STUDIES ON BIURET UTILIZATION IN SHEEP USING *IN VITRO* VFA KINETICS AND *IN VIVO* INFUSION TECHNIQUES

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CHAPTER I

INTRODUCTION

In recent years there has been much interest in the use of non-protein-nitrogen as a supplement to forage-based rations. In many areas protein supplementation is a necessity for adequate production from livestock under range conditions. Biuret and urea are presently being used as nitrogen sources; however, the use of the latter is limited because of its high solubility and rapid release of ammonia. The lack of toxicity of biuret has been demonstrated, and some research results indicate that it is comparable to urea and natural proteins for promoting weight gains under range conditions. It has been shown that a period of adaptation to biuret is needed for optimum performance by ruminants.

The mechanism of utilization of biuret in the ruminant is not clearly understood. Recent research has demonstrated hydrolysis of biuret by rumen microorganisms; however, other studies suggest metabolism of biuret by the tissues.

The purpose of this study was to use an *in vitro* VFA production technique to evaluate nitrogen sources and to use *in vivo* infusion techniques to study biuret metabolism in sheep.

CHAPTER II

REVIEW OF LITERATURE

Introduction

Biuret and urea are presently being used as non-protein-nitrogen sources for ruminants. The mechanism of urea and ammonia utilization by rumen microorganisms is rather clearly understood; however, until recently, the mechanism of utilization of biuret has been poorly understood. To study the mechanism of utilization of biuret, it is necessary to review the factors affecting its utilization in the ruminant animal. The following review will include a discussion of the effect of biuret on nitrogen balance and growth in comparison with urea and natural protein sources. A discussion of the fate of injected biuret will also be included. Reference will be made to the production of volatile fatty acids as a technique for evaluating various dietary regimens.

Utilization of Non-Protein Nitrogen

Toxicity of Biuret

Many workers, including Berry (1955), Meiske et al. (1955) and Hatfield et al. (1959), have demonstrated that biuret is not toxic to ruminants. Meiske et al. (1955) drenched sheep with either urea or biuret. Toxicity appeared with urea at about 25 g/100 lbs body weight, but the biuret-drenched sheep showed no signs of toxicity at 31 g/100

lbs body weight. Berry (1955) fed "crude" biuret at a level to provide three percent biuret and two percent urea in the diet of lambs. The addition of this level of crude biuret did not affect feed consumption or gains, and no signs of toxicity appeared. Repp et al. (1955) concluded that clinical symptoms of ammonia toxicity did not occur until the blood NH_3-N levels rose about 1000 µg/100 ml. When the blood NH_3-N level rose above 1158 µg/100 ml, acute toxicity resulted and the animal died. The administration of 40 g of urea to lambs resulted in toxicity and death. Biuret was shown to be nontoxic at this level in these experiments; and there was little, if any, increase in blood ammonia and urea nitrogen with the administration of biuret.

Growth and Nitrogen Balance

Using an artificial rumen, Belasco (1954) compared several sources of nitrogen for use by rumen microorganisms. In these experiments cellulose digestion was utilized as an index of nitrogen availability. It was concluded that biuret was not utilized to any extent as a nitrogen source for cellulose digestion using urea as a comparison. Strained steer rumen contents were used as an inoculum for the in vitro flasks.

Meiske et al. (1955) found supplementation of a fattening ration for lambs with urea, biuret or crude biuret resulted in gains comparable to gains from soybean meal supplementation. Hatfield et al. (1959) obtained positive nitrogen balances with sheep feed rations supplemented with large amounts of biuret. The average weight gains for biuret-fed sheep lagged behind urea-fed sheep, but at the end of the trial weight gains were similar. Biuret supplementation promoted weight gains and feed efficiency similar to urea supplementation for growing heifers on

a roughage diet (Campbell et al., 1963); however, nitrogen retention was lower for animals fed biuret rations than for those fed urea rations.

Anderson et al. (1959) stated that nutrient digestibility and nitrogen utilization were not significantly influenced by crude biuret when fed in a high-roughage ration to lambs. When pure biuret was the only source of supplemental nitrogen, nutrient digestibility was significantly reduced and a negative nitrogen balance resulted. Rations containing urea or soybean protein resulted in positive nitrogen balances. The lambs on these trials were on the NPN diet for only 10 days prior to the digestion and balance trials.

Adaptation

The length of time on the ration affects the utilization of biuret. McLaren et al. (1959) reported that retention of absorbed nitrogen by lambs increased as length of time on a biuret ration increased up to 30 to 40 days. The apparent digestibility of protein increased as time on the biuret ration increased, but time did not affect organic matter of crude fiber digestibility.

When lambs fed a biuret diet were inoculated with rumen contents from lambs on a biuret diet for 20 months (Ewan, Hatfield and Garrigus, 1958), the retention of nitrogen was higher than in lambs inoculated with rumen contents from animals fed diets not containing biuret. Thus the adaptation period for biuret can be shortened by inoculating the animal with rumen contents from an adapted animal. Dry matter and organic matter digestion was slightly lower when biuret was the nitrogen source than when urea was used. According to Clark, Barrett and

Kellerman (1963), a long period is not needed for biuret utilization. Positive nitrogen retention was obtained during the first five days of biuret feeding; however, maximum retention was not attained until six to eight weeks on a biuret-supplemented hay diet. After the adaptation period it was concluded that biuret was as efficient as urea when judged by nitrogen retention, apparent nitrogen absorption and stimulation of hay consumption.

In contrast, Johnson and McClure (1964) observed that sheep adapted to biuret in terms of increased digestibilities of dry matter, organic matter and nitrogen, but not in terms of nitrogen retention or biological value. In this trial biuret feeding had no appreciable effect on rumen ammonia or blood urea, whereas urea feeding raised both considerably. However, Schaadt, Johnson and McClure (1966) noted that in a 72-day metabolism trial the amount of urinary-apparent biuret decreased after day 36 with a corresponding increase in urinary urea ammonia nitrogen. Lambs on a urea diet showed increased nitrogen balance and biological value, while lambs on a biuret diet did not show increased nitrogen balance or biological value. Oltjen, Williams and Richardson (1968) found nitrogen balance data indicated that steers fed a high-roughage diet were adapted to urea within seven days, while it took 21 days for steers to adapt to biuret.

Ruminal Hydrolysis of Biuret

The utilization of urea by its hydrolysis to ammonia and CO_2 has led investigations to the method of utilization of biuret by the ruminant. Using an *in vitro* starch digestion technique, Johnson and McClure (1964) found that rumen bacteria taken from adapted sheep failed to

utilize biuret, and the organisms from the adapted animals did not release ammonia from biuret *in vitro*. The rumen inoculum used in these studies was strained and centrifuged to remove large coarse debris. Waite and Wilson (1968) observed that the rumen fluid of cows fed biuret had higher concentrations of NPN and lower concentrations of ammonia N than did cows fed urea. However, the concentration of ammonia N in the rumen liquor of these cows fed biuret increased progressively up to the fifth and eighth week. Recently it has been demonstrated that ruminal flora obtained from sheep on a biuret ration will utilize biuret. Gilchrist, Potgieter and Voss (1968) measured the biuretolytic activity of rumen ingesta from sheep receiving a biuret diet by the rate of disappearance of biuret in an *in vitro* fermentation system. It was found that the biuretolytic activity was only present when biuret was included in the diet. The addition of maize meal to a poor-quality hay diet increased the biuretolytic activity. Schroder and Gilchrist (1969) have shown the biuretolytic activity of rumen microorganisms was increased when starch was added to the ration. It was suggested that the multiplication of biuretolytic organism was favored by the addition of a readily-available carbohydrate. The rate of increase of biuretolytic activity was related to the protein content of the ration. Very low, medium and higher protein diets were found to take 15, 30 and 70 days respectively to reach maximum biuretolytic activity. It was also demonstrated by these studies that there was a stoichiometric conversion of biuret-N to NH_3-N *in vitro*, but this was not reflected by the nitrogen levels *in vivo*. The majority of the biuretolytic activity is present in the rumen (Schroder, 1970). Biuretolytic activity could be demonstrated in the lower intestinal tract of sheep adapted to biuret by

in vitro techniques. The amount of activity was small in comparison to that in the rumen. Bauriedel (1971) demonstrated the incorporation of ^{14}C of biuret into $^{14}CO_2$, $^{14}CH_4$ and ^{14}C incorporated into the bacterial mass. Using ^{15}N- ^{14}C- biuret (Bauriedel *et al.*, 1971), the hydrolysis of crude biuret to $^{14}CO_2$ and $^{15}NH_3$ has been demonstrated.

Bauriedel (1971) isolated crude biuretase preparations from rumen microorganisms from lambs fed a biuret-supplemented ration. The greatest biuretolytic activity was associated with the top layer of plant debris when rumen contents were allowed to stand for a period of two hours. A cell-free extract of the ruptured microorganisms was shown to possess biuretolytic activity, indicating that the enzyme is intracellular.

Biuret in High-Roughage Diets

The need for a nitrogen supplement for ruminants utilizing roughage rations has increased the interest in non-protein nitrogen compounds. The fact that biuret is not toxic when fed in large amounts makes it practical for use in these conditions. Mackenzie and Altona (1964) compared biuret, urea and mixtures of these two chemicals for sheep and cattle on hay diets. Animals on a low-quality hay diet without supplement lost weight, while those supplemented with one of the sources of NPN maintained their weight throughout the feeding period. Hay consumption by sheep was increased by the addition of biuret or biuret and urea to the diet.

