UREA ADAPTATION IN RUMINANTS

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ABSTRACT

The effect on calves of a semipurified diet containing 100%, 50% or 0% of total nitrogen as urea was investigated. Three fistulated bull calves averaging 240 kg were used in a latin square design. The calves were fed with a continuous feeder to provide 7 kg of feed daily. Nitrogen retention, rumen ammonia, blood urea and ammonia were determined. Amino acid concentration in the liver, rumen epithelium, rumen microorganisms and blood plasma were also investigated. The activity of glutamate dehydrogenase, glutamine synthetase and carbamyl phosphate synthetase (carbamyl phosphokinase) were estimated in the liver, rumen epithelium and rumen microorganisms.

The digestibility of the protein increased significantly with time on the experiment. The urea containing diets increased protein digestibility when compared to the soybean diet.

Dry matter digestibility was depressed by urea. Blood urea-N concentration was increased significantly on the urea containing diets. There were no significant differences among diets in nitrogen retention or blood ammonia-N concentrations. There was no difference among diets in the amino acid concentrations of the rumen micro-

organisms, but the essential amino acids were decreased and the nonessential were increased in the plasma, liver and rumen epithelium of the urea-fed animals when compared to the soybean-fed animals.

The activity of glutamate dehydrogenase in the liver or rumen epithelium was not affected by the diets, however, the urea-fed animals had a higher activity of this enzyme in the rumen microorganisms when compared to soybean-fed animals.

Glutamine synthetase activity in liver and rumen epithelium was also not affected by the diet, but the 50% urea-N diet had a significantly higher ($P<0.05$) activity of this enzyme in the rumen microorganisms than either 100% or 0% urea diets.

Carbamyl phosphate synthetase activity was very low in either rumen epithelium or microorganisms, however, the activity was high in the liver and increased with time on the experiment for all diets.

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Mn	Manganese
NAD	Nicotinamide-adenine dinucleotide
NADH	Nicotinamide-adenine dinucleotide (reduced form)
NADP	Nicotinamide-adenine dinucleotide phosphate
NADPH	Nicotinamide-adenine dinucleotide phosphate (reduced form)
NFE	Nitrogen free extract
NH	Ammonia
NMN	Nicotinamide mononucleotide
NMNH	Nicotinamide mononucleotide (reduced form)
NPN	Non-protein nitrogen
PEP	Phospho-enol-pyruvate
Pi	Inorganic phosphate
μM	Micromole/liter
UMP	Uridine monophosphate (uridulic acid)

Research has been oriented towards improving the utilization of urea-N taking different approaches: (i) feeding the urea over longer periods of time to give the animal a chance to adapt to this N source, or (ii) by trying to slow the hydrolysis rate of urea in the rumen using urease inhibiting materials, or (iii) feeding small amounts of urea several times a day.

Most of the work cited in the literature has indicated that urea utilization is synonymous with ammonia utilization. Ammonia is utilized in the animal body by the action of three major enzyme systems. These enzyme systems are glutamate dehydrogenase, glutamine synthetase, and carbamyl phosphate synthetase. They are present in both animal tissue and rumen microorganisms.

This investigation was initiated to examine the changes in the activities of these enzymes during the time in which urea was fed, in an attempt to elucidate on the area of urea adaptation.

CHAPTER II

REVIEW OF LITERATURE

Nitrogen Metabolism in Ruminants

Although the use of urea as a protein substitute in ruminant nutrition has become a common practice, its use as the major or sole source of nitrogen is limited by inefficient utilization and the danger of ammonia toxicity. The entrance of large quantities of urea into the rumen and its subsequent rapid hydrolysis results in the release of ammonia at a rate which does not permit efficient use of the nitrogen by rumen micro-organisms for the synthesis of cellular protein. Ammonia not utilized by the rumen flora or fauna is rapidly absorbed into the blood stream where it may reach toxic levels.

These adverse effects of urea might be partially eliminated through (i) the use of more slowly hydrolyzed non-protein nitrogen compounds like biuret; (ii) by trying to slow the hydrolysis rate of urea in the rumen; or (iii) by prolonging the period in which the animal receives the urea containing diets (adjustment period) to give the animal a chance to adapt to this type of nitrogen source.

