

# Metabolism of Glycine by Rumen Microorganisms

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## ABSTRACT

Rumen microorganisms rapidly metabolized glycine at rates varying from 0.014 to 0.241  $\mu$ mole of glycine per ml per min. The main metabolic products were carbon dioxide, acetic acid, and ammonia; little glycine was incorporated into bacterial protein. Use of carboxyl or methylene-labeled glycine showed that the carbon dioxide came mainly from the carboxyl of glycine, whereas both carbons of acetic acid were derived partly from the methylene carbon of glycine and partly from carbon dioxide. The ratio of carbon-14 to nitrogen-15 in glycine isolated from the protein of rumen bacteria incubated in the presence of  $N^{15}$ - and  $C^{14}$ -labeled glycine indicated that most of the extracellular glycine incorporated into protein was incorporated without intervening deamination.

Studies on the metabolism of amino acids by rumen microorganisms have demonstrated the conversion of amino acids to ammonia, carbon dioxide, volatile fatty acids, and microbial protein (1, 5-8, 11, 15, 16; A. V. Portugal, Ph.D. Thesis, Univ. Aberdeen, Aberdeen, Scotland, 1963). With the exception of Portugal's work, most of these studies have used nonphysiological conditions involving washed suspensions or pure cultures of rumen bacteria, high substrate concentrations, or extended incubation times. Since amino acids are present in rumen contents only in low concentrations (1, 4, 21; Portugal, Ph.D. Thesis, 1963), quantitative data on the metabolism of amino acids will be meaningful only if experimental conditions are similar to those existing *in vivo*.

Many rumen bacteria preferentially use ammonia rather than extracellular amino acids for the synthesis of microbial protein (18; A. C. I. Warner, Ph.D. Thesis, Univ. Aberdeen, Aberdeen, Scotland, 1955). Portugal and Sutherland (13) found that aspartic and glutamic acids were largely degraded to carbon dioxide and volatile fatty acids, with only a small incorporation into bacterial protein. This supports the idea that most of the amino acids in rumen microbial protein are synthesized *de novo*.

Studies on the metabolism of glycine are reported in this paper. This amino acid was selected for several reasons. It occurs in rumen fluid in measurable concentrations (19; Portugal, Ph.D. Thesis, 1963), the availability of both carbon-14

and nitrogen-15 labels permits comparisons between the fate of the carbon and of the nitrogen, and metabolic studies (16) using glycine incubated with washed suspensions of rumen bacteria allow comparisons with results of the present study, in which whole rumen contents were used rather than the liquid alone.

## MATERIALS AND METHODS

*Preparation of samples.* Rumen contents were collected from a rumen-fistulated Holstein cow fed once daily on alfalfa hay. Samples of rumen liquor were incubated at 39 C under  $O_2$ -free  $CO_2$  or  $N_2$ . Radioactive solutions were added by injecting them through rubber enclosures. Metabolism was stopped by adding 0.05 volume of 10 N  $H_2SO_4$ . In some experiments,  $CO_2$  was collected by bubbling  $N_2$  through the acidified sample and collecting the  $CO_2$  in 1 M Hyamine. After acidification, samples were centrifuged at  $600 \times g$  for 2 min to remove protozoa and plant material. A portion of the supernatant liquid was centrifuged at  $20,000 \times g$  for 30 min at 2 C and the deposited bacteria were washed with 0.9% NaCl and extracted by the method of Roberts et al. (14). The bacterial protein was uniformly suspended in water, and samples were counted or hydrolyzed in a sealed tube with 6 N HCl for 16 hr at 110 C. Portions of the clarified rumen fluid were taken for estimating glycine and volatile fatty acids.

*Pool size measurements.* The difficulty in obtaining accurate measurements of the concentrations of amino acids in extracellular fluid are discussed in the preceding paper (19). The technique of adding 0.05 volume of 10 N  $H_2SO_4$  to kill rumen microorganisms has been used in these experiments since it was not possible to use the ultrafiltration technique with small volumes (15 to 20 ml) and brief incubation times (15 to 240 sec). The total content of glycine

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was estimated by sampling rumen contents acidified at zero-time, with the understanding that the values were too high but could be corrected. Carrier glycine was added to each acidified sample. The amino acids were converted to their 2,4-dinitrophenyl (DNP) derivatives and DNP-glycine was isolated by chromatography (10, 19; Portugal, Ph.D. Thesis, 1963) for counting.

