulating effect as measured at 4.5 hr of fermentation. Cystine, cysteine and methionine individually stimulated bacterial growth similar to that of MHA. These observations indicated that any of the sulfur-containing amino acids or MHA can be rapidly converted to the other sulfur-containing amino acids, but the synthesis of the sulfur-containing amino acids from inorganic sulfur is a rate-limiting process for growth of rumen bacteria metabolizing a readily available carbohydrate.

1. Barth, K. M., McLaren, G. A., Anderson, G. C., Welch, J. A., and Smith, G. S., J. Anim. Sci. **18**, 1521 (1959).

2. McLaren, G. A., Anderson, G. C., and Barth, K. M., J. Anim. Sci. **24**, 231 (1965).

3. Polan, C. E., Chandler, P. T., and Miller, C. N., J. Dairy Sci. **53**, 607 (1970).

4. Salsburg, R. L., Marvin, D. K., Woodmansee, C. W., and Henlein, G. F. W., J. Dairy Sci. **54**, 390 (1971).

5. Gil, A., and Shirley, R. L., J. Anim. Sci. **32**, 12 (Abstr.) (1971).

6. Gil, A., and Shirley, R. L., J. Anim. Sci. **34**, 359 (Abstr.) (1972).

7. McDougall, E. I., Biochem. J. **43**, 99 (1948).

8. Shultz, T. A., and Shultz, E., J. Dairy Sci. **53**, 781 (1970).

9. Kaplan, A., Methods Clin. Chem. **5**, 245 (1965).

10. Kirk, P. L., Anal. Chem. **24**, 1806 (1950).

11. Hoffman, W. S., J. Biol. Chem. **120**, 51 (1937).

12. Kjeldgaard, N. O., Maaloe, O., and Schaechter, M., J. Gen. Microbiol. **19**, 607 (1958).

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