Comparison of Urea and Other NPN Compounds

Studies by Anderson *et al.* (1959), with digestion and nitrogen metabolism trials, were conducted with lambs to compare uramite (a urea formaldehyde condensation compound), crude biuret, biuret, and creatine with purified soybean protein and urea as a source of nitrogen. It had previously been reported by Kralovec and Morgan (1954) that uramite was not rapidly hydrolyzed in the rumen. It was hoped, however, that the rate of hydrolysis would be much less when uramite was fed with urea and the efficiency of nitrogen would be greater than obtained with urea alone. This study indicated that much of the nitrogen in uramite did not become available and was excreted in the feces. The apparent digestibility of crude protein was significantly reduced when 4, 8, 16, and 32% of the supplemental nitrogen was supplied by uramite. Nitrogen utilization and nutrient digestion were not significantly changed when 50% of the supplemental nitrogen was supplied by crude biuret in comparison with 100% urea. Apparent digestibility of protein was significantly depressed when all supplemental nitrogen was supplied by crude biuret. When pure biuret supplied 100% of the supplemental nitrogen instead of urea, nutrient digestion (organic matter, crude fiber and nitrogen free extract NFE) and nitrogen utilization were significantly depressed. Nutrient digestibility was improved when purified soybean protein or equivalent nitrogen mixture of urea and creatine replaced

urea as the source of supplemental nitrogen (Welch *et al.*, 1957).

Hatfield *et al.* (1959) compared three sources of supplemental nitrogen (urea, a mixture of urea and biuret and biuret) at two levels of feed intake (maintenance and 150% of maintenance). They found that utilization of biuret nitrogen in comparison with urea nitrogen appeared to be influenced by the level of feed intake. Hatfield *et al.* (1955) in a comparison study using urea, biuret, and soybean oil meal as a source of nitrogen for steers found that less nitrogen was retained with the biuret diet than the urea or soybean oil meal diets. Also a high amount of biuret was excreted in the urine on the biuret diet and this may have been the reason for the low nitrogen balance with biuret as compared to urea or soybean diets. The biuret diet also resulted in a low apparent digestion coefficient for the nitrogen.

Meiske *et al.* (1955), in a feeding trial involving fifty 75 lb lambs, fed a 7.13% crude protein basal diet. They found that adding either urea, biuret or crude biuret significantly increased the average daily gain and feed efficiency of the lambs. No significant differences were noted among lambs fed the various supplemental non-protein nitrogen sources. It was concluded that, under the conditions of their experiment, urea, biuret and crude biuret were satisfactory sources of supplemental nitrogen for growing-

fattening lambs. In addition biuret appeared to be less toxic than urea when fed in large amounts. Campbell *et al.* (1963) reported that biuret promoted slightly but not significantly lower growth and feed efficiency than urea in studies with growing Holstein heifers. Biuret supplemented diets resulted in lower nitrogen retention when compared to urea diets for Holstein bull calves.

Ewan *et al.* (1958) compared urea with biuret as a nitrogen source for lambs by feeding a basal diet supplemented with urea or biuret. The data which they obtained indicated that the apparent digestion coefficients for organic matter and dry matter were depressed when the basal plus biuret was compared to basal plus urea. Although nitrogen balance was higher when the basal plus urea was compared to the basal plus biuret, the difference was not significant. Inoculation, however, of the biuret-fed lambs with rumen contents from sheep maintained on biuret increased nitrogen balance.

Oltjen *et al.*(1968) carried out an experiment to evaluate urea, biuret, urea phosphate and uric acid as non-protein nitrogen sources for cattle. They reported that ruminal microorganisms were essentially unable to degrade biuret to ammonia but degradation of uric acid to ammonia occurred. Metabolism results indicated that the apparent digestibility of dry matter and gross energy were significantly greater when uric acid was compared with biuret.

Acid detergent fiber digestibility was significantly less when steers were fed biuret as compared with other NPN sources (Ewan *et al.*, 1958). Percent nitrogen retained for steers fed urea phosphate or biuret was lower than those receiving urea or uric acid. From this data they observed that the amount of biuret excreted in urine daily was much higher in comparison with the other NPN sources and that could be the reason for less nitrogen being retained in the body. Farlin *et al.* (1968) conducted an experiment to compare urea, biuret using urea-^{14}C and biuret-^{14}C. They concluded that carbon of biuret and urea did not equilibrate with the CO_2 pool. Failure to achieve equilibration suggested that biuret and urea were metabolized in the rumen without complete hydrolysis to CO_2 and NH_3. Also they found that 95% of the biuret-^{14}C injected intravenously was excreted in the urine.

Johnson and McClure (1964) reported that biuret feeding had no appreciable effect on rumen ammonia or blood urea; whereas, urea raised both considerably. Biuret was utilized very poorly by starch digesting rumen bacteria taken from sheep adapted to biuret. Furthermore, microorganisms taken from urea or biuret adapted animals failed to release ammonia from biuret when incubated *in vitro*.

Schaadt *et al.* (1966) in a palatability trial with lambs reported that both urea and soybean meal supplemented diets were equally preferred over diammonium phosphate or

diammonium phosphate plus urea. Diammonium phosphate plus urea was preferred over diammonium phosphate. Oltjen *et al.* (1963) reported that diammonium phosphate (DAP) was a satisfactory source of phosphorus, but the nitrogen of this compound was not retained as well as that supplied by urea or soybean meal. Higher urinary nitrogen excretion was found in sheep fed DAP. Considerable difficulty was experienced in obtaining adequate feed consumption on diets containing DAP. The results of this experiment were in agreement with those of Russell *et al.* (1962) with steers and Perez *et al.* (1957) with lambs.