**Chromatography.** Volatile fatty acids collected from the steam distillation of rumen fluid were separated on Celite at pH 6.5 (3). Amino acids were separated by paper chromatography with butanol-acetic acid-water (100:21:50, v/v) and phenol-water (100:39, v/v). A 0.1% solution of ninhydrin in acetone containing 2% collidine was used to locate the amino acids on the chromatograms. Glutamic acid and glycine were isolated from hydrolysates of bacterial protein on a column (55 by 2.5 cm) of Dowex-50 X12 washed with 1.5 N HCl and 2.5 N HCl (17).

Ammonia was collected by steam distillation into boric acid containing a mixed indicator of methyl red and bromocresol green and titration with standard HCl. Analyses for nitrogen-15 were kindly done by S. St. John of the Kearney Foundation for Soil Science.

The distribution of carbon-14 in acetic acid was determined by the Schmidt degradation method (9). Radioactivity was estimated by standard liquid scintillation techniques. Glycine- $U\text{-}C^{14}$ , glycine- $1\text{-}C^{14}$ , and glycine- $2\text{-}C^{14}$  were obtained from the New England Nuclear Corp., Boston, Mass. Glycine- $N^{15}$  (97 atoms % excess) was a product of Isomet Corp., Palisades Park, N.J.

## RESULTS

A preliminary experiment was run to determine the approximate rate of glycine metabolism and whether carbon dioxide was a metabolic product. The results in Table 1 show that carbon dioxide and volatile fatty acids were labeled, together accounting for 47% of the added radioactivity. Under the conditions in this experiment, nearly 60% of the added glycine- $U\text{-}C^{14}$  was utilized in 120 sec; about 12% appeared in the bacterial cells.

The rate of glycine metabolism was determined

TABLE 1. *Metabolism of glycine- $U\text{-}C^{14}$  by rumen contents<sup>a</sup>*

Product	Radioactivity	Percentage of added radioactivity
	<i>counts/min</i>	
CO <sub>2</sub> .....	690,000	25
Volatile fatty acids....	610,000	22
Bacteria.....	344,600	12

<sup>a</sup> Rumen contents were collected 2 hr after feeding. The sample (16.6 g) was incubated with 0.2 ml of glycine- $U\text{-}C^{14}$  solution (2  $\mu$ g,  $2.7 \times 10^6$  counts per min) for 120 sec.

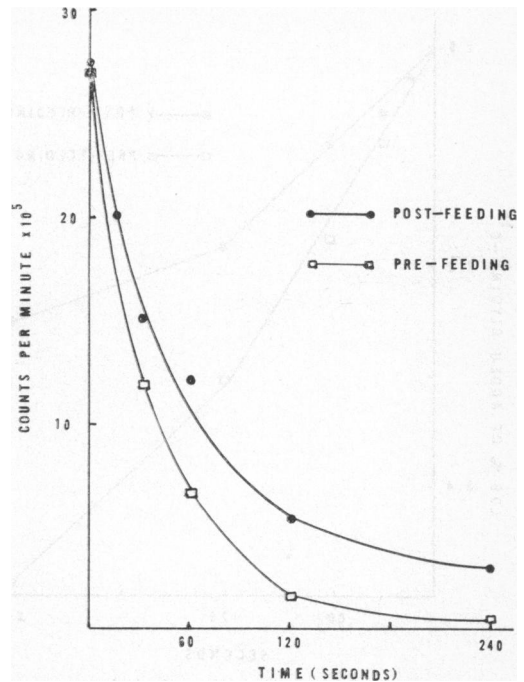


FIG. 1. *Disappearance of glycine- $U\text{-}C^{14}$  from rumen contents.*

in samples of rumen contents collected before feeding and 2 hr after feeding (Fig. 1). Glycine disappeared more rapidly in the prefeeding samples than in the postfeeding samples. By plotting the logarithms of the percentage of radioactive glycine remaining (Fig. 2), rate constants for glycine utilization were calculated. The plots in both experiments were nearly linear up to 120 sec. For the prefeeding sample, the 60-sec  $K$  value was 1.36/min and the 120-sec  $K$  value was 1.41/min; for the postfeeding sample, the 60-sec  $K$  value was 0.81/min and the 120-sec  $K$  value was 0.84/min. Pool size measurements gave values of 0.025 and 0.110  $\mu$ mole/ml, respectively, in the pre- and postfeeding samples. If the extracellular pool was 0.030/0.053 of the total glycine (19; from the average value), the turnover rates for glycine in these two samples were 0.014 and 0.038  $\mu$ mole of glycine per ml per min, respectively.