Digestion and nitrogen balance trials with sheep (Tomlin, Harris and Butcher, 1967) indicate that biuret is comparable to urea and natural proteins when used to supplement a low-protein native hay ration.

Many other workers have found biuret to be satisfactory in low-quality, high-roughage rations (Mies, Thomas and Newman, 1967; Turner and Raleigh, 1969; Tollet et al., 1969; Raleigh and Turner, 1968). Steers wintered on a 50 percent straw ration (Mies, Thomas and Newman, 1967) gained faster than the nonsupplemented steers when urea or biuret was added to the ration, but gained less than steers supplemented with soybean meal. Turner and Raleigh (1969) fed steers a winter growing ration supplemented with either urea, biuret or cottonseed meal. Steers receiving cottonseed meal gained faster than those receiving urea or biuret. Van Horn et al. (1969) supplemented ewes with a 20 percent pellet containing biuret or urea. Weight gains of ewes and pounds of lamb weaned per ewe were not affected by treatment when 0, 12.5, 25 or 50 percent of the equivalent crude protein was replaced with biuret or urea. When heifers on mature native range were supplemented with biuret, urea or cottonseed meal, Raleigh and Turner (1968) noted that the biuret- and cottonseed meal- supplemented heifers gained faster than the urea-supplemented animals when on a high-energy supplement. When fed a low-energy supplement, heifers receiving biuret gained 0.11 kg more per day than heifers receiving cottonseed meal or urea, while the latter two treatments gained 0.23 kg more per day than control animals.

It can be concluded that biuret is comparable to urea and natural protein supplements for ruminants consuming low-quality, high-roughage diets when weight gains are used as criteria for evaluation.

Metabolism of Nitrogen in the Ruminant

Recycling of Urea

Since the postulation of the nitrogen cycle (McDonald, 1948), the metabolism of nitrogen in the ruminant has been studied extensively. McDonald (1948) noted that ammonia present in the rumen can be absorbed into the venous blood and pass to the liver by the portal vein; here the ammonia is converted to urea which can be secreted in the saliva and thus returned to the rumen. In comparing the ammonia levels in blood, it was observed that only traces were present in the general circulation of the sheep, while the venous blood contained larger amounts. The addition of ammonium acetate to the rumen increased the ammonia concentration in the blood draining the rumen.

Transfer of Urea From Blood to Rumen

The utilization of injected urea was investigated by Houpt (1959). Fifty-two percent of the injected urea was not recovered, indicating that blood urea was absorbed by the rumen and utilized for microbial protein synthesis. When rumen contents were replaced with saline, ammonia accumulated after urea was injected intravenously, thus suggesting that urea transferred across the rumen wall and was hydrolyzed to ammonia. Weston and Hogan (1967) infused urea into the abomasum and found a transfer of nitrogen to the rumen similar to the amount infused per abomasum up to 2.5 to 3.5 g/day. The infusion of urea into the rumen or abomasum resulted in a linear increase in blood urea concentration. Changes in the body urea pool (Packett and Groves, 1965) were observed to decrease during feeding and increase during nonfeeding,

suggesting that an energy source stimulates the uptake of rumen ammonia for protein synthesis. Cocimano and Leng (1967) reported a linear relationship between the amount of urea transferred from blood to the rumen and the plasma urea concentration. This relationship was also observed by Ford and Milligan (1969). The ration influenced urea recycling only by its effect on blood urea concentrations.

During normal conditions of the rumen the transfer of urea from the blood to the rumen is characterized by an increase in rumen ammonia. Houpt and Houpt (1968) found that removal of rumen urease by repeatedly rinsing the rumen epithelium with saline resulted in an increase of urea in the rumen. However, when the urease activity was undisturbed, nearly all urea transferred was hydrolyzed to ammonia. It was hypothesized that urea transfers by diffusion and that ruminal urease penetrates the rumen epithelium to hydrolyze the urea to ammonia, which is diffused faster than urea. The net effect is to enhance urea transfer across the rumen wall. Weston and Hogan (1968) estimated the net transfer of nitrogen across the rumen wall of sheep to be approximately 4-5 g/day when blood urea nitrogen levels were 16-18 mg/100 ml. Work with steers (Vercoe, 1968) indicated that the net transfer of urea was 17-20 g/day when plasma urea concentrations were about 12 mg N/100 ml. In the later study the increase in rumen ammonia concentration was proportional to the plasma urea concentration until plasma urea reached about 12 mg N/100 ml. McDonald (1948) estimated absorption of ammonia by the rumen of sheep to be 4.3 g N/day.

Salivary Secretion of Nitrogen

McDonald (1948) calculated that there was 0.5 g N/day returned to the rumen via the saliva. Houpt (1959), as cited by Phillipson (1964), found 1.6 g N/day returned to the rumen via the saliva of sheep. Waldo (1968) noted salivary nitrogen secretion between 0.4 and 0.9 g N/day. The total secretion seemed dependent upon total nitrogen intake of the diet.

It can be concluded that nitrogen transfer across the rumen wall is more important than the nitrogen returned to the rumen by the saliva when total amounts of nitrogen recycled per day are considered.

Excretion of Urea Nitrogen

Sheep on a low-protein, high-roughage diet excreted all of the intravenously-infused urea (McIntyre and Williams, 1970), while sheep receiving an energy source excreted only about 50 percent of the infused urea. An increase in microbial protein is suggested as the site of utilization of the unexcreted urea. Ford and Milligan (1970) noted an increase in urine excretion as plasma urea concentrations increased. A correlation between urine flow rate and urea excretion and between plasma urea concentration and urine flow rate was noted (Cocimano and Leng, 1967). Thornton (1970) also reported that the blood urea levels and urine urea excretion were related. It was suggested that the urine flow rate was influenced by urinary urea excretion, which in turn was influenced by the plasma urea concentration and, therefore, by the filtered load of urea.

Infused Biuret

The known recycling of urea in the ruminant has stimulated research to discover the pathways of biuret when infused either intraruminally or intravenously. Gray and Clark (1964) dosed two wethers with biuret through a rumen fistula and observed that 20 to 30 percent of the biuret was excreted in the urine within 24 hours. Farlin, Brown and Garrigus (1968a) injected ^{14}C labeled biuret intravenously to a wether adapted to dietary biuret and noted that 50 percent was recovered in the urine in eight hours, 87 percent in 24 hours and 94 percent in 48 hours. The rumen fluid and saliva also contained small amounts of ^{14}C activity and 1.4 percent of the ^{14}C was recovered as CO_2 in expired air. When the same sheep was infused with ^{14}C-biuret intraruminally, only 3 percent of the initial dose of ^{14}C was found in the urine and a negligible amount was found in the feces over a 56-hour period. Farlin (1968b) indicated that the amount of biuret excreted in the urine of sheep on a biuret diet decreased as length of time on the ration increased. Schroder (1970) recovered 95 percent of intravenously-injected biuret in the urine within 24 hours. Feces from the sheep infused intravenously contained no biuret. In the same study it was observed that there was some biuretolytic activity in the lower intestinal tract, but that this activity was small compared to the activity of the rumen. There was no evidence to suggest recycling of biuret, and it was suggested that all biuret absorbed into the blood stream was quantitatively excreted in the urine.

It appears that the site of utilization of biuret is in the rumen and the extent of utilization depends upon the presence of biuretolytic organisms. Possibly small amounts of biuret are hydrolyzed in the

lower intestinal tract. Any biuret in the blood stream is excreted in the urine and not recycled or utilized by the tissues.

In Vitro Techniques

In vitro fermentation techniques have been used to measure the rate of fermentation of various substrates by rumen microorganisms. Gas production, cellulose digestion, dry matter disappearance and volatile fatty acid production have often been used as criteria for evaluation of the microbial activity. Adams and Hungate (1950) used the increase in yeast count and sugar disappearance to measure rate of fermentation. Gas production was used (el-Shazly and Hungate, 1965) to estimate rate of fermentation. The rate of production of volatile fatty acids in the rumen has been studied by in vitro techniques (Belasco, 1954; Stewart, Stewart and Schultz, 1958; Hungate, Mah and Simesen, 1961). This technique eliminates the problem of absorption of the volatile fatty acids and movement of digesta through the digestive tract, which occurs in in vivo experiments. Adams and Hungate (1950) suggested that the yeast growth curve or sugar utilization curve could be used to predict rates of fermentation. el-Shazly et al. (1961) observed that in vivo and in vitro volatile fatty acid production were somewhat parallel up to 12 hours. It was also noted that the in vitro rate of production was greater when whole rumen contents were used as the inoculum. It was suggested that similar rates of cellulose digestion by the in vitro and in vivo methods support the volatile fatty acid data. It was observed that little morphological change in the microbial population occurred in periods of 24-30 hours. Carroll and Hungate (1954) have indicated that if anaerobiosis, temperature, pH and buffering capacity are

controlled, the removal of rumen contents need not modify the microbial activity.

Carroll and Hungate (1954) constructed a curve of volatile fatty acids and incubation time and suggested the slope of this curve would represent the rate of production of volatile fatty acids. The slope at zero time would be the rate of production at the time of removal of rumen contents from the animal. Hungate, Mah and Simesen (1961) found that the zero time rate technique was more reliable than manometric estimates, and results from the zero time rate technique were the same order of magnitude as isotope data. Stewart, Stewart and Shultz suggested that in vitro and in vivo volatile fatty acid production differed during long term incubations. This indicates that in vitro studies must be of short duration and conditions must be kept similar to that found in the rumen.