Cowman and Thomas (1962) evaluated some non-protein nitrogen compounds as a source of nitrogen for ruminants by feeding a basal diet supplemented with various sources of NPN compounds. They also compared NPN sources with soybean oil meal and found that average daily gain and pounds of feed per 100 lb gain were as follows: Monoammonium phosphate (MAP), 2.81, 648; urea, 2.97, 665; soybean oil meal, 3.07, 628.

Repp *et al.* (1955) designed an experiment to determine the comparative feeding value of urea, ammonium acetate, ammonium propionate, ammonium formate, formamide and propionamide as protein replacement for lambs. They found that formamide was inferior to the other compounds studied. The remaining NPN compounds appeared to be of about equal value in supporting growth as measured by weight gains of

lambs. At 50% nitrogen replacement level, the NPN compounds did not support as rapid gain as conventional protein. However, at 15% and 30% replacement levels, weight gain with propionamide and urea were equal to those with conventional protein.

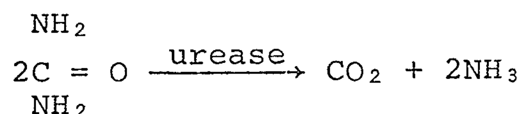
Hoar *et al.* (1968) conducted an experiment to compare utilization of soybean meal, urea, and sodium nitrate when added to a low protein diet, and urea-nitrate interaction. The control diet was corn and corn silage. They found that sodium nitrate (2.5% of the diet) tended to reduce weight gain when fed with a diet which contained soybean meal (7% air-dry basis) but it had only a slight effect in diets with an equivalent amount of crude protein from urea (1% air-dry basis) where gains were already below that of lambs fed soybean meal. In these instances, lambs fed urea gained 16.5 - 19.5% less than lambs fed soybean meal. In another trial the crude protein content of an 8.04% protein ration was increased to 9.54% using soybean meal, urea or sodium nitrate to furnish equivalent amounts of nitrogen. Under these conditions the three nitrogen sources were utilized equally well as sources of crude protein.

Acord *et al.* (1966) studied the effectiveness of some selected ammonium salts, amino acids, amides and amidines as nitrogen sources for *in vitro* starch digestion by rumen microorganisms and compared them with urea. Each nitrogen source was added to provide 3, 6, and 9 mg of nitrogen per

20 ml of an incubation mixture containing washed rumen microorganisms, minerals, buffer and approximately 100 mg of purified corn starch. Ammonium sulfate, ammonium chloride, ammonium acetate, and ammonium phosphate were equivalent to urea at all levels tested. Aspartic acid was inferior to urea but significantly more effective than the other amino acids tested. Arginine, serine, methionine, valine, and glutamic acid additions resulted in moderate to slight increases in starch digestion. Lysine was not effectively utilized. Acetamide, propionamide, butyramide, succinamide, malonamide, guanidine acetate, or aminoguanidine bicarbonate did not consistently stimulate starch digestion. High ammonia levels after 4 - 8 hrs of fermentation were associated with stimulation of starch digestion.

Urea Hydrolysis

The reaction for the enzymatic degradation of urea to ammonia and carbon dioxide is given below:



$$2C(NH_2)_2 = O \xrightarrow{\text{urease}} CO_2 + 2NH_3$$

The breakdown of urea to ammonia and carbon dioxide in the rumen was reported as a result of rumen microorganisms urease activity (Jones, 1967; Hill *et al.*, 1962; Houpt and Houpt, 1968 and Abdel Rahman and Decker, 1966). Pearson and Smith (1943b) found that urea hydrolysis increased as temperature increased to a maximum of 49°C, then decreased

steadily. Optimum pH for hydrolysis was 7 to 9 with little activity below 3 or above 9.5. Because urease activity in the dietary constituents was small and it was not secreted into the rumen, they concluded that rumen microorganisms possess the ability to degrade urea. They observed that 100 g of rumen contents could convert 100 mg urea to ammonia in 1 hr. Brent and Richardson (1967) also indicated that rumen urease was intracellular in nature.

Little is known about the organisms responsible for the bulk of urease activity. Hungate (1966) concluded that many bacterial types contribute to the process. Gibbons and Doetsch (1959) have reported that many species of facultative anaerobic rumen bacteria exhibit ureolytic activity and that this activity was limited among the anaerobes. Carroll (1961) has shown urease to be a constitutive enzyme of ruminants fed either natural or synthetic diets. Jones *et al.* (1964a) reported that a limited number of the viable bacteria in strained rumen fluid were associated with urease production in the rumen contents of urea-fed sheep. The production of ammonia in the rumen by protozoa has not been extensively investigated.