The conversion of glycine- $U\text{-}C^{14}$  to bacterial protein and volatile fatty acids is shown in Fig. 3. Nearly one-third of the added label was found in the volatile fatty acid fraction. After 240 sec, the prefeeding sample contained 1% of the label in the microbial protein and the postfeeding sample, 3.4%.

The distribution of radioactivity in the volatile fatty acids was found by chromatography. Acetic

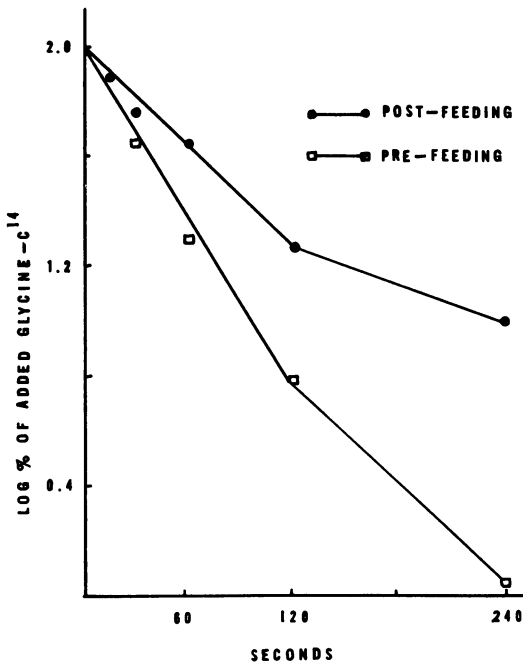


FIG. 2. Radioactivity in residual glycine.

acid contained nearly 90% of the label in this fraction (Table 2).

By paper chromatography of amino acids in the rumen fluid or in acid-hydrolyzed bacterial protein, it was found that glycine was the only radioactive amino acid in the extracellular fluid, whereas, in the protein, glycine contained about 90% of the label, with nearly 10% in serine and a trace in alanine. These results were confirmed by fractionating hydrolysates of protein on Dowex-50 X12 and counting the fractions.

The fate of the carboxyl and methylene carbons of glycine was determined by adding glycine-1-C<sup>14</sup> or glycine-2-C<sup>14</sup> to samples of rumen contents collected on successive days. Table 3 shows that a greater proportion of the glycine-1-C<sup>14</sup> than of the glycine-2-C<sup>14</sup> was used. Since the latter compound had a lower specific activity, it was added in nearly twice the concentration of the glycine-1-C<sup>14</sup>.

The carboxyl carbon was converted mainly to CO<sub>2</sub> (71%), with 8.1% in the volatile fatty acids and 12.4% in the bacteria. The methylene carbon from glycine-2-C<sup>14</sup> was recovered in the volatile acids (43.2%) and in the bacteria (19.4%), with only a trace (0.6%) in the carbon dioxide. Although most of the carbon-14 in the bacteria was found in the protein, the lipid fraction was also heavily labeled.

Schmidt degradation of acetic acid formed from glycine-1-C<sup>14</sup> showed that 82.8% of the label in acetic acid from glycine-1-C<sup>14</sup> was in the carboxyl

and 17.2% in the methylene. The acetic acid from glycine-2-C<sup>14</sup> contained 43.5% of its radioactivity in the carboxyl and 56.5% in the methylene.

