Proline Utilization during Cellulose Fermentation by Rumen Microorganisms

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The metabolism of proline during *in vitro* cellulose digestion by rumen microorganisms was studied. Tracer amounts of uniformly labeled ^{14}C-proline were used with stimulatory levels of unlabeled proline, valeric acid, or distillers dried solubles (DDS). Decreases in the ^{14}C-activity and concentration of free proline were accompanied by increases in the concentration and ^{14}C-activity of valeric acid. Carbon from proline was recovered in both bacterial proteins and lipids. Proline was the only amino acid containing appreciable label in the bacterial protein. The lipid fraction with all treatments contained more ^{14}C-activity than did the protein fraction. The addition of valeric acid or the DDS extract tended to increase the amount of label recovered in the lipid fraction of the microbial cells when compared to a similar proline treatment.

Proline has been shown to stimulate the *in vitro* fermentation of cellulose by rumen microorganisms (Dehority *et al.*, 1957; Potter *et al.*, 1966) and has been implicated as one of the factors in distillers dried solubles which stimulate *in vitro* cellulose digestion (Potter *et al.*, 1966). Valeric acid has also been identified as a stimulant to rumen microbial digestion of cellulose (Bentley *et al.*, 1955; Potter *et al.*, 1966). A possible interrelationship of these two compounds is suggested by the observations that valeric acid can be formed by the reductive deamination of proline (Strickland, 1934) and that initial increases of valeric acid levels in the fermentation medium to which proline has been added are followed by decreases during periods of rapid cellulose digestion (Potter *et al.*, 1966). This work was initiated to follow the metabolism and utilization of proline during *in vitro* cellulose digestion by rumen microorganisms, and to study the relationship between proline and valeric acid.

EXPERIMENTAL AND RESULTS

Washed cell suspensions of rumen microorganisms were cultured *in vitro* to study the metabolism of proline. Ruminal ingestum was obtained 3 to 4 hr post prandial from a steer receiving 6 kg of alfalfa hay and 3 kg of a corn, distillers dried solubles, and soybean meal concentrate mixture daily. The concentrate mixture was supplemented with vitamins A, D, and E, and minerals, calcium, and phosphorus. The ingestum was processed and microorganisms were cultured according to the procedure of Cheng *et al.* (1955) using a chemically defined buffered medium and purified wood cellulose as the energy substrate.

Uniformly labeled ^{14}C-proline, obtained from Tracer Lab., Inc., Waltham, Mass., with a specific activity of 170 mCi per mM, was used to trace the metabolism of proline. In experiments 1 and 2, the rumen microorganisms were incubated separately in 500 ml Erlenmeyer flasks with 250 ml medium for 0, 6, 12, 18, or 24 hr. Approximately 2.0 μCi in experiment 1 or 10.0 μCi in experiment 2 of labeled ^{14}C-proline and 125 μM of unlabeled proline were added to each flask. In experiment 3, 8.9 μCi of labeled proline and either 125 μM of proline, 125 μM valeric acid, or a hot water extract equivalent to 625 mg of DDS (Potter, 1965) were added to each 250 ml of the fermentation medium. Fermentation times for experiment 3 were 0, 12, or 24 hr.

Following incubation, cellulose was determined using the procedure given by Crampton and Maynard (1938), as modified by Donefer *et al.* (1960). The ^{14}C-activity was separated into water soluble and cell bound fractions by centrifuging the fermented mixture at 25,000 *g* for 30 min. The soluble ^{14}C-activity was further separated into free proline, δ-amino valeric acid, and acetic, propionic, butyric, and valeric acids. The cellular bound ^{14}C-activity was further divided into bacterial protein and lipids. The concentrations of free proline, δ-aminovaleric acid, and the amino acids present in cellular components were determined by the procedure of Hamilton (1963). The fermentation medium was centrifuged at 25,000 *g* for 30 min and washed three times in 0.1 *M* phosphate buffer at a pH of 6.8, then hydrolyzed in 6*N* NCl to determine total amino acids. A fraction of the column eluent was collected and ^{14}C-activity determined by liquid scintillation techniques using a Packard series 4000 spectrometer and the scintillation fluid described by Bruno and Christian (1961).