Effect of Nitrogen on VFA Production

Belasco (1954) compared the rate of production of volatile fatty acids when urea or natural proteins were included in the diet. It was noted that urea increased the amount of propionic acid and decreased butyric and valeric acids when compared to equivalent amounts of protein feed meals. The total quantity of acids was not affected by nitrogen source. Orskov and Oltjen (1967) noted that acetic acid proportions were lower and butyric acid proportions higher when urea was included in the diet compared to isolated soy-protein and a natural diet. In another experiment urea, urea phosphate, uric acid and biuret were compared. The nitrogen source had no effect on total concentration of volatile fatty acids in vivo. Urea produced higher concentrations of

propionic than did biuret, while biuret resulted in a greater proportion of valeric acids. Waite and Wilson (1967) noted that supplementation with biuret or urea resulted in no treatment differences in the concentration of total volatile fatty acids of rumen fluid of cows. The proportions of acetic, propionic and butyric were not changed by nitrogen source.

CHAPTER III

MATERIALS AND METHODS

Introduction

Four rations consisting of a weathered bermudagrass hay plus isonitrogenous supplements of (I) cottonseed meal (CSM), (II) biuret, (III) biuret + corn meal (CM) and (IV) urea + CSM + CM were fed to lambs. Twelve lambs fitted with rumen cannulae were alloted to the four treatments with three sheep per treatment.

Rumen contents were removed at intervals throughout the trial for use in the in vitro VFA rate studies. After completion of the rate study, the animals were retained on their respective rations and were used for the biuret infusion study. Various nitrogen fractions of the blood, urine and rumen contents were obtained.

Animals, Equipment and Diets

Twelve wether lambs fitted with permanent rumen cannulae were alloted to four treatments of three lambs each and placed in metabolism stalls. During an adjustment period the lambs were fed ration I, consisting of bermudagrass hay plus 150 gm of cottonseed meal daily. After the adjustment period, the lambs were assigned to the supplementary treatments shown in Table I. The bermudagrass hay was harvested in February from an ungrazed plot to obtain a weathered hay similar to that consumed by animals on pasture during the winter months. As a result

TABLE I

COMPOSITION OF RATIONS AND DAILY FEED ALLOWANCE

	Daily Feed Allowances (g)			
	Ration 1	Ration 2	Ration 3	Ration 4
Low Quality Bermudagrass Hay	450	450	450	450
Supplement 1	168	---	---	---
Supplement 2	---	45	---	---
Supplement 3	---	---	195	---
Supplement 4	---	---	---	179
Supplement Composition	1	2	3	4
	%	%	%	%
Cottonseed Meal	89.3	---	---	41.9
Ground Corn	---	---	77.0	41.9
Biuret	---	60.0	13.8	---
Urea, 281	---	---	---	6.1
Dicalcium Phosphate	3.6	13.3	3.1	3.3
Limestone	3.6	13.3	3.1	3.3
Trace Mineralized Salt	3.6	13.3	3.1	3.3

of high nitrogen fertilization, the hay contained about 9.0% crude protein. One lamb in supplement II died during the trial.

Rumen Sampling and In Vitro Procedure

The rate of production of volatile fatty acids was determined by an *in vitro* technique similar to that of Carroll and Hungate (1954). *In vitro* volatile fatty acid production trials were conducted on days 34, 63 and 82 after the start of the feeding of the supplemental nitrogen. Rumen ingesta for *in vitro* acid production was removed at four and eight hours postfeeding. Sampling of rumen contents was accomplished by applying suction to a hose inserted through the fistula and attached to a collecting flask. Ingesta was transferred to warmed jars and taken to the laboratory as quickly as possible and placed in a water bath at 39°C. A zero time sample was taken at this time for VFA analysis. Carbon dioxide was then bubbled through the flask to displace the air, and the flask was stoppered with a bunsen valve. Samples of rumen fluid were removed at one, two and three hours after the beginning of the fermentation, acidified with 25 percent meta phosphoric acid and frozen until GLC analysis could be performed. Samples of rumen fluid were removed from the flask after mixing by inserting a syringe fitted with a filter of glass wool and withdrawing a 2 ml sample.

The pH of the ingesta was taken at the time of removal from the rumen and at the end of the *in vitro* fermentation.

Chromatographic Analysis

Acidified rumen fluid samples for VFA analysis were allowed to thaw and were then centrifuged at 11,000 x g for ten minutes and the

supernate removed for analysis. Five microliters of the supernate were injected directly into a six-foot U-shaped column of 28 percent carbowax, 20M TPA on 60/80 chromasorb W.[1] A Bendix, series 2500, chromatograph[2] was used for the VFA analysis. The detector, injection and column temperatures were maintained at 250, 225 and 140°C, respectively. Calculation of VFAs was by the rectangular method suggested by Carroll (1961).

Analysis of Data

The rate of VFA production for each treatment and each period was determined at four and eight hours postfeeding. Using actual VFA values, an example of how typical data appears when plotted against time is shown in Figure 1. The values plotted in this figure are averages of the three sheep per treatment. There is a slight trend for these to be curvilinear; however, the curvilinearity was determined to be nonsignificant. It was determined by the use of orthogonal comparisons (Snedecor and Cochran, 1965) that a line fitted through these points was linear.

To further demonstrate how data were obtained, Figure 2 illustrates the calculated regression line plotted along with the change in concentration of acetic acid in the in vitro flask from 0 to 3 hours. The rate of production of volatile fatty acid is the slope of the regression line at zero time. Comparisons between treatments and sampling dates were then determined by comparing the slopes of these lines.

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[1]Wilkens Instrument and Research, Inc., Walnut Creek, California.

[2]Bendix Process Instrument Division, Ronceverte, West Virginia.

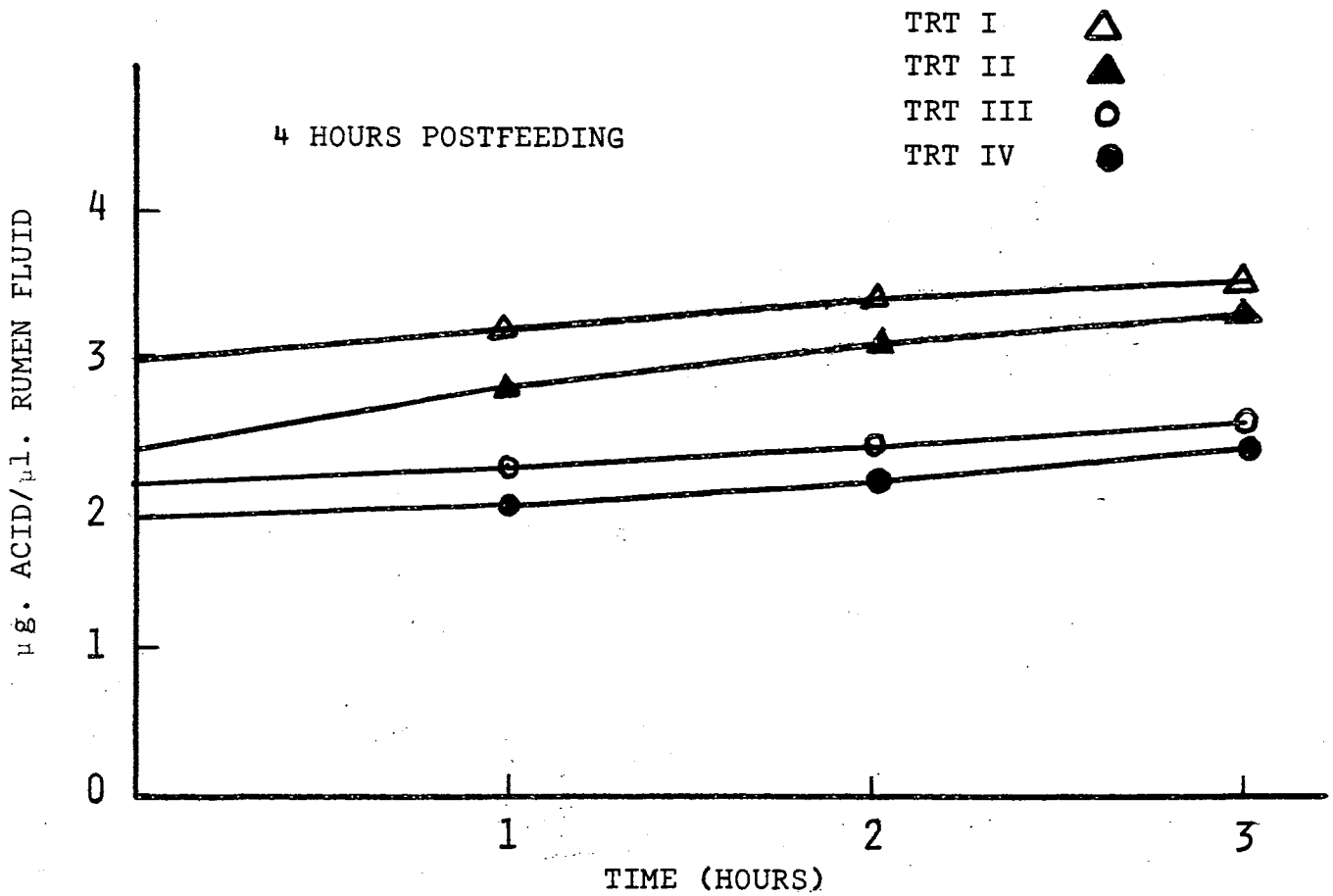

Figure 1. Example of how Typical Data Appear When Actual VFA Levels in the in Vitro Flask are Plotted Against Time

