

# Amino Acid Concentrations in Rumen Fluid

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## ABSTRACT

Methods using dialysis or ultrafiltration are described for the collection of extracellular fluid in rumen contents for analysis of amino acids. Marked differences in the concentration of aspartic acid, glutamic acid, and alanine were found in samples of either diffusate or ultrafiltrate and in clarified acidified rumen liquor. Concentrations are given for aspartic acid, glutamic acid, alanine, glycine,  $\gamma$ -aminobutyric acid, valine,  $\delta$ -aminovaleric acid, and leucine.

To investigate the *in vivo* metabolism of amino acids by rumen microorganisms, it is necessary to know the concentration of these compounds in rumen contents. Although values for  $\alpha$ -amino nitrogen in rumen liquor have long been known (1, 3, 5), only recently has an attempt been made to measure the concentrations of individual amino acids (A. V. Portugal, Ph.D. Thesis, Univ. Aberdeen, Aberdeen, Scotland, 1963).

Portugal's method of determining the concentration of several amino acids in rumen liquor by centrifugation to obtain cell-free fluid and subsequent analysis of the fluid by the preparation and chromatography of the dinitrophenyl derivatives of the amino acids can be criticized in two respects. First, his methods do not necessarily measure the amino acids in the extracellular fluid since centrifugation at low temperature combined in some instances with acidification of rumen contents will most likely result in the leakage of intracellular amino acids from microbial cells. This would cause elevated concentrations in the analyzed fluid. Second, chromatographic separations based entirely on the analysis of dinitrophenyl-amino acids found in protein hydrolysates may not always be applicable to the separation of amino acids from rumen contents containing non-protein amino acids such as  $\delta$ -aminovaleric acid and  $\gamma$ -aminobutyric acid formed by the microbial metabolism of protein amino acids (4, 6).

The difficulty of obtaining samples of intracellular rumen fluid has been overcome in two ways: (i) by suspending salt solution in dialysis sacs in rumen contents, a technique which has been modified for the analysis of small volumes of *in vitro* rumen samples and (ii) by direct ultrafiltration

from rumen contents. The results of these experiments are reported in this paper.

## MATERIALS AND METHODS

*Collection of samples for amino acid analysis.* All samples of rumen contents were collected from a rumen-fistulated Holstein cow stall-fed once daily on alfalfa hay.

*Rumen diffusates.* Three sacs prepared from Visking dialysis tubing no. 27, each half filled with 30 ml of salt solution, were placed inside the rumen. The sacs, attached to a metal sinker by nylon thread, were withdrawn after 1 to 2 hr, and their contents were removed with a 50-ml syringe and needle. The salt solution prepared just before use contained 0.05%  $\text{KH}_2\text{PO}_4$ , 0.05%  $\text{K}_2\text{HPO}_4$ , 0.1%  $(\text{NH}_4)_2\text{SO}_4$ , 0.01%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.1%  $\text{NaCl}$ , and 0.5%  $\text{NaHCO}_3$ .

*Rumen ultrafiltrates.* In experiments using small volumes of rumen contents (100 to 200 ml), it was impractical to use the diffusate method. Instead, a dialysis bag tightly stretched over a polypropylene support to form an ultrafilter was evacuated on a water pump and immersed in the sample of rumen contents incubated anaerobically at 39 C. After 10 min, 4 to 5 ml of liquid had accumulated inside the filter.

*Acidified rumen fluid.* Comparisons were made between the amino acid concentrations in diffusates or ultrafiltrates and in acidified rumen contents. Three 100-ml samples of rumen liquor were taken from the rumen at the same time as the dialysis sacs were withdrawn. The samples were taken from approximately the same location where the sacs were found. The three samples were pooled immediately, and 15 ml of 10 N  $\text{H}_2\text{SO}_4$  was mixed with the sample which was centrifuged at  $2,000 \times g$  for 5 min, and then at  $20,000 \times g$  for 60 min. When ultrafiltrates were collected, a 20-ml sample of the rumen contents in which the ultrafilter had been placed was removed directly after removal of the filter. A 0.1-ml amount of 10 N  $\text{H}_2\text{SO}_4$  was added, and the sample was centrifuged as above.