These experiments show the extensive degradation of glycine to acetic acid and carbon dioxide with a small incorporation into bacterial protein. Although the concentration of the carbon-14 chiefly in glycine suggests that this amino acid arises from extracellular labeled glycine, it is possible that it arises by de novo synthesis of glycine from a two- or three-carbon pool formed from the metabolism of radioactive glycine. If this were so

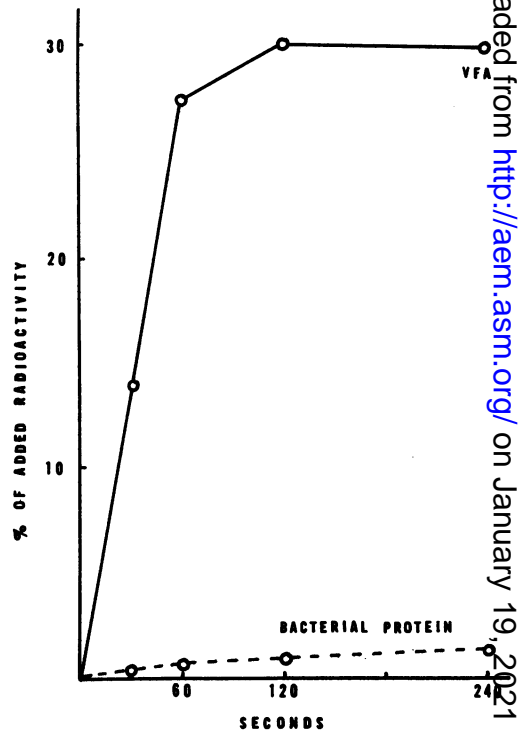


FIG. 3. Conversion of glycine-U-<sup>13</sup>C to volatile fatty acids and bacterial protein by rumen contents collected before feeding.

TABLE 2. Radioactivity in volatile fatty acids<sup>a</sup>

Volatile fatty acid	Percentage of total volatile fatty acids	Percentage of radioactivity in total volatile fatty acids
Acetic.....	68.0	89.8
Propionic.....	18.6	6.1
Butyric and higher...	10.3	4.1

<sup>a</sup> Incubation conditions as shown in Table 1. The total volatile fatty acid concentration was 17.4 mmoles per 100 ml.

the amino nitrogen of glycine would be converted to ammonia and mix with the pool of ammonia. By using glycine labeled with carbon-14 and nitrogen-15, it would be possible to compare the ratio of  $C^{14}$  to  $N^{15}$  in the added glycine with that in the protein glycine, and thus determine whether glycine is incorporated unchanged from extracellular glycine.

In the two experiments with doubly labeled glycine, it was necessary to add larger amounts of glycine to the samples and to incubate them for longer periods to obtain enough nitrogen-15 in the bacterial protein for mass spectrometry.

In the first experiment, rumen contents were taken 5 hr after feeding. The results in Table 4 show that 165  $\mu$ moles of added  $N^{15}$ -glycine- $U$ - $C^{14}$  was used by 108 g of rumen contents incubated for 100 min, giving a rate of glycine utilization of 0.176  $\mu$ mole per ml per min. Less than 1% of the carbon-14 was found in the bacterial protein.

Nitrogen-15 determinations showed that glycine had the greatest concentration of nitrogen-15 among the protein amino acids. Ammonia in the rumen fluid contained nearly 3 atoms % excess nitrogen-15; the glycine had been extensively deaminated.

The glycine solution added to the rumen sample had a radioactivity giving 44,300 counts per min per  $\mu$ mole of nitrogen-15, whereas the glycine isolated from bacterial protein had a specific activity of 49,500, a slight relative increase in the  $C^{14}$  label.

The second experiment used  $N^{15}$ -glycine- $I$ - $C^{14}$  (sample 1), and included additions of glucose (sample 2) or glucose plus a mixture of amino acids (sample 3) to determine whether these substrates affected the incorporation of glycine into

TABLE 3. *Metabolism of glycine-1- $C^{14}$  and glycine-2- $C^{14}$  by rumen microorganisms<sup>a</sup>*

Product	Substrate	
	Glycine-1- $C^{14}$	Glycine-2- $C^{14}$
	counts/min	counts/min
Volatile fatty acids. . . . .	1,020,000	2,890,000
Carbon dioxide. . . . .	9,000,000	94,000
Bacteria		
Lipid. . . . .	312,000	380,000
Nucleic acids. . . . .	114,000	85,000
Protein. . . . .	1,140,000	820,000
Residual glycine. . . . .	2,320,000	8,300,000

<sup>a</sup> Rumen contents were collected 3 hr after feeding. Samples of rumen contents (16.2 to 17.7 g) were incubated for 15 min at 39 C with 2.1  $\mu$ moles of glycine-1- $C^{14}$  or 4.0  $\mu$ moles of glycine-2- $C^{14}$ . The total counts added were  $15 \times 10^6$  counts per min.