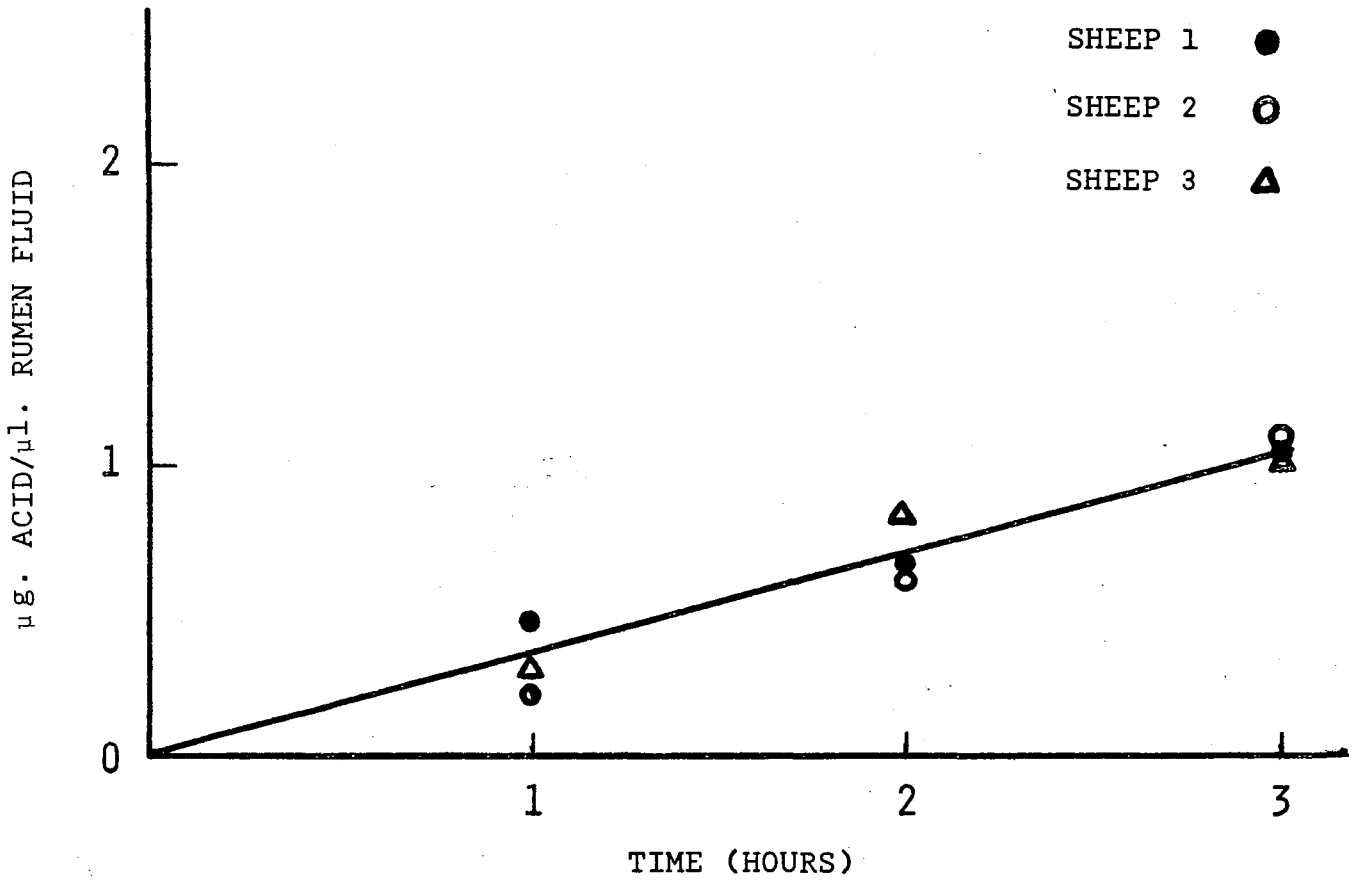

Figure 2. Example of Method Used to Calculate Rate of VFA Production. The line is the average slope for the three sheep in each treatment.

Biuret Infusions

Lambs from treatments I and III were used for intraruminal and intravenous infusions of biuret in an attempt to study the metabolism of biuret by the biuret-adapted and unadapted ruminant. For the intravenous infusions indwelling polyethylene catheters[3] were placed in each jugular vein. A one percent solution of biuret in 0.6 percent saline was infused through one catheter and blood samples withdrawn through the other catheter. A Harvard reciprocal action pump[4] was used for the first two sheep. At this time a peristaltic action pump[5] was used to reduce labor required for the other pump. The rate of infusion and amount of biuret infused are listed in Table II.

For the intraruminal infusions, one catheter was placed in the jugular vein to facilitate blood sampling and the biuret infused through the rumen fistula.

Urine was collected prior to beginning of infusions and at 8, 14, 20, 24 and 48 hours after the start of the infusion. Blood was sampled at 0, 2, 4, 6, 8, 10, 12, 14, 20 and 24 hours from the start of the infusion. Rumen fluid samples were obtained at 0, 4, 8, 14, 20 and 24 hours when biuret was infused intravenously and at 0, 7, 10, 14, 20 and 24 hours when biuret was infused intraruminally.

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[3]Intramedic Polyethylene Tubing, Clay-Adams, Inc., New York, New York.

[4]Harvard Apparatus Co., Inc., Millis, Massachusetts.

[5]New Brunswick Scientific Co., New Brunswick, New Jersey.

TABLE II

TOTAL BIURET INFUSED AND RATE OF INFUSION

Ration	Sheep No.	Type of Infusion			
		Intravenous		Intraruminal	
		g Biuret	Rate g/hr	g Biuret	Rate g/hr
Cottonseed Meal	29	44.0[a]	3.67	--	--
	36	46.4[a]	3.87	23.3	3.88
	39	24.4	4.07	25.6	4.27
Biuret + CM	35	26.5	4.42	25.2	4.25
	38	25.0	4.17	24.2	4.03
	40	25.2	4.20	24.9	4.15

[a]Lambs 29 and 36 were infused intravenously for 12 hours. All other infusions were 6 hours in duration.

Data Obtained and Method of Analysis

Whole rumen contents were removed as previously described in the VFA study and strained through two layers of cheesecloth. Twenty-five ml of strained rumen fluid were added to a tube containing 0.5 ml of $HgCl_2$, centrifuged[6] at 11,000 x g for ten minutes and the supernate frozen for ammonia analysis by the Conway (1957) micro-diffusion method.

Samples of mixed urine were acidified to a pH of less than three and frozen. Urine urea, ammonia and total nitrogen were determined (AOAC, 1960). The biuret content of the urine was determined by a procedure adapted from Gilchrist, Potgieter and Voss (1968). The biuret procedure is given in detail in the Appendix.

Forty milliliters of blood were withdrawn into a heparinized syringe and 20 ml placed into a centrifuge tube containing 20 ml of 20% TCA. After mixing by inversion, this was centrifuged at 11,000 x g and the supernate saved for ammonia analysis. The remaining 20 ml of blood were centrifuged and the plasma removed and frozen for urea analysis. Blood urea was obtained by the Hycel blood urea nitrogen method.[7] Hematocrit was determined on the heparinized blood using a Micro-Capillary Centrifuge.[8]

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[6]International High-Speed Refrigerated Centrifuge Model HR-1, International Equipment Co., Needham Heights, Mass.

[7]Hycel, Inc., Houston, Texas.

[8]Clay-Adams, Inc., New York, New York.

CHAPTER IV

RESULTS AND DISCUSSION

Zero Time Rate Study

The following data are the slopes of the lines representing the increase in VFA over time after the start of the in vitro fermentation. The reader will recall that these lines were linear, so they represent the rate of VFA production at the time of removal of rumen ingesta from the experimental animals. The rate of VFA production for each treatment and period was determined at both four and eight hours postfeeding.

The rates of production of acetic acid at both four and eight hours postfeeding are presented in Table III. There are significant differences between treatments for each sampling date for both four and eight hours postfeeding. On day 34 the rate of VFA production from the cottonseed meal ration was significantly greater ($P < .05$) than from the other three treatments at four hours postfeeding. The situation is somewhat reversed on days 63 and 82 when the rate of production of acetic acid from the urea ration was significantly greater ($P < .05$) than from the other treatments. On day 63 the biuret ration had a lower rate of production than the biuret + CM and cottonseed meal rations. However, on day 83 there were no differences in acetic acid production between the biuret, biuret + CM and cottonseed meal ration.