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*Preparation and chromatography of dinitrophenyl-amino acids.* Matheson (7, 8) and Portugal, Green, and Sutherland (10) have published methods for the estimation of dinitrophenyl-amino acids. By using a combination of their methods it was possible to separate dinitrophenyl-aspartic and dinitrophenyl-glutamic acids on a 16-g column of acid-washed Celite 545 equilibrated with ethyl acetate-isoamyl alcohol-sodium acetate, pH 4.8 (10); dinitrophenyl-glycine and dinitrophenyl-alanine were separated on a similar column at pH 7.4 (8). Dinitrophenyl- $\gamma$ -aminobutyric acid ( $R = 0.16$ ) could be separated from dinitrophenyl-leucine ( $R = 1.0$ ) and a mixture of dinitrophenyl-valine and dinitrophenyl- $\delta$ -aminovaleric acid ( $R = 0.50$  to  $0.53$ ) at pH 9.10 (7). It was later found that dinitrophenyl-valine ( $R = 0.44$ ) could be separated from dinitrophenyl- $\delta$ -aminovaleric acid ( $R = 1.0$ ) at pH 12.0 (7). Fractions from the columns were dried in vacuo and dissolved in 1% (w/v) NaHCO<sub>3</sub> for spectrophotometry at 360 m $\mu$ .

Provided the preparation and chromatography of the dinitrophenyl-amino acids was done in subdued light, recoveries of standard amino acid mixtures or glycine-2-C<sup>14</sup> added to rumen diffusates ranged from 86 to 91%. These recoveries were considered adequate, considering the low concentration present in most samples.

*Paper chromatography.* Amino acids were identified by use of the following solvent systems: phenol-water (100:39, w/v), *n*-butanol-acetic acid-water (100:21:50, v/v), methanol-water-pyridine (80:20:4, v/v).

*Thin-layer chromatography on Silica Gel G.* Dinitrophenyl-amino acids were separated with the following solvents: chloroform-benzyl alcohol-acetic acid (70:30:3, v/v), benzene-pyridine-acetic acid (80:20:2, v/v), chloroform-methanol-acetic acid (55:5:1, v/v), and 1.5 M phosphate buffer (pH 6.0). Amino acids were separated with phenol-water (75:25, v/v) and chloroform-methanol-17% ammonium hydroxide (2:2:1, v/v).

*High-voltage electrophoresis.* Amino acids were separated by use of formic acid-acetic acid (pH 1.85) buffer (2).

*Spray reagent.* Chromatograms were sprayed with 0.1% ninhydrin in acetone containing 2% collidine or with ninhydrin-cadmium reagent (2).

*Preparation of samples for chromatography or electrophoresis of amino acids.* Samples (2 to 10 ml) acidified to pH 1 with 2 N H<sub>2</sub>SO<sub>4</sub> were treated with Amberlite IR-120 cation-exchange resin; the resin was washed with water until the eluate was neutral, and then the absorbed amino acids were washed off with 2 N ammonia. After removing the ammonia solution in vacuo, the samples were dissolved in 0.2 to 0.5 ml of water for analysis.

*Radioactivity measurements.* Samples were counted for radioactivity by standard liquid scintillation procedures.

## RESULTS AND DISCUSSION

*Identification of the amino acids in rumen fluid.* The amino acids present in greatest concentration in samples of rumen diffusate, centrifuged rumen

liquor, or clarified acidified rumen liquor were aspartic acid, glutamic acid, glycine, alanine,  $\delta$ -aminovaleric acid,  $\gamma$ -aminobutyric acid, valine, and leucine. Traces of proline, serine, and methionine were sometimes present.

*Testing dialysis methods.* Since the use of dialysis sacs for obtaining extracellular amino acid concentrations is only valid if there is equilibration between the amino acids in the fluid on both sides of the membrane, the method was tested by removing two samples of 2 liters of rumen contents and killing the microorganisms either by steaming for 30 min or acidification with H<sub>2</sub>SO<sub>4</sub> to pH 1 for 30 min followed by neutralizing to pH 6.5. A solution of glycine-1-C<sup>14</sup> (2  $\mu$ g/2  $\mu$ c) was added to each 2-liter sample and well mixed; four dialysis sacs, each containing 20 ml of salt solution, were then suspended in each sample of rumen contents. The sacs were slowly rotated with a stirrer to simulate the mixing in the rumen. At intervals, sacs were removed and their contents were counted for radioactivity and were compared with the radioactivity present in the samples of clarified rumen contents. Paper chromatography of the amino acids in the samples followed by radioactive scanning showed that glycine was the only labeled material present in the fluid.