TABLE 4. *Metabolism of  $N^{15}$ -glycine- $U$ - $C^{14}$ <sup>a</sup>*

Product	Radioactivity measurements	
	Counts/min	Percentage of $C^{14}$ added
Volatile fatty acids. . . . .	1,350,000	11.3
Bacterial protein. . . . .	117,000	0.98
Residual glycine. . . . .	4,920,000	41.0
	Nitrogen measurements	
	Amt ( $\mu$ moles) of N	Atoms % excess $N^{15}$
Ammonia in rumen fluid. . . . .	2,120	2.957
Glycine in protein. . . . .	42	2.786

<sup>a</sup> Rumen contents were collected 5 hr after feeding. The sample (108 g) was incubated for 100 min with a solution of glycine (21 mg of glycine- $N^{15}$  (97 atoms % excess) containing  $12 \times 10^6$  counts/min. The solution was added at the rate of 0.25 ml per 5 min.

bacterial protein. The rumen contents were collected before feeding to ensure low concentrations of soluble sugars and amino acids. As noted previously (Fig. 1), glycine was rapidly metabolized by rumen contents collected before feeding; less than 3% remained after 110 min (Table 5).

In these experiments, the volatile fatty acids contained about 10% and the bacterial protein less than 0.4% of the added carbon-14, with samples 2 and 3 containing more label in these fractions than did sample 1. The rates of utilization of the added glycine in samples 1, 2, and 3 were 0.233, 0.223, and 0.241  $\mu$ mole of glycine per ml per min, respectively.

The ammonia pool contained much more nitrogen-15 than in the first experiment, as would be expected from the longer duration and more rapid metabolism of the added glycine. The addition of glucose increased the nitrogen-15 in the glycine and in the glutamic acid in bacterial protein. The nitrogen-15 concentration in the glutamic acid was much lower than in glycine. The specific activity of the glycine had changed from 75,200 counts per min per  $\mu$ mole of nitrogen-15 in the added glycine to 54,400, 56,700, and 58,300 in the bacterial protein from samples 1, 2, and 3, respectively.

## DISCUSSION

Van den Henden et al. (16) found that washed suspensions of rumen bacteria fermented glycine to ammonia and acetic acid, without the release of any carbon dioxide. They concluded that these

TABLE 5. Metabolism of  $N^{15}$ -glycine-1- $C^{14}$  by rumen microorganisms<sup>a</sup>

Sample	Radioactivity (counts/min)			Nitrogen measurements				
	Volatile fatty acids	Bacterial protein	Residual glycine	Amt of ammonia ( $\mu$ moles)	Atoms % excess $N^{15}$			Specific activity of protein glycine (counts per min per $\mu$ mole of $N^{15}$ )
					Ammonia	Protein glycine	Protein glutamic acid	
1	1,888,000	50,000	565,000	3,300	5.436	0.542	0.082	54,400
2 <sup>b</sup>	2,270,000	65,000	455,000	3,070	5.099	1.140	0.116	56,700
3 <sup>c</sup>	2,230,000	69,000	414,000	2,840	5.000	1.230	0.162	58,300

<sup>a</sup> Rumen contents collected prefeeding. Each sample of rumen contents incubated for 100 min. Labeled glycine solution (20 mg of glycine- $N^{15}$ ) containing  $19.5 \times 10^6$  counts/min injected at the rate of 0.25 ml per 5 min.

<sup>b</sup> Glycine solution also contained 400 mg of glucose.

<sup>c</sup> Glycine solution also contained 400 mg of glucose and a mixture of amino acids containing L-alanine (4 mg), L-arginine (1.2 mg), L-aspartic acid (2 mg), L-glutamic acid (2.5 mg), L-histidine (0.25 mg), L-isoleucine (0.8 mg), L-leucine (2.3 mg), L-lysine (1 mg), L-phenylalanine (0.9 mg), L-proline (1 mg), L-serine (1.3 mg), L-threonine (1.2 mg), L-tyrosine (0.5 mg), L-valine (1.6 mg), L-methionine (1.2 mg), L-cystine (0.5 mg), and L-tryptophan (0.5 mg).