At eight hours postfeeding the rate of production of acetic acid had different trends than at four hours postfeeding. The urea ration

TABLE III

IN VITRO ACETIC ACID PRODUCTION

Treatment	Sampling Date		
	34	63	82
4 Hours Postfeeding--µg acid produced/µl rumen fluid			
Cottonseed Meal	0.286^{abx}	0.237^{ay}	0.342^{bx}
Biuret	0.155^{ay}	0.072^{ax}	0.275^{bx}
Biuret + CM	0.138^{ay}	0.187^{ay}	0.276^{bx}
Urea + CSM + CM	0.176^{ay}	0.352^{bz}	0.446^{cy}
8 Hours Postfeeding			
Cottonseed Meal	0.198^{x}	0.231^{x}	0.276^{x}
Biuret	0.241^{xy}	0.246^{xy}	0.324^{x}
Biuret + CM	0.275^{xy}	0.266^{xy}	0.348^{xy}
Urea + CSM + CM	0.289^{ay}	0.321^{ay}	0.414^{by}

^{abc}Values in a row not having the same letter differ significantly ($P < .05$).

^{xyz}Values in a column not having the same letter differ significantly ($P < .05$).

promoted a greater rate of production than the cottonseed meal ration ($P < .05$) while the two biuret rations were intermediate for all sampling dates.

The length of time on the ration had varying effects for the different rations at both sampling times. At four hours postfeeding, acetic acid production was greater on day 82 than for day 34 and 63 for the biuret ration ($P < .05$). For the biuret + CM and the urea ration, there was a progressive increase ($P < .05$) in the rate of acetic acid production as time on the ration increased. At eight hours postfeeding there was no increase in the rate of acetic acid production as time on the ration increased for the two biuret rations. However, the urea ration showed a progressive increase across sampling dates.

Propionic acid production (Table IV) at four hours postfeeding followed similar patterns to those of acetic acid. On day 34 the rate from the cottonseed meal ration was significantly greater ($P < .01$) than from the other rations. For day 63 the rate from the urea ration was significantly greater ($P < .05$) than from the other rations, and the rate from the biuret ration was less ($P < .05$) than from the cottonseed meal and biuret + CM rations. Day 82 was similar to day 63 in that the rate of propionic acid production from the urea ration was significantly ($P < .05$) greater than from the biuret-containing rations and that from the cottonseed meal ration was significantly ($P < .05$) greater than from the all-biuret ration. The two biuret rations showed a significant ($P < .05$) increase in the rate of propionic acid production for day 82 over days 34 and 63. The urea ration again showed an increase in rate as length of time on the ration increased; however, the increase in rate from day 63 to day 82 was not significant ($P > .05$).

TABLE IV

IN VITRO PROPIONIC ACID PRODUCTION

Treatment	Sampling Date		
	34	63	82
4 Hours Postfeeding--µg acid produced/µl rumen fluid			
Cottonseed Meal	0.103^{ax}	0.079^{by}	0.112^{ayz}
Biuret	0.042^{a}	0.027^{ax}	0.086^{bx}
Biuret + CM	0.053^{a}	0.059^{ay}	0.098^{bxy}
Urea + CSM + CM	0.044^{a}	0.059^{ay}	0.124^{bz}
8 Hours Postfeeding			
Cottonseed Meal	0.062	0.070	0.080^{y}
Biuret	0.062	0.082	0.093^{xy}
Biuret + CM	0.106^{x}	0.091	0.105^{xy}
Urea + CSM + CM	0.077^{a}	0.082^{a}	0.108^{bx}

^{abc}Values in a row not having the same letter differ significantly ($P < .05$).

^{xyz}Values in a column not having the same letter differ significantly ($P < .05$).

At eight hours postfeeding on day 34, the rate of production of propionic acid was greatest ($P < .05$) for the biuret + CM ration. There was no difference in rate on day 62 for the four rations. On day 82 the rate of VFA production from the urea ration was greater ($P < .05$) than from the cottonseed meal ration. The urea ration showed an increase in rate of propionic acid from day 34 to day 82 ($P < .05$).

Table V presents the rates of production of butyric acid at four and eight hours postfeeding. At four hours postfeeding there were no significant differences between rations on day 34. On days 63 and 82 the rates of production of butyric acid were greater ($P < .05$) from the urea and biuret + CM rations than from the biuret and cottonseed meal rations. The urea and biuret + CM rations supported progressively increasing rates of production as days on the respective rations increased. These increases were all significant ($P < .05$) except for the increase from day 63 to day 82 on the urea ration.

At eight hours postfeeding, butyric acid production rates on day 34 from the biuret + CM and urea rations were greater than the rates from the cottonseed meal and biuret rations ($P < .05$). The biuret ration supported a greater rate ($P < .01$) than the cottonseed meal ration. On day 63 and 82 the biuret + CM ration supported a higher rate of production of butyric acid than did the urea ration ($P < .01$). The urea ration had a greater rate of production ($P < .05$) than the biuret and cottonseed meal rations.

The pH of the rumen ingesta during the *in vitro* fermentation (Table VI) decreased from 0 time to 3 hours *in vitro*. This is evident in all treatments and reflects the acid production by the rumen microorganisms. These observations are in agreement with results of Stewart,

TABLE V

IN VITRO BUTYRIC ACID PRODUCTION

Treatment	Sampling Date		
	34	63	82
4 Hours Postfeeding--µg acid produced/µl rumen fluid			
Cottonseed Meal	0.038	0.030^{x}	0.041^{x}
Biuret	0.023	0.010^{x}	0.027^{x}
Biuret + CM	0.030^{a}	0.048^{by}	0.071^{cy}
Urea + CSM + CM	0.040^{a}	0.058^{by}	0.069^{by}
8 Hours Postfeeding			
Cottonseed Meal	0.019^{x}	0.016^{x}	0.025^{x}
Biuret	0.035^{ay}	0.017^{bx}	0.035^{ax}
Biuret + CM	0.062^{z}	0.064^{y}	0.068^{y}
Urea + CSM + CM	0.052^{z}	0.046^{z}	0.053^{z}

^{abc}Values in a row not having the same letter differ significantly ($P < .05$).

^{xyz}Values in a column not having the same letter differ significantly ($P < .05$).

TABLE VI

IN VITRO pH

Ration	Time of Rumen Sampling			
	4 Hrs Postfeeding		8 Hrs Postfeeding	
	0 Time	3 hrs In Vitro	0 Time	3 hrs In Vitro
Day 34				
CSM	6.77	6.23	6.57	6.13
B	6.87	6.50	6.77	6.33
B + CM	6.67	6.37	6.70	6.33
Urea	6.73	6.40	6.60	6.20
Day 63				
CSM	6.57	6.33	6.50	6.20
B	6.80	6.80	6.65	6.50
B + CM	6.77	6.60	6.57	6.47
Urea	6.57	6.43	6.43	6.23
Day 82				
CSM	6.73	6.20	6.40	6.03
B	6.80	6.50	6.60	6.40
B + CM	6.60	6.30	6.47	6.30
Urea	6.57	6.33	6.47	6.07

Stewart and Shultz (1958) who reported that the pH was lowest during times of greatest VFA production.

The rate of fermentation in the rumen is affected to a great extent by the time since the last feeding. It was expected that the low-quality, high-roughage rations fed would have a slow rate of fermentation. Therefore, rate studies were determined at four and eight hours postfeeding in an attempt to demonstrate differences in the rate of fermentation at these times. The data presented clearly indicate that differences in the rate of VFA production were greater at four hours postfeeding than at eight hours postfeeding. It appears that rate studies with this type of ration should be determined before eight hours postfeeding if differences between rations are to be demonstrated. This suggests that if the zero time rate technique is to be used to evaluate various dietary regimens, the rate must be determined at several times during the day, or a constant rumen fermentation rate must be maintained in the experimental animals.

At four hours postfeeding the biuret ration supported the lowest rate of VFA production, followed by the biuret + CM and cottonseed meal ration, with the urea + CSM + CM ration supporting the highest rate of production. It is evident that rations containing a source of readily-available carbohydrate supported the highest rate of fermentation shortly after feeding. Rations I, III and IV were similar in energy value; however, the addition of an energy source to the biuret ration did little to enhance its ability to produce VFAs.

The cottonseed meal ration supported the lowest rate of VFA production at eight hours postfeeding. Apparently, the high rates of fermentation observed for the cottonseed meal ration and the urea + CSM

ration at four hours postfeeding were supported by the presence of small amounts of available carbohydrates which disappeared rather quickly and were no longer participating in the fermentation to an appreciable degree at eight hours.

Where as the rates at eight hours were generally the same or less than the rates at four hours for rations I and IV. The rates increased from four to eight hours for rations II and III. Thus it appears that biuret may support cellulolytic activity at eight hours as well as or better than the other sources of nitrogen, especially after adaptation. Both biuret rations tended to have a greater rate of VFA production at four hours postfeeding on day 82 than on day 34, suggesting that the lambs were adapting to the ration. By day 82 the biuret rations were beginning to support a rate of fermentation comparable to that of the other rations at both four and eight hours postfeeding.

The data presented in this study indicate that supplementation of low quality forages with biuret may result in livestock production comparable to that from natural protein sources. The addition of an energy source to the biuret supplement improved the rate of VFA production slightly; however, the length of time needed for adaptation to the ration raises question as to its use under range conditions.

Biuret Infusion Study

The mechanism of biuret metabolism in the ruminant is not clearly understood. Bauriedel *et al.* (1971) have recently demonstrated hydrolysis of biuret to ammonia and carbon dioxide by ruminal biuretase preparations; however, the fate of biuret absorbed from the digestive tract is not well known. In an attempt to answer this question, the biuret

adapted and unadapted lambs in the present study were infused with biuret intravenously and intraruminally.