After 15 min, the glycine content of the diffusate was 62% that of the rumen fluid; after 30 min, 80%; after 60 min, 100%; and after 120 min, 99%. Identical results were obtained with either heat- or acid-treated rumen contents. Another experiment in which sacs were stirred by slowly rotating a bottle containing heat-killed rumen contents showed equilibration between the sac contents and the rumen liquor after 60 min.

Smith and Mah (11) have used a similar approach to determine pool sizes of acetic acid in extracellular fluid in sludge digestors. By immersing a collodion sac containing distilled water for 2 hr in sludge, they obtained acetic acid concentrations similar to those in the supernatant liquid prepared by centrifuging sludge.

The ultrafiltration method was tested by filtering heat-treated rumen contents containing glycine-1-C<sup>14</sup>. Duplicate analyses showed that the ultrafiltrate contained 95 and 97% of the concentration of glycine-C<sup>14</sup> in cell-free rumen liquor.

The results in Tables 1 and 2 show that acidification of rumen contents to stop microbial metabolism results in amino acid concentrations higher in the clarified rumen liquor than in rumen diffusates or ultrafiltrates. It seems likely that these higher levels are due to the inclusion of intracellular pools of amino acids.

The high levels of aspartic acid, glutamic acid, and alanine found in the acidified rumen liquor

support this idea, since these amino acids involved in the utilization of the ammonia pool usually found in the rumen would be expected to be prominent intracellular intermediates in the nitrogen economy of rumen microorganisms.

$\gamma$ -Aminobutyric acid, in concentrations up to 0.15  $\mu$ mole/ml, presumably is formed by the

decarboxylation of glutamic acid. Several reports on the metabolism of glutamic acid by rumen microorganisms have described low recoveries of glutamate carbon. Otagaki et al. (9) recovered only 37% of added carbon, Van den Hende, Oyaert, and Bouckaert (12) found 75% of the radioactivity from glutamate- $1-C^{14}$  in carbon dioxide or volatile fatty acids, whereas Portugal recovered between 52.9 and 72.0% of the counts from glutamate- $U-C^{14}$ . It is possible that part of the missing carbon was  $\gamma$ -aminobutyric acid.

Another product of microbial amino acid metabolism which accumulated is  $\delta$ -aminovaleric acid. El-Shazly (4) demonstrated the formation of  $\delta$ -aminovaleric acid by rumen contents. Lewis and Emery (6) showed that washed suspensions of rumen bacteria metabolized L-arginine, L-ornithine, and L-lysine to several products, including  $\delta$ -aminovaleric acid.

As reported by Lewis (5), amino acid concentrations increased after feeding. Notable is the accumulation of alanine which was the major amino acid found in most samples (Tables 1 and 2).

The concentrations of amino acids found in these experiments show more variation than those reported by Portugal, who sampled sheep on a continuous-feeding regime. Nevertheless, some of the values are comparable as shown in Table 3. Aspartic and glutamic acids are very similar, although fourfold differences in concentrations are found for glycine, valine, and alanine. Since acid treatment of rumen contents increases the measured concentration of amino acids, it is surprising that Portugal's results are generally lower. A

TABLE 1. Amino acid concentrations in rumen diffusates and acidified rumen liquor<sup>a</sup>

Amino acid	Sampling time				
	Prefeeding (expt 1)		60 min postfeeding (expt 2)		120 min post-feeding (expt 3)
	D	RL	D	RL	D
Aspartic acid . . . . .	0.009	0.045	0.050	0.090	0.030
Glutamic acid . . . . .	0.009	0.170	0.005	0.250	0.042
Glycine . . . . .	0.008	0.015	0.042	0.060	0.080
Alanine . . . . .	0	0.090	0.290	0.390	0.240
$\gamma$ -Amino-butyric acid . . . . .	0	0	0.060	0.080	0.050
Valine . . . . .	0	0.015	0.110	0.170	0.170
$\delta$ -Amino-valeric acid . . . . .	0	0.009	0.060	0.060	0.042
Total . . . . .	0.026	0.344	0.617	1.100	0.654

<sup>a</sup> D = diffusate; RL = acidified and clarified rumen liquor. Results are expressed in micromoles per milliliter.