The ^{14}C-activity of the bacterial lipids was determined on an aliquot of the incubation flask contents using a chloroform-methanol extraction procedure (Schmidt and Wynne, 1965). One milliliter of this extract was evaporated to dryness, resuspended in 0.5 ml petroleum ether, and the ^{14}C-activity determined as described previously.

The ^{14}C-activity present in short-chain fatty acids was determined by steam distilling an acidified aliquot of the supernatant resulting from centrifuging the entire fermentation medium at 25,000 *g* for 30 min and collecting the distillate in 5 ml 0.5*N* NaOH. The distillate was dried over steam, resuspended in 4 ml 0.6*N* H_2SO_4, and the pH adjusted to approximately 2.5 with 1 ml of 25% metaphosphoric acid. The ^{14}C-activity present in acetic, propionic, butyric, and valeric acids was obtained after separation using a Packard series 7820 flame ionization gas chromatograph, equipped with a 4.0 mm × 1.5 m coiled glass column packed with 20% PEG 1500 and 2% H_3PO_4 on fire-brick. The chromatograph was operated with an injection temperature of 200° C, column temperature 145° C, detector temperature 155° C, and a nitrogen carrier gas flow rate of 50 ml per min. The eluted fatty acids were oxidized to CO_2 and H_2O in a Packard model 325 combustion furnace. The activity in each fatty acid was counted as CO_2 in the previously described Packard spectrometer using an anthracene continuous gas flow cell. Total counts were obtained from the counter printout system, and efficiency of counting determined using standard ^{14}C-toluene.

Carbon dioxide was determined by trapping all CO_2 released during fermentation in Packard Hydroxide of Hy-

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amine 10-x (*p*-diisobutylcresoxyethoxyethyl-dimethylbenzyl ammonium chloride) and counting the activity by liquid scintillation techniques.

The percentage cellulose digestion at each fermentation time for experiment 1 is presented in Table I. The results indicate a continuous increase in the percentage of cellulose digested throughout the 24-hr fermentation period. The percentage distribution of ^{14}C-activity in the various fermentation components is given in Table II. The percentage ^{14}C-activity as free proline decreased with fermentation time and accounted for only 1.9% of the total activity present in the media at the end of 24 hr. The decrease in free proline was accompanied by an increase in ^{14}C-activity present as valeric acid. At the end of 24 hr, 50.9% of the ^{14}C-activity initially added as proline was recovered in valeric acid. Cellular lipid ^{14}C-activity increased throughout the 24 hr fermentation period. Protein ^{14}C-activity reached a maximum after 18 hr fermentation and decreased slightly at 24 hr. After 24-hr fermentation, 19.3 and 6.8% of the ^{14}C-activity was recovered in lipid and bound proline, respectively. Only traces of ^{14}C-activity were recovered as carbon dioxide at any fermentation time. The component listed as other VFA varied considerably with fermentation time, but does indicate some conversion of proline or degradation products to short-chain fatty acids of less than five carbons in length.

The percentages of total hydrolyzate activity recovered in individual amino acids are shown in Table III. Approximately 75% of the total hydrolyzate ^{14}C-activity was present as proline, with varying percentages present in the other amino acids at the end of 25 hr fermentation. No ^{14}C-activity was recovered in amino acids other than proline, lysine, arginine, and glutamate. The ^{14}C-activity represented by glutamate, lysine, and arginine accounted for less than 1% of the original ^{14}C-activity at 6, 12, and 18 hr and only 1.8% of the ^{14}C-activity initially added as proline at the end of 24 hr fermentation; however, glutamate and arginine accounted for 11.8 and 13.0% of the total ^{14}C-carbon activity recovered from the hydrolyzate in amino acids. The percentage ^{14}C-activity in lysine was highest after 12 hr fermentation and decreased thereafter.