The hydrolysis of urea in the rumen is considered to be a major problem in practical feeding of urea. Bloomfield *et al.* (1960) indicated that the hydrolysis of urea in the rumen occurred four times faster than uptake of the liberated ammonia and thereby, resulted in an eventual loss of N

available for microbial protein synthesis. Feeding a urea purified diet was reported to elevate ruminal ammonia concentrations (Chalupa *et al.*, 1964; Oltjen and Putnam, 1966; Oltjen *et al.*, 1968; Little *et al.*, 1963 and Tagari *et al.*, 1962) resulting in a high urinary N loss which accounted for a large amount of the difference in performance of ruminants fed protein versus urea containing diets. Urea toxicity may also be caused by an elevated blood ammonia concentration. Wang and Tarr (1955) indicated that ammonium carbamate was an intermediate during urea hydrolysis, which was a highly toxic compound. However, Soejima *et al.*(1959) presented evidence to exclude ammonium carbamate as an intermediate in the hydrolysis of urea by urease. Research has also been directed to overcome the problem through the inhibition of urease activity. Pearson and Smith (1943b) found that quinone and sodium chloride had an inhibitory effect on rumen urease activity. Streeter *et al.* (1969) conducted an experiment to study the inhibitory effect of three levels of acetohydroxamic acid (AHA) (90, 180, and 360 mg AHA/kg of body weight) on urease activity in the rumen content of urea-fed sheep. They found that rumen ammonia peaks were depressed with all levels of AHA as compared to that of the same animals when no AHA was administered. However, only the high level of AHA depressed the ammonia peaks for the entire experimental period and resulted in a relatively sustained release of ammonia. The animals

receiving AHA retained a significantly ($P<0.05$) larger percent of the dietary N than the controls. AHA had little effect on the digestibility of the diet or on bacterial or protozoal numbers. Kobashi, Hase and Uehara (1962) reported that AHA was both a potent and specific inhibitor of plant urease. Fishbein (1966 and 1967) found that AHA had a low toxicity when administered orally to mice.

Most of the work previously cited has indicated that urea is rapidly hydrolyzed to ammonia and carbon dioxide making urea utilization virtually synonymous with ammonia utilization. However, work by Farlin *et al.* (1968) questions the theory that all urea was converted to ammonia before utilization. By infusing either ^{14}C-labeled urea or sodium bicarbonate into the rumen, they observed that the carbon of urea did not equilibrate with the carbon dioxide pool, which suggests that urea was metabolized in the rumen without complete hydrolysis to carbon dioxide and ammonia, and that it need not be hydrolyzed for utilization of the nitrogen.

Ammonia Absorption

Ammonia absorption was first demonstrated as the disappearance of nitrogen from the rumen by McDonald (1948). He estimated that the quantity of ammonia-N absorbed from the rumen may be 4 to 5 g per day in sheep. Later studies by Lewis *et al.* (1957) raised that estimate to as high as 14 g ammonia-N in 24 hr. Hogan (1961) showed that ammonia

absorption depends on the concentration gradients at pH 6.5, and that absorption was nil at pH 4.5.

McLaren *et al.* (1961) indicated that some ammonia may be utilized by the rumen mucosa in synthesizing L-glutamate. Hoshino *et al.* (1966) confirmed that rumen ammonia was incorporated into glutamate and that a portion of glutamate was absorbed into circulation after it was converted to glutamine in rumen mucosa.

Urea Toxicity

The toxicity of urea as a non-protein nitrogen source in ruminant rations was reviewed by Lewis (1960) and Ward *et al.* (1969); the biochemical and metabolic changes which occurred during urea toxicity were reported by Coombe *et al.* (1960), Repp *et al.* (1955). Thillman and Sidhu (1969), Visek (1968) and Clifford and Prior (1970) investigated the intermediary metabolic manifestations of ammonia toxicity. The source of non-protein nitrogen has been limited almost entirely to urea, and many compounds are either more toxic or contain less available nitrogen than urea (Hale, 1956; Russell *et al.*, 1962). The urea level required to obtain toxic symptoms as well as the blood ammonia nitrogen levels associated with toxicity varied considerably (Repp *et al.*, 1955; Russell *et al.*, 1962). Conditions protecting the animal from urea toxicity were also quite variable, for urea administered with the ration was less toxic than when administered separately (Dinning *et al.*,

1948); in addition, protein, carbohydrates and fasting apparently influenced the toxicity of urea (Church, 1969; Clark *et al.*, 1951; McBarron and McInnes, 1968).