Urinary Nitrogen Excretion

Urinary nitrogen excretion patterns expressed as a percentage of the total 24-hour nitrogen excretion are presented in Figure 3. Differences between the adapted and unadapted lambs for total 24-hour excretions of biuret, urea and ammonia nitrogen were nonsignificant when biuret was infused intravenously. However, lambs on the biuret + CM ration tended to have a greater excretion of urinary urea nitrogen than lambs on the cottonseed meal ration. The percentage of the total urinary nitrogen excreted as urea nitrogen was 31.5 and 23.2 for the respective rations.

Urinary nitrogen fractions were different when biuret was infused intraruminally. Lambs on the cottonseed meal ration excreted 30.5% of the total urinary nitrogen as biuret compared to 17.9% excreted as biuret for lambs on the biuret + CM ration. The difference in percent biuret excreted was significant ($P < .10$). This is in agreement with data from Farlin, Garrigus and Hatfield (1968) and Schaadt, Johnson and McClure (1966) which indicated a reduced urinary excretion of biuret as the animal became adapted to biuret. Urea nitrogen excretion as a percent of the total urinary nitrogen was 47.4% and 73.1% for lambs on the cottonseed meal and biuret + CM rations, respectively. The higher ($P < .10$) level of urinary urea excretion for lambs adapted to biuret suggests that the biuret infused intraruminally was hydrolyzed to NH_3 and CO_2 in the rumen. The proportion of urinary nitrogen excreted as ammonia was also greater ($P < .05$) for the lambs on the biuret ration.

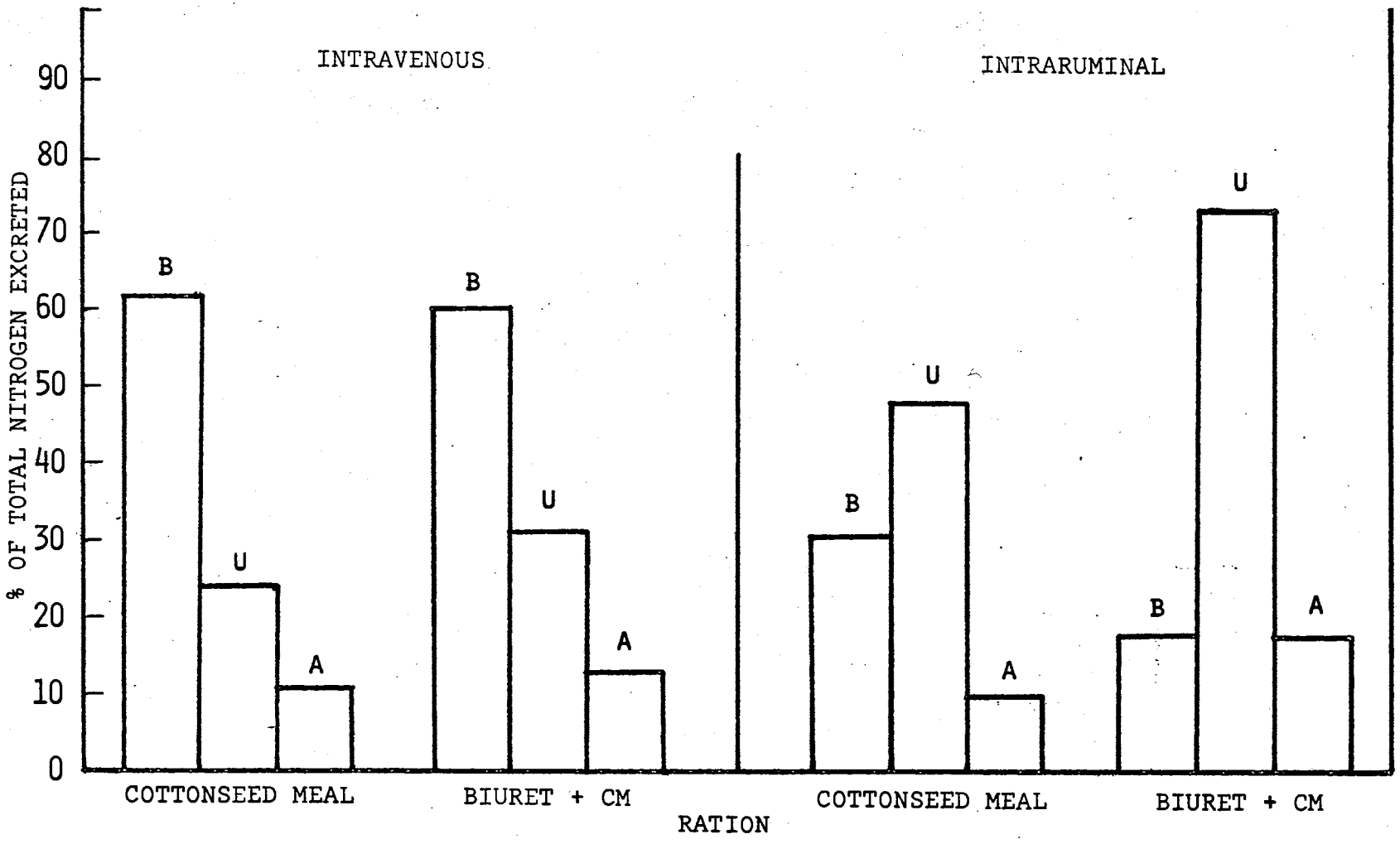

Figure 3. Components of Total Urinary Nitrogen Obtained During Intraruminal and Intravenous Biuret Infusions. B = Biuret Nitrogen; U = Urea Nitrogen; A = Ammonia Nitrogen.

These differences, although not highly significant, suggest biuret was hydrolyzed to a greater extent in the rumen of the biuret-adapted animals. These data also suggest that if biuret is metabolized by the tissues, the percentage utilized is small.

Urinary Biuret Recovery

The percent of intravenously-infused biuret nitrogen recovered in the urine in 24 and 48 hours was not statistically different for lambs on the cottonseed meal and biuret + CM rations (Figure 4). Percent recovery was 52.4 and 64.5 within 24 hours and 70.4 and 80.3 within 48 hours for the cottonseed meal and biuret + CM rations, respectively. This seems contradictory to the amount of biuret excreted when expressed as a percent of total urinary nitrogen; however, the lambs on the cottonseed meal ration excreted more total urinary nitrogen than lambs on the biuret + CM ration. This is a lower recovery rate than reported by Schroder (1970) and Farlin, Brown and Garrigus (1968). However, the above workers infused smaller amounts of biuret than those used in the present study. Schroder (1970) infused 0.025 g of biuret in 5.0 ml of physiological saline and recovered approximately 95% within 24 hours. Farlin, Brown and Garrigus (1968) infused small amounts of ^{14}C-biuret intravenously and recovered 87% and 94% of injected ^{14}C in the urine in 24 and 48 hours, respectively, although the ^{14}C was not identified as biuret. The rate of excretion of biuret in this study may have been changed due to the large volume of saline infused which resulted in a high rate of urine excretion.

Although not statistically significant, there was a greater recovery of biuret in the urine from lambs on the cottonseed meal ration