The second experiment in the study was conducted as outlined for experiment 1, except for the additional labeled proline added initially. The percentage cellulose fermented in this experiment (Table I) was considerably greater than that in experiment 1, with the greatest increase occurring during the last 12 hr of fermentation. Figures 1, 2, and 3 show the observed changes in the concentrations of free proline, δ-aminovaleric, and valeric acid, and the ^{14}C-activity of each of these components at each respective fermentation period. A very close relationship between the disappearance of free proline on a concentration basis and the ^{14}C-activity present as free proline during the first 12 hr fermentation is evident (Figure 1). The free proline concentration had decreased below detectable amounts at 12 hr fermentation, although small quantities of ^{14}C-activity were present at both 12 and 18 hr fermentation.

Figure 2 shows that the highest concentration and ^{14}C-activity of δ-aminovaleric acid was present at the end of 12 hr fermentation, corresponding to the time when almost all free proline had disappeared from the fermentation media. The δ-aminovaleric acid decreased between 12 and 18 hr fermentation and was accompanied by an increase in valeric acid concentration and ^{14}C-activity present in valeric acid, as shown in Figure 3.

The specific activity of free proline, δ-aminovaleric acid, and valeric acid at several selected fermentation times is shown in Table IV. There is a close relationship between the specific activities of each component at each fermentation time except the valeric acid values at 6 and 24 hr of fermentation. Apparently, these lowered specific activities indicate that some other carbon source in the medium contributed to the production of valeric acid.

The percentage cellulose digestion from the third experiment, in which proline, valeric acid, and the DDS extract

Table I. Percentage Cellulose Digestion Obtained with Stimulatory Levels of L-Proline

Fermentation Time (hr)	Cellulose Fermented (%)
Experiment 1	
6	5.1
12	11.6
18	28.1
24	45.3
Experiment 2	
6	1.7
12	3.7
18	47.1
24	70.6

Table II. Percentage Distribution of ^{14}C-Activity from Uniformly Labeled Free Proline after Fermentation by Rumen Microorganisms (Experiment 1)[a]

	Fermentation Time (hr)			
	%			
Component	6	12	18	24
Free Proline	52.3	13.1	17.8	1.9
Valeric	15.6	30.9	30.3	50.9
Other VFA	11.3	6.0	9.0	17.1
Lipid	3.9	6.4	14.6	19.3
Bound Proline	2.4	2.8	8.1	6.8
Carbon Dioxide	0.1	0.5	1.0	2.1

[a] At 0-time all ^{14}C-activity was present as free proline.

Table III. Percentage Distribution of Hydrolyzate ^{14}C-Activity in Glutamate, Proline, Lysine, and Arginine at Each Fermentation Time (Experiment 1)

	Fermentation Time (hr)			
	%			
Component	6	12	18	24
Glutamate	24.1	20.9	8.9	11.8
Proline	71.6	76.3	88.3	75.4
Lysine	...	2.8	1.1	0.3
Arginine	4.3	...	1.6	13.0

Table IV. Specific Activity of Proline, δ-Aminovaleric Acid, and Valeric Acid in the Fermentation Medium (Experiment 2)

Component	Time (hr)	^{14}C-Activity 10^6 DPM	Concentration (μM/250 ml)	Specific Activity (10^3 DPM/μM)
Free Proline	0	21.1	151.8	138
Free Proline	6	13.2	99.8	132
δ-Aminovaleric	12	17.5	146.9	119
Valeric	6	4.9	80.6	60
Valeric	12	2.5	22.0	116
Valeric	18	8.9	75.4	118
Valeric	24	8.0	32.0	72