Kromann *et al.* (1971) investigated urea toxicity, as influenced by various nutritional and physiological factors, with 160 sheep. The variables (energy levels, protein levels, age, period of fasting and urea levels) were studied in a 2^5 factorial design. The two energy levels were a low energy (15% concentrate) and a high energy (85% concentrate) ration; and the two digestible protein levels were 3.8 and 8.8%. Lambs 5 months of age, and ewes approximately 5 years of age were the two age groups. The sheep were administered either 44 or 176 g urea per 100 kg live weight at 1 or 24 hours after feeding. The effect of these various factors was determined by blood NH_3-N levels at 0, 30, 60, 90 and 120 minutes after treatment and by mortality. The animals survival and blood NH_3-N levels were influenced by all factors studied. The highest mortalities and highest blood NH_3-N levels occurred with animals fed the high-energy low protein diet; thus, the ratio of energy to protein was most important. The lambs were more susceptible to urea toxicity than the ewes. The period of fasting, *per se*, had very little effect on the toxicity of urea. The mortality and blood NH_3-N levels were directly related to the urea dose level. The LD_{50} of urea was 1.45 ± 0.07 log urea dose (g/100 kg body weight).

The increase in blood pyruvate and αketoglutarate and liver glucose in urea-fed sheep indicates altered carbohydrate metabolism (Prior *et al.*, 1970). In lambs given 0.33 g urea/kg body weight, concentrations of blood glucose, pyruvate, αketoglutarate, nonesterified fatty acids and acetate were elevated in urea-fed lambs (Prior *et al.*, 1970 and Chalupa and Opliger, 1969). In rats acutely intoxicated with ammonia, blood glucose, pyruvate, citrate and αketoglutarate have also been elevated (Clifford *et al.*, 1969 and Prior *et al.*, 1969). This evidence signifies that ammonia or the products of urea hydrolysis alter the glycolytic and tricarboxylic acid pathways of metabolism.

Katunuma *et al.* (1966) have suggested that ammonia inhibits the conversion of isocitrate to αketoglutarate. These workers have shown that ammonia *in vitro* activates pyridine nucleotide oxidase which oxidizes NADH and NMNH (reduced form of nicotinamide mononucleotide) to NAD and NMH (nicotinamide mononucleotide), respectively. Matsuda and Katunuma (1969) have shown that ammonia also activates a pyridine nucleotide pyrophosphatase in rat liver mitochondria or microsomes which cleave NADH and NADPH to NMNH. Thus, if ammonia activates the pyrophosphatase pathway of NADPH breakdown, a decrease in the total NADP + NADPH would be expected and this was observed in the liver of urea-fed lambs by Prior *et al.* (1970).

Tryptophan , nicotinic acid and nicotinamide can

serve as precursors for the *in vivo* synthesis of pyridine nucleotides (Mahter and Cordes, 1966; and Dietrich *et al.*, 1968). Ruminal concentrations of nicotinic acid and the synthesis of this vitamin were decreased in lambs fed urea as the primary nitrogen source (Buziassy and Tribe, 1960).

All the data listed above show that urea in ruminant diets can cause marked changes in intermediary carbohydrate and nitrogen metabolism. Many of the alterations in carbohydrate metabolism may be mediated through altered pyridine nucleotide metabolism, which would undoubtedly be due to products of urea hydrolysis arising in the digestive tract. The possibility exists that there was increased need for precursors of pyridine nucleotides to partially overcome the decreased performance of urea-fed animals.

Urea Recycling

Not all blood formed urea was eliminated by the kidney. Houpt (1959) indicated that 52% of urea injected intravenously into mature sheep receiving a low-protein, carbohydrate-supplemented ration was not recovered in urine, did not remain in body fluids, and presumably was recycled to the rumen for utilization by rumen microorganisms in protein synthesis. Part of this urea returns to the rumen by way of saliva. McDonald (1948) demonstrated the presence of salivary urea. Baily and Balch (1961) found that urea-N represented 77% of the total nitrogen in mixed

cattle saliva. Higher salivary concentrations of total N resulted in higher percentages of urea-N. The urea content of mixed saliva was about 65% of plasma urea concentration which ranged from 4 to 19 mg urea-N/100 ml. Somers (1961) found that urea represented 60 to 70% of total nitrogen in mixed and parotid saliva. Concentrations of urea-N in blood and saliva were significantly positively correlated.

Urea also returns to the rumen by influx across the rumen wall. Packett and Groves (1965) reported that ruminant venous blood concentration was sufficiently high to suggest a direct transfer of blood urea into the rumen. They also suggest that more urea passes directly from the blood to the rumen through the rumen epithelium than enters by the way of saliva. This hypothesis was proven correct when Houpt (1959) replaced the rumen contents of sheep with saline and measured the return of urea-N to the rumen. He found that of the 5.2 mmoles urea-N/hour returned to the rumen. Only 0.3 mmoles was found in the saliva. The remaining 4.9 mmoles/hour were attributed to absorption through the rumen wall.