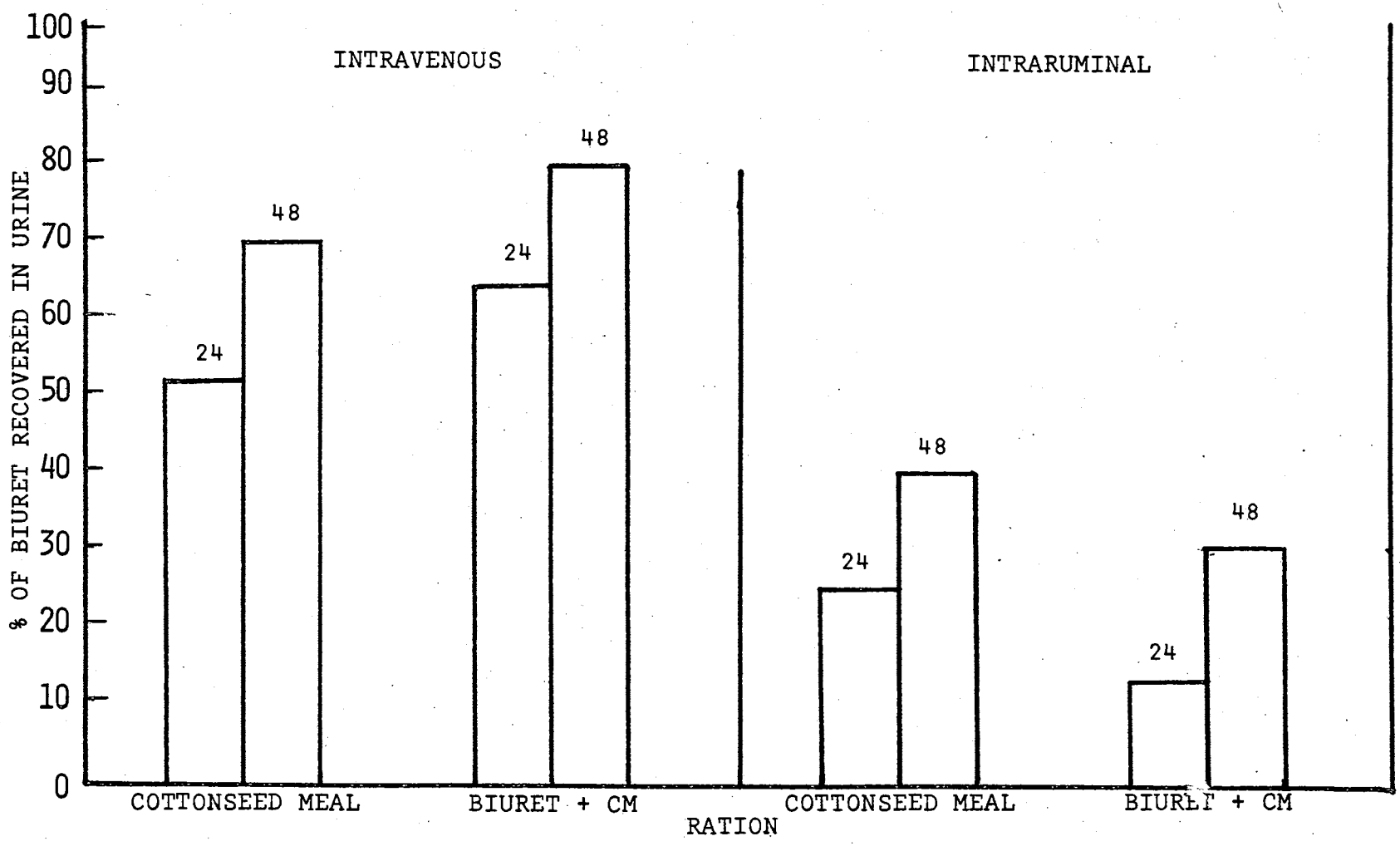

Figure 4. Percent of Infused Biuret Recovered in Urine Within 24 and 48 Hours

than from lambs on the biuret + CM ration when biuret was infused intraruminally. Percent recovery was 25.3 and 13.3 for 24 hours and 38.8 and 30.0 for 48 hours for the cottonseed meal and biuret rations, respectively. Farlin, Garrigus and Brown (1968) found that urinary excretion of biuret decreased as sheep became adapted to the ration. These results are also in agreement with those of Gray and Clark (1964) were 20 to 30 percent of the intraruminally-infused biuret was excreted as such in the urine.

Blood Urea Nitrogen

Blood urea nitrogen levels were not statistically different during the 24-hour period studied (Figure 5). Lambs on the cottonseed meal and biuret rations had a decrease in blood urea levels from the start of the intravenous infusions until 8-10 hours. Within 24 hours, blood urea nitrogen levels had returned to near the original level. The decrease in blood urea nitrogen levels was coincident with a decrease in blood hematocrit values (Table VII) and so could well be a result of hemodilution caused by the large volume of infused biuret solution.

Blood urea nitrogen levels during the intraruminal infusions showed trends that are of interest. The blood urea nitrogen levels decreased for the lambs on the cottonseed meal ration similar to those for the intravenous infusions, although the hematocrit (Table VIII) did not decrease to the extent that occurred during the intravenous infusions. In contrast, the blood urea levels for the lambs on the biuret + CM ration increased slightly up to eight hours. Thus, despite a slight hemodilution, the blood urea nitrogen increased, suggesting the hydrolysis of biuret by the rumen microorganisms.

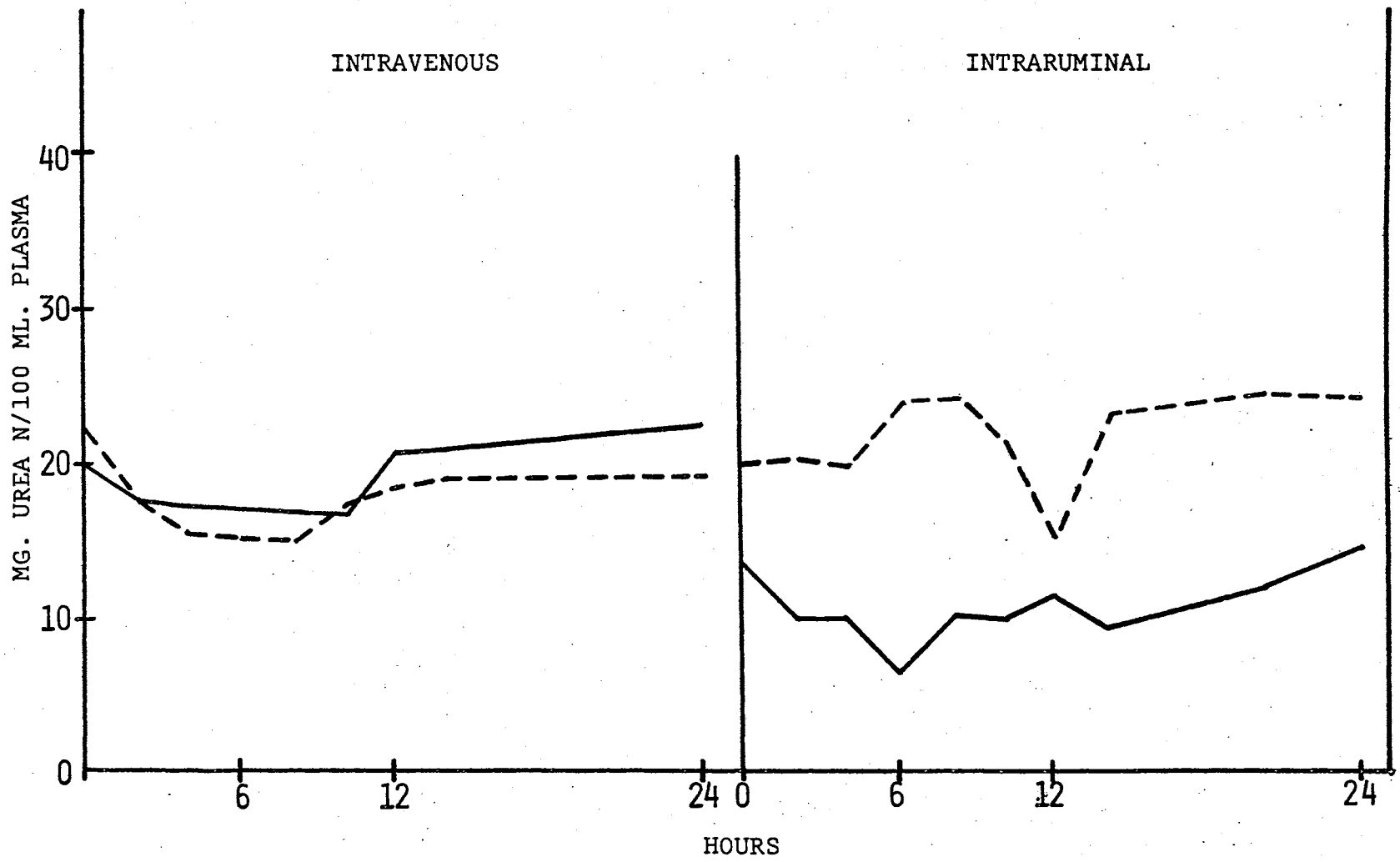

Figure 5. Blood Urea Nitrogen Levels During Intraruminal and Intravenous Biuret Infusions.
—— Cottonseed Meal Ration; – – – Biuret + CM Ration.

TABLE VII

HEMATOCRIT DURING INTRAVENOUS INFUSIONS

	Ration					
	Cottonseed Meal			Biuret + CM		
Sheep No.	36	39	29	35	38	40
Sampling Time (hrs)	% RBC					
00	24.2	22.4	27.5	20.0	21.6	23.0
02	19.5	21.0	24.0	18.5	20.7	19.5
04	17.8	20.2	21.0	18.4	19.0	17.8
06	16.3	18.8	19.4	16.4	19.5	16.8
08	16.3	20.0	16.8	16.8	18.4	16.3
10	15.6	18.4	16.0	16.8	17.5	17.2
12	16.3	18.5	16.3	16.2	18.3	16.0
14	16.9	18.8	17.2	16.8	19.0	16.0
20	18.2	18.6	20.5	17.0	22.0	17.2
24	21.2	19.0	25.5	17.2	21.0	17.0

TABLE VIII

HEMATOCRIT DURING INTRARUMINAL INFUSIONS

Sheep No.	Ration: Cottonseed Meal 36	Cottonseed Meal 39	Cottonseed Meal 29	Biuret + CM 35	Biuret + CM 38	Biuret + CM 40
Sampling Time (Hrs)			% RBC			
00	16.5	16.0	----	28.1	26.1	28.0
02	15.8	----	----	24.1	24.8	24.8
04	16.2	14.8	----	23.5	24.2	23.5
06	----	14.8	----	22.6	----	21.5
08	21.5	14.3	----	22.1	21.5	22.2
10	15.2	16.2	----	25.6	20.6	20.0
12	16.0	13.0	----	22.5	21.5	20.1
14	16.8	13.8	----	20.7	22.4	21.0
20	18.2	15.0	----	21.1	24.6	22.0
24	15.8	11.2	----	20.5	22.0	21.0

Rumen Ammonia Levels

Rumen ammonia levels for lambs infused intravenously were not statistically different (Figure 6). There was a decrease in rumen ammonia levels for lambs on the cottonseed meal ration up to the eighth hour. This decrease in rumen ammonia levels suggests that fluids were transferred into the rumen during the intravenous infusions. Farlin, Brown and Garrigus (1968) found 2.5% of ^{14}C infused intravenously as ^{14}C-biuret in the rumen of sheep; however, the purity of their biuret was not stated and the ^{14}C was possibly recycled as urea.

Rumen ammonia levels for the intraruminally-infused lambs were highly variable and differences were not statistically significant. There was a sharp increase in ammonia levels in the rumen fluid of lambs on the biuret ration. Ammonia levels increased from 213 µg/ml to 378 µg/ml during the eight-hour infusion. Previous data from these lambs (Johnson, 1971) demonstrated their ability to release ammonia from biuret *in vitro*. The same *in vitro* data for the lambs on the cottonseed meal ration showed little ability to release ammonia from biuret. The rumen ammonia levels for the lambs on the cottonseed meal ration were so variable that it is difficult to suggest any trends.

The data from this study suggest that biuret was metabolized in the rumen and not at the tissue level. Intravenous biuret infusions resulted in nonsignificant differences in urinary biuret, urea and ammonia nitrogen or percent recovery of infused biuret. Blood urea nitrogen and rumen ammonia levels did not change significantly during the infusion period. Although nonsignificant, rumen ammonia levels began to rise after four hours of biuret infusion for the lambs on the biuret + CM ration. This might suggest transfer of biuret across the rumen

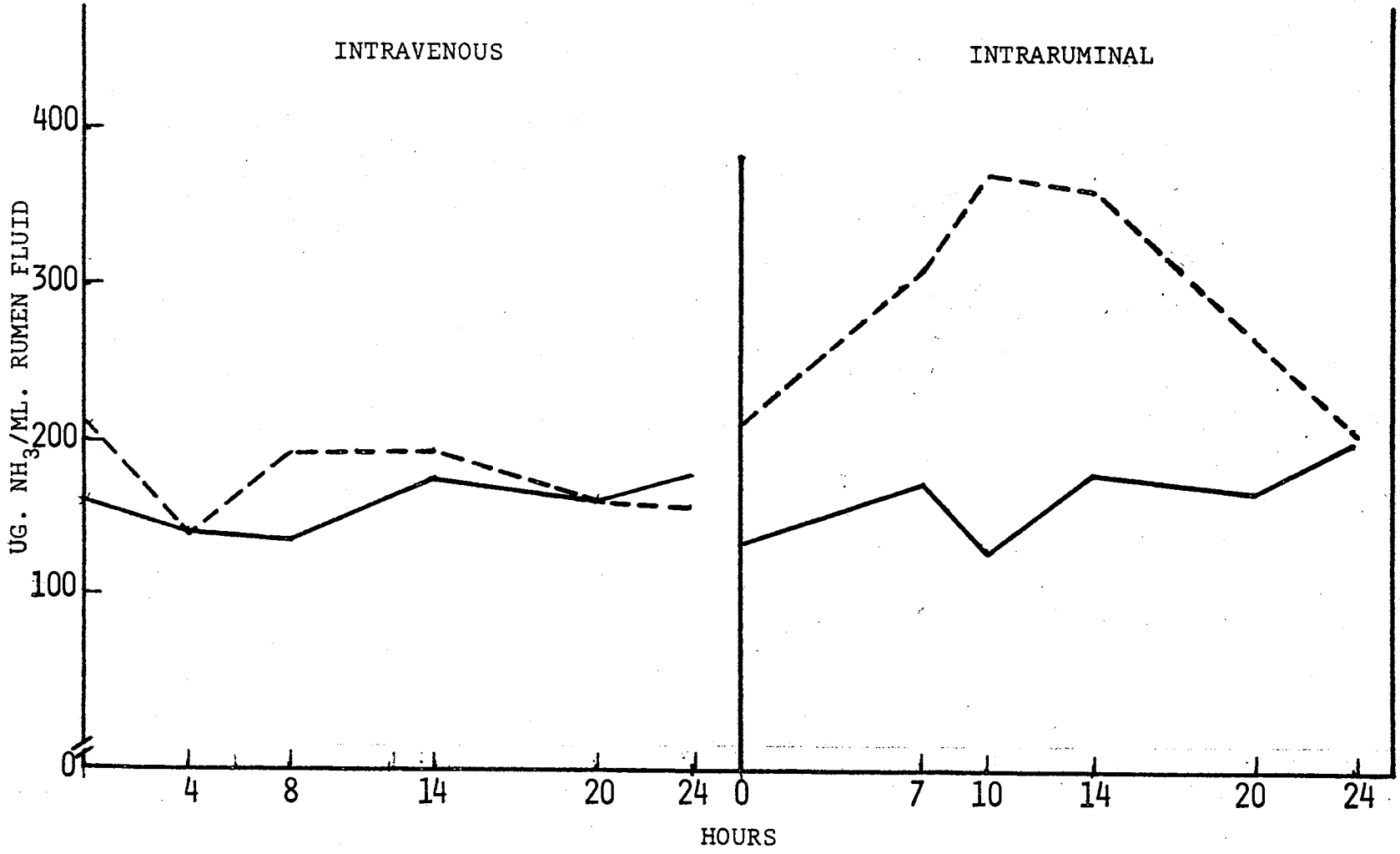

Figure 6. Rumen Ammonia Levels During Intravenous and Intraruminal Biuret Infusions.
—— Cottonseed Meal Ration; – – – Biuret + CM Ration.

wall and hydrolysis by microorganisms in the rumen. Farlin et al. (1968) suggested recycling of biuret similar to urea; however, Schroder (1970) found no evidence for the transfer of biuret from the bloodstream to the rumen.

The intraruminal biuret infusion data indicate that hydrolysis of biuret occurs in the rumen. An increase in rumen ammonia and blood urea nitrogen levels for the biuret-adapted lambs suggests that biuret was hydrolyzed to ammonia by the rumen microorganisms. This is also supported by the decreased urinary excretion of biuret from these lambs.

CHAPTER V

SUMMARY AND CONCLUSIONS

This study was conducted to evaluate the utilization of biuret in lambs as related to *in vitro* VFA production and nitrogen metabolism *in vivo*.

Four rations consisting of a weathered bermudagrass hay plus isonitrogenous supplements of (I) cottonseed meal (CSM), (II) biuret, (III) biuret + corn meal (CM) and (IV) urea + CSM + CM were fed to lambs. The rate of VFA production was measured on days 34, 63 and 82 from the start of the feeding of the nitrogen supplements. Differences in the rate of VFA production between treatments were more evident at four hours postfeeding than at eight hours postfeeding. The biuret-containing rations tended to support a greater rate of VFA production at eight hours than at four hours postfeeding. The rate of VFA production from the biuret rations and the urea ration increased as days on the ration increased. The cottonseed meal and urea rations supported a higher rate of VFA production than the biuret rations.

Lambs on the cottonseed meal and biuret + CM rations were retained for studies with intravenous and intraruminal infusions of biuret. A one percent solution of biuret was infused and various nitrogen fractions of the blood, urine and rumen contents were determined. There were no statistically significant differences in urinary biuret, urea and ammonia nitrogen excretions between the biuret- and cottonseed meal-

fed lambs when biuret was infused intravenously.

When biuret was infused intraruminally, the biuret-adapted animals excreted more urinary urea and ammonia nitrogen and less biuret than the unadapted lambs. Blood urea nitrogen was higher for the biuret-adapted lambs, and rumen ammonia levels increased steadily during the infusion period. The data indicate that biuret was metabolized in the rumen and not at the tissue level.

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APPENDIX

TABLE IX

DETERMINATION OF BIURET

Reagents

Sulfuric acid solution (10 N).

Sodium hydroxide solution (10%). Should be adjusted so 4 ml neutralizer 1 ml of the 10 N sulfuric acid solution.

Zinc sulfate (7%).

Barium hydroxide solution (5%). Adjusted so 10 ml exactly neutralizer 7 ml of zinc sulfate solution. Use CO_2 free water.

Alkaline NaK tartrate solution: Dissolve 110 gm of NaOH pellets in 500 ml of distilled water. Add 100 gm of $NaKC_4H_4O_6 \cdot 4H_2O$ and dissolve. Dilute to 1 liter.

Copper sulfate solution: Dissolve 35 gm of $CuSO_4 \cdot 5H_2O$ in distilled water and dilute to 1 liter.

Color reagent: Mix equal quantities of the tartrate and $CuSO_4$ solutions. Let stand 48 hours before using.

Biuret standard: One percent biuret in water.

Activated charcoal: Powered willow charcoal (pyramid brand, Ehrmann-Strauss Co., New York).

Procedure

Strain rumen fluid through two layers of cheescloth and pipet 25 ml of the strained fluid into a centrifuge tube. Add 0.5 ml of 10 N H_2SO_4 as a preservative. Samples may be preserved at this point by storing at 4°C. Centrifuge at 10,000 r.p.m. for 15 minutes and pipet 10 ml of supernatant fluid into another centrifuge tube. Add 5.0 ml of distilled water, 0.8 ml of 10% NaOH, 7.0 ml of $ZnSO_4$ and 10.0 ml of $Ba(OH)_2$. Stopper and shake and let stand for ten minutes. Centrifuge at 10,000 r.p.m. for 15 minutes, decant supernatant fluid into a flask containing 0.3 gm of activated charcoal. Swirl and let stand for 10 minutes. Filter through Whatman #40 filter paper, add 2 pellets of NaOH to the filtrate and swirl to dissolve. Let stand for 20 minutes. Filter through Whatman #42 filter paper and pipet 15 ml of the filtrate into a test tube. Add 5 ml of color reagent and allow to stand for 15 minutes at room temperature. Read optical density at 550 mμ.

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