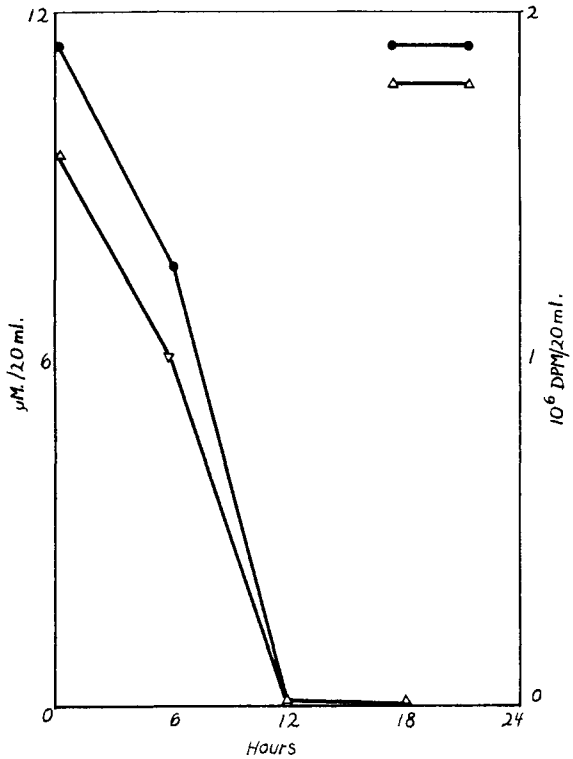

Figure 1. Changes in free proline concentration and ^{14}C-activity with time

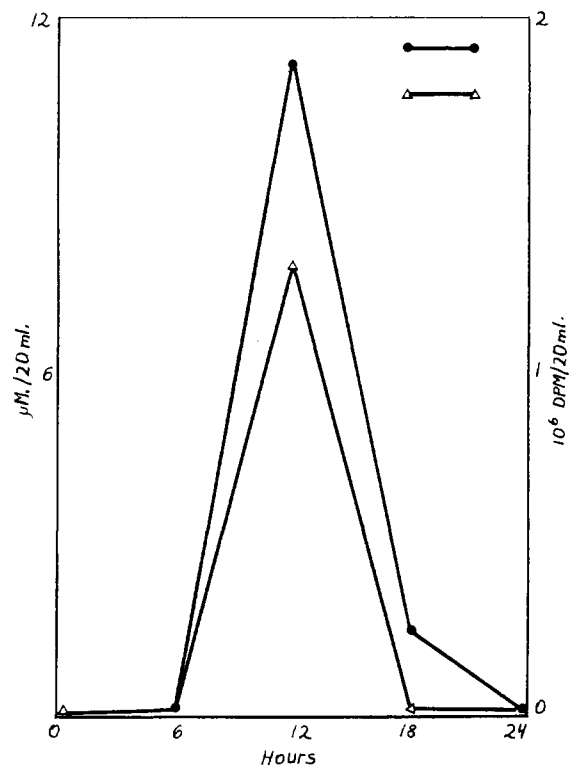

Figure 2. Changes in δ-aminovaleric acid concentration and ^{14}C-activity with time

additions were made, is shown in Table V. The data indicate a similar cellulose digestion between the proline and valeric acid treatments at the end of 24 hr, with a somewhat higher percentage cellulose digestion for the DDS treatment at 12 and 24 hr.

The percentage distribution of ^{14}C-activity from proline

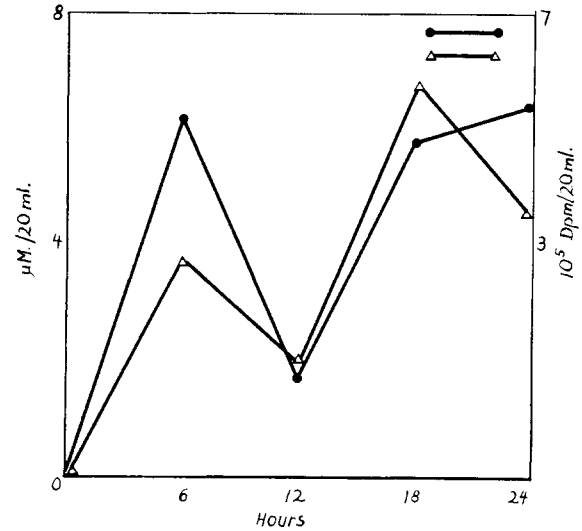

Figure 3. Changes in valeric acid concentration and ^{14}C-activity with time

Table V. Percentage Cellulose Digestion for Valeric Acid, Proline, and DDS Extract Treatments (Experiment 3)