Ammonia Metabolism in Ruminants

Although urea as a protein supplement has been one of the major developments in ruminant nutrition and its function in this respect has been well established, our knowledge of the mechanisms involved in the breakdown and

utilization of urea by ruminants is still incomplete. Urea is rapidly hydrolyzed to ammonia and carbon dioxide in the rumen, making urea utilization virtually synonymous with ammonia utilization.

The importance of rumen mucosal urease, which was of bacterial origin, was recently discussed by Houpt and Houpt (1968) and Abdel Rahman and Decker (1966). These workers hypothesized that urea passed from the circulatory system into the basal cells of rumen epithelium by diffusion. Bacterial urease can also penetrate the rumen epithelium and hydrolyze urea to ammonia and carbon dioxide, and ammonia molecules being smaller and more lipid-soluble were able to penetrate the horny cells of the rumen mucosa more readily. Thus urease in rumen mucosa enhances the rate of N transfer to the rumen. They also found that rumen mucosa from urea-fed sheep had less ureolytic activity than sheep fed no urea and this was probably a reflection of decreased ureolytic activity of rumen bacteria limiting N transfer across the rumen mucosa to the rumen. Merino and Raun (1964) and Chalupa *et al.* (1970) also reported the presence of urease in rumen mucosa.

Ammonia was found to be the most important source of N for ruminal bacteria (Bryant and Robinson, 1962), and obtaining comparable rates of hydrolysis and fixation of liberated ammonia into cellular protein remains a major factor limiting efficient use of urea-N (Chalupa *et al.*

1968; and Bloomfield *et al.*, 1960).

Chalupa *et al.* (1970) investigated the potential pathways of ammonia metabolism in rumen bacteria and rumen mucosa using enzymatic criteria in sheep fed purified diets in which the sole nitrogen source was either urea or soy protein. They found that amination and transamination reactions appear to be the major mechanisms for ammonia assimilation by rumen bacteria. Carbamyl phosphokinase was found to play a minor role, while glutamine synthetase which was suggested to be of substantial importance in utilization of ammonia by rumen bacteria was not affected by N source. Extracts of rumen bacteria obtained from urea-fed sheep exhibited lower specific activities of urease, glutamate-oxaloacetate and glutamate-pyruvate transaminases, and glutamate dehydrogenases than comparable preparations from soy protein fed animals. This may be the result of dietary nitrogen sources altering rumen microbial populations or rumen environments or both. Although activities of enzyme systems which utilize ammonia exceeded those which produce ammonia, the higher rumen ammonia concentrations in urea-fed sheep supports the postulation that the rate of ammonia production from urea was greater than the rate of ammonia utilization. Since actual rates of products formation are not controlled by enzyme activities alone, insufficient amounts of substrates, cofactors, energy and cell permeability may be largely responsible

for ammonia utilization in the rumen to occur at a lower rate than its production from urea.

Hoshino *et al.* (1966) found increases in rumen glutamine concentration with urea feeding and suggested that glutamine was quite labile in the rumen. Production of glutamine utilizes an additional molecule of ammonia, thus providing at least a temporary reservoir for this metabolite which may then be used in subsequent metabolism either by a glutaminase reaction or by participation in a transaminase reaction. Bryant and Robinson (1961) and Warner (1964) demonstrated the presence of glutaminase in microbes obtained from the sheep rumen.

Carbamyl phosphokinase was found in many microorganisms, and Niederman and Wolin (1967) indicated that carbamyl phosphokinase was involved in the synthesis of arginine by rumen microorganisms. Beckwith *et al.* (1962) and Thorne and Jones (1963) indicated that the substrate concentration and pH required to satisfy the thermodynamic and kinetic needs of the forward reaction (carbamyl phosphate synthesis) of this reversible system would almost have to be unphysiological.

Since ammonia ions as well as urea have been reported to influence either directly or indirectly the activities of urease (Wall and Laidler, 1953) and glutamate dehydrogenase (Inagaki, 1959), the N source *per se* may have been responsible for changing enzyme activities

especially with urea fed animals. This may result from a deficiency of amino acids which could decrease enzyme synthesis.

Amino Acids of Rumen Microbial Population and Tissue

The amino acid composition of rumen bacteria and protozoa has been studied by Weller (1957), Bergen *et al.* (1967) and Meyer *et al.* (1967). Purser and Buechler (1966) analyzed 22 strains of rumen bacteria grown in pure culture. The organisms used were selected to present some of the predominant strains found in the rumen when either concentrates or roughages were fed. Similarities in amino acid composition of mixed populations of rumen bacteria or protozoa were remarkable when one considers the environment, experimental, feed, and species variables involved. However, Bergen *et al.* (1967) found considerable variation among individual strains by studying protein quality of individual rumen bacteria using an *in vitro* enzymatic digestion system. Furthermore, the proportion of essential amino acids released during the digestion of different bacterial strains varied markedly, which suggested that modification of the bacterial population may be an important factor in the nitrogen status of an animal and its response to dietary changes.

Analysis for essential amino acids by Weller (1957) showed that rumen protozoa contained greater amounts of

iso-leucine, leucine, phenylalanine, and lysine than bacteria. Meyer *et al.* (1967) also found differences between amino acid compositions of protozoa and of bacteria.

Evidence that the ruminant was capable of converting urea-N into animal protein has been obtained through isotope studies. Watson *et al.* (1949) using urea labelled with ^{15}N and Land and Virtanen (1959) using an ammonium salt labelled with ^{15}N, showed that urea and ammonium nitrogen can be incorporated into body proteins and milk proteins, respectively.

Loosli *et al.* (1949) fed a purified diet containing urea as the N source to sheep and goats. Rumen contents of the animals contained 9 to 20 times more amino acids than the ration fed, and all 10 essential amino acids were synthesized in large amounts. Similar results were obtained by Virtanen and Lampila (1962) and Duncan *et al.* (1953).

Virtanen (1966) fed lactating cows purified carbohydrates with urea and ammonium salts as the sole sources of nitrogen. He found that the concentrations of most of the free amino acids, particularly the essential amino acids, in the plasma and whole blood of the lactating cows fed the purified diet were lower than the concentrations in plasma and blood of cows fed a normal diet. Histidine decreased relatively more than the decrease for any other amino acid in the plasma of urea-fed cows.

Little *et al.* (1966) determined the free plasma

amino acid concentrations of steers fed rations supplemented with either soybean meal or urea and found that substituting urea for soybean meal decreased plasma levels of lysine, iso-leucine, valine, proline, and methionine. A similar study by Freitag *et al.* (1968) showed that the concentrations of valine, iso-leucine, lysine, leucine, phenylalanine, arginine, and glutamic acid were significantly higher in the plasma of steers fed a soybean meal-supplemented diet than in plasma of steers fed a urea-supplemented diet. Theurer *et al.* (1968) fed lambs semi-purified diets supplemented with either soybean meal, corn gluten meal, or urea. Blood plasma amino acid patterns differed considerably among lambs fed nitrogen from different sources. Oltjen and Putnam (1966) detected serine and glycine in significantly greater quantities, and valine, iso-leucine, leucine, and phenylalanine in significantly smaller quantities in blood plasma of steers fed a urea-containing diet than in one containing isolated soy protein.

McLaren *et al.* (1961) and Hoshino *et al.* (1966) found that rumen mucosa exhibits the ability to synthesize glutamic acid.

Factors Affecting Utilization of Urea Nitrogen

Effect of Carbohydrates

The effect of carbohydrates on the conversion of urea nitrogen to protein has been studied by Mills *et al.* (1942). They measured the amounts of protein and ammonia nitrogen in the rumen ingesta at different times after the feeding of various rations. When timothy hay was fed with urea, the level of protein in the rumen ingesta was the same as that when the hay was fed alone or in combination with starch. These findings suggested that timothy hay alone did not provide a suitable medium for the bacterial synthesis of protein from urea. The addition of starch to the timothy hay - urea ration resulted in an increase in the protein content of the rumen ingesta.

In other experiments Mills *et al.* (1944) studied the influence of molasses upon the synthesis of protein from urea. The addition of urea to a ration of molasses and timothy hay increased the true protein level of rumen contents from 7.0 to 9.3%. However, the addition of starch to a ration of timothy hay, molasses and urea resulted in a further increase in true protein content to 11.0%.

The value of urea as a source of nitrogen in rations containing different high-carbohydrate feeds was studied by Bell *et al.* (1951) in a series of digestion and nitrogen balance trials conducted with steers. A greater improve-

ment in nitrogen retention resulted from the addition of urea to a corn containing ration than from the addition of urea to a ration containing cane molasses. The other carbohydrate sources (sucrose, glucose and lactose) appeared to be about equal to corn (Gallup *et al.*, 1954).

The value of dextrose, sucrose, starch, cellulose, molasses and ground corn cobs as sources of energy for the conversion of urea N to protein N were examined *in vitro* by Arias *et al.* (1951). Each source of energy aided urea utilization when cellulose was digested. However, when relatively large amounts of readily available carbohydrates were added to the media, the extent of cellulose digestion or urea utilization was reduced. On the other hand, small amounts of readily available carbohydrates seemed to stimulate the digestion of cellulose and the utilization of urea. They concluded that urea utilization *in vitro* was greatest when medium amounts of both readily available and complex carbohydrates were present. Mills *et al.* (1944) however, suggested that the presence of cellulose and other carbohydrates more complex than starch did not result in appreciably increased utilization of urea.