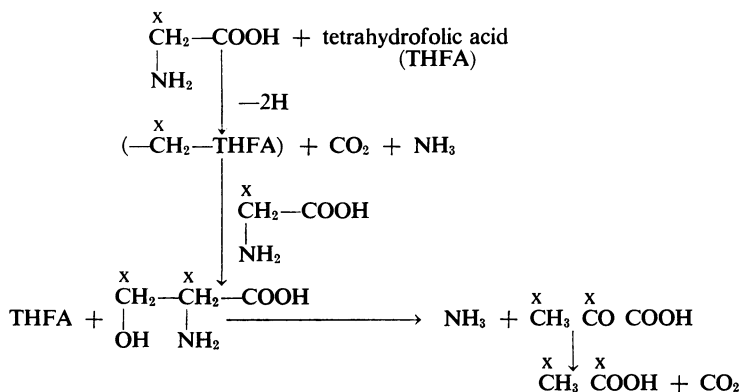


FIG. 4. Proposed pathway for metabolism of glycine.

products were formed in a glycine reductase system.

The rapid release of carbon dioxide, principally from the carboxyl carbon of glycine, and the distribution of carbon-14 in acetic acid formed from glycine, show that rumen microorganisms incubated under physiological conditions metabolize glycine very differently from the manner proposed by Van den Hende et al. (16).

*Diplococcus glycinophilus* ferments glycine to carbon dioxide, ammonia, and acetic acid (2). By using specifically labeled glycine, it was found that the carbon dioxide is derived entirely from the carboxyl of glycine, whereas both carbons of acetate are derived partly from the methylene carbon of glycine and partly from carbon dioxide. The proposed pathway is shown in Fig. 4. It is not known how carbon dioxide is converted into acetate carbon.

The labeling pattern in acetic acid formed from

the fermentation of glycine by rumen contents is consistent with this scheme. It is possible that part of the glycine is fermented by the more direct glycine reductase system, but most of the acetate from glycine presumably is formed by the pathway outlined in Fig. 4.

The rate of metabolism of glycine estimated for rumen contents was 0.014 and 0.038  $\mu$ mole per ml per min. In these samples, the addition of labeled glycine did not appreciably alter the glycine concentration. The  $K$  values for glycine metabolism (0.81 to 1.36/min) and the turnover rates (0.014 to 0.038  $\mu$ mole of glycine per ml per min) are comparable with those found by Portugal (Ph.D. Thesis, Univ. Aberdeen, Aberdeen, Scotland, 1963) for aspartic acid ( $K = 0.76/\text{min}$ , 0.012  $\mu$ mole per ml per min) and glutamic acid ( $K = 0.53/\text{min}$ , 0.011  $\mu$ mole per ml per min), but appear to be slightly higher.

The incorporation of glycine carbon into bac-

terial protein was low in all experiments, the highest value of 3.4% of the added radioactivity being considerably less than the 17 and 8% found by Portugal and Sutherland (13) for glutamic and aspartic acids.

The experiments with doubly labeled  $N^{15}$ - and  $C^{14}$ -glycine suggest that part of the glycine label is incorporated into bacterial protein after a partial degradation and resynthesis. In the postfeeding sample, the ratio of carbon-14 to nitrogen-15 (49,500 counts per min per  $\mu$ mole of  $N^{15}$ ) in the protein glycine is only slightly higher than in the added glycine (44,300). These values are sufficiently similar to suggest that most of the glycine was incorporated without degradation. Little label was derived from separate pools of carbon-14 and nitrogen-15 compounds. This was not so in the prefeeding samples using  $N^{15}$ -glycine- $I$ - $C^{14}$  where the carbon-14 to nitrogen-15 ratios in the protein glycine (54,400 to 58,300) were considerably lower than in the added glycine (75,200). In this experiment, it appears that part of the intracellular glycine arose from precursors with a carbon-14 to nitrogen-15 ratio much lower than 75,200. Since most of the radioactivity from the carboxyl of glycine is found in carbon dioxide, it is reasonable to assume that there would be present in these cells pools of carbon-14 with relatively lower specific activity.

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