Fermentation Time (hr)	Treatment		
	Proline	Valeric	DDS
12	7.9	4.4	15.3
24	54.4	54.2	57.9

Table VI. Percentage Distribution of ^{14}C-Activity from Uniformly Labeled Free Proline After Fermentation with Proline, Valeric Acid, and DDS Extract Treatments (Experiment 3)

Component	Hr	Treatment		
		Proline	Valeric	DDS
Free Proline	12	64.9	0.2	0.6
	24	0.6	0.1	0.7
δ-Aminovaleric	12	3.2	...	0.6
	24	0.5	...	0.6
Valeric	12	22.2	80.1	51.9
	24	43.5	50.1	36.9
Other VFA	12	1.8	20.7	20.2
	24	5.5	12.1	17.4
Bound Proline	12	7.3	0.9	1.7
	24	10.8	7.5	2.8
Lipid	12	0.7	0.8	4.3
	24	11.1	20.3	27.3

for each treatment is shown in Table VI. Free proline tended to disappear more rapidly from the DDS and valeric acid treatments than from the proline treatment. The faster rates of proline disappearance for the DDS and valeric acid treatments were reflected in much more ^{14}C-activity being present as valeric acid or other VFA at the end of 12 hr in these treatments than was present at this time in the proline treatment. More proline carbon was incorporated into bacterial protein in the proline treatment than in the DDS extract or valeric acid treatments; however, the addition of the DDS extract or valeric acid resulted in a higher level of ^{14}C-activity in the bacterial lipids for these treatments than was recovered in bacterial lipids from the proline treatment.

DISCUSSION

The results from these experiments verify that proline is metabolized *in vitro* to valeric acid by rumen microorganisms. In each experiment a decrease in the concentration and ^{14}C-activity of free proline was followed by an increase in the concentration and ^{14}C-activity of valeric acid. The data from experiment 2 suggest that δ-aminovaleric acid is an intermediate in this microbial conversion of proline to valeric acid. Based upon the percentage of activity appearing as valeric acid at 12 hr fermentation, apparently the conversion of proline to δ-aminovaleric or valeric acids occurred prior to the time when cellulose digestion was greatest. The specific activities calculated for free proline, δ-aminovaleric acid, and valeric acid indicate that one proline molecule is converted to one molecule of valeric acid by the rumen microorganisms.

The data presented indicate that proline apparently is used in several pathways in rumen microbial metabolism. Appreciable quantities of proline carbon were incorporated into bacterial proteins and lipids. The critical nature of proline in these synthetic pathways of cellulolytic microorganisms is uncertain; however, Potter (1965) has shown that proline stimulates *in vitro* cellulolytic activity of rumen microorganisms and similar but nonadditive responses result with equal molar additions of valeric acid.

The fact that proline stimulates *in vitro* rumen microbial cellulose digestion may be explained partially by its conversion to valeric acid, since Allison *et al.* (1959) and Bentley *et al.* (1955) have shown that certain rumen cellulolytic microorganisms are stimulated by or actually require a straight-chain fatty acid 5 to 8 carbons in length. Further work by Wegner and Foster (1963) has shown that carboxyl labeled valeric acid was incorporated primarily into 13 and 15 carbon fatty acids by *Bacteroides succinogenes*. The almost complete disappearance of free proline by the end of 12 hr fermentation was indicative, although not conclusive, that most of the proline added initially was metabolized to valeric acid before being utilized by the microorganisms for protein or lipid synthesis. Apparently most of the proline carbon used in synthesis of amino acids in microbial protein is used primarily for the resynthesis of proline. Proline was the only amino acid present in bacterial protein which contained appreciable ^{14}C-label.

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