The addition of animal fat to cattle ration has become common and results have been generally satisfactory (Esplin *et al.*, 1963). In a study of the relationship between animal fat and urea in complete mixed rations Bradley *et al.* (1966) reported that rate of gain was

consistently depressed by simultaneous additions of fat and urea. Adding fat to a control diet reduced ($P < 0.01$) the digestibility of dry matter, energy and NFE. When urea was added along with fat, digestibility of crude protein was also reduced ($P < 0.01$). The addition of calcium, lysine or corn distillers dried grains with solubles to rations containing both fat and urea did not improve feedlot performance.

Meiske and Goodrich (1966) concluded that with high energy feeds, cattle fed high-urea supplements consume slightly less feed and gain slightly less than cattle fed preformed protein supplements.

It has been suggested by Bloomfield *et al.* (1958) that the ratio of amylose to amylopectin might influence the utilization of urea nitrogen by rumen microorganisms. Nitrogen balance data obtained by Smith *et al.* (1957b, 1958) with lambs fed corn cobs, wheat straw, bagasse pith or oat-mill feed in rations in which 67% of the nitrogen was furnished by urea, indicated that nitrogen utilization was influenced by the source of roughage carbohydrate. Nitrogen utilization was increased by corn cobs and decreased by bagasse pith and oat-mill feed when compared with the results obtained with lambs fed wheat straw. These workers also showed that replacement of various amounts of wheat straw with equal quantities of a mixture of dextrose and starch improved nitrogen utilization in lambs fed rations

containing urea.

Barth (1962) determined the influence of supplementary readily available carbohydrates on the utilization of nitrogen by lambs fed all-urea supplemented ration. The reduced regression equation was

$$Y = 10.08 + 0.0354\ X_4$$

where Y represents regression of percent nitrogen utilization and X_4 represents the gross energy from readily available carbohydrates. Increasing the readily available carbohydrate from 1200 cal. per day to 1900 cal. per day resulted in a linear increase in nitrogen utilization of approximately 60%. This type of increase was in agreement with the report of Fontenot *et al.* (1955) on the favorable influence of added dextrose on nitrogen utilization by steers fed wintering-type rations containing at least 10% preformed protein. However, it was contradictory to the conclusion of Drori and Loosli (1961) that indicated ration glucose or starch may be responsible for the poor utilization of urea by ruminants.

McDonald (1966) reported that a purified diet containing 25% cellulose was optimal for urea nitrogen utilization and that gain and feed efficiency was 73% for lambs fed urea as compared with isolated soy protein.

Oltjen and Putnam (1966) found that replacing glucose in the purified diet with additional starch resulted in a

significant improvement in nitrogen (either urea or isolated soy protein) digestibility, but also caused a significant increase in urinary nitrogen losses which resulted in little net effect on nitrogen balance. Nitrogen retention of steers fed urea was 60% of that of steers fed the isolated soy protein diet.

Effect of Protein

According to the literature, the ability of ruminants to utilize simple nitrogenous compounds as a source of protein can be explained by the synthesizing ability of the microorganisms of the forestomachs. The activity of the microorganisms depends upon the kind of ration fed to the host animal. As a result of an early experiment, Wegner *et al.* (1941) reported that when a ration in which urea composed 1 - 5% of the dry matter was ingested, urea disappeared from the rumen within 4 - 6 hours. Pearson and Smith (1943b) found that certain amino acids promoted and others depressed the *in vitro* synthesis of protein from urea by rumen microorganisms. It was suggested that the form of protein in a ration may affect the efficiency with which non-protein nitrogen will be utilized as protein. These workers pointed out the possibility that when the ration contains insoluble protein the amount of ammonia formed from the protein may be small, favoring a more efficient utilization of urea. In *in vitro* experiments, McDonald

(1952) demonstrated that large amounts of ammonia were formed from casein and gelatin but that the addition of zein caused no change in the ammonia content of the rumen liquor. Because of the apparent complexity of the conditions in the rumen and the problems encountered in attempting to simulate these conditions *in vitro*, it was difficult to apply the results of experiments conducted *in vitro* to the feeding of the intact animals. It has been found that ruminal flora and fauna were much different microbiologically after an incubation period of even one day from those examined prior to incubation. In many of the early experiments the poor utilization of urea was attributable to the relatively high level of true protein in the rations in which urea was added.

Two feeding trials and a metabolism trial were conducted by Lowrey and McCormick (1969) to compare cottonseed meal and urea as nitrogen supplements in steers, calves, and yearling steers finishing diets. Results of the feeding trials indicated that yearling steers and steer calves could utilize high concentrate diets in which most or all of the supplemental nitrogen came from urea, but that calves seemed more sensitive to the urea than yearlings. That data was in agreement with Kirk *et al.* (1958) in which the response to the addition of urea to a fattening diet was partially controlled by the age of the animals. The inclusion of alfalfa meal in the high urea diets improved gains

for steer calves but not for yearling steers. Beeson *et al