TABLE 2. Concentration of amino acids in ultrafiltrates and acidified rumen liquor<sup>a</sup>

Amino acid	Sampling date and time after feeding							
	11 March 1966						9 March 1966, 120 min	
	60 min		200 min		480 min		UF	RL
	UF	RL	UF	RL	UF	RL		
Aspartic acid . . . . .	0.021	0.050	0.016	0.060	0.011	0.030	0.040	0.090
Glutamic acid . . . . .	0.021	0.270	0.016	0.100	0.011	0.180	0.028	0.210
Glycine . . . . .	0.004	0.005	0.006	0.016	0.006	0.022	0.016	0.004
Alanine . . . . .	0.016	0.090	0.028	0.070	0.016	0.070	0.060	0.230
$\gamma$ -Aminobutyric acid . . . . .	0.008	0.004	0	0.060	0.006	0.016	0.028	0.056
Valine . . . . .								
$\delta$ -Aminovaleric acid . . . . .	0.004	0.016	0.006	0.016	0.006	0.028	0.050	0.090
Leucine . . . . .	0.004	0.004	0.006	0.060	0.006	0.011	0.006	0.016
Total . . . . .	0.078	0.439	0.078	0.382	0.062	0.357	0.228	0.696

<sup>a</sup> UF = ultra-filtrate; RL = acidified, clarified rumen liquor. Results are expressed in micromoles per milliliter.

TABLE 3. Comparison of the mean amino acid concentrations in diffusate-ultrafiltrates and acidified rumen contents in comparison with the values reported by Portugal

Amino acid	Diffusate-ultrafiltrates		Acidified rumen liquor		Portugal's data	
	No. of tests	Concn (range)	No. of tests	Concn (range)	No. of tests	Concn (range)
		<i>μmoles/ml</i>		<i>μmoles/ml</i>		<i>μmoles/ml</i>
Aspartic acid.....	9	0.026 (0.009-0.050)	9	0.065 (0.020-0.150)	4	0.022 (0.020-0.023)
Glutamic acid.....	9	0.024 (0.009-0.069)	9	0.190 (0.100-0.270)	4	0.028 (0.027-0.029)
Glycine.....	9	0.030 (0.004-0.080)	12	0.053 (0.005-0.130)	3	0.008 (0.007-0.009)
Alanine.....	9	0.102 (0-0.290)	10	0.252 (0.060-1.130)	3	0.025 (0.024-0.026)
γ-Aminobutyric acid.....	8	0.038 (0-0.150)	9	0.046 (0-0.180)		
Valine-δ-amino-valeric acid.....	8	0.070 (0-0.220)	8	0.074 (0-0.180)	3	0.021 <sup>a</sup> (0.020-0.021)
Leucine.....	7	0.018 (0-0.060)	7	0.025 (0.009-0.060)		

<sup>a</sup> Recorded as valine concentration.

probable explanation is his continuous feeding regime. This would give a constant fermentation system in which the rates of amino acid formation and utilization would be identical, and the concentration of amino acids would be relatively low.

Converting the total amino acid concentration into milligrams of α-amino nitrogen per 100 ml, values range from 0.038 to 0.915 mg for diffusates and ultrafiltrates, and between 0.480 and 1.540 mg for acidified rumen fluid. The latter figures are comparable with the 0.1 to 1.4 mg reported by Lewis (5), 0.4 to 4.0 by Chalmers and Synge (3), and 0.3 to 1.5 mg by Annison (1).

On the assumption that analyses of acidified rumen contents give values for the total amino acids, whereas the dialysis bag methods measure only the extracellular pool, it is concluded that the extracellular amino acids usually represent less than half of the total amino acids demonstrable in liquid from killed samples of rumen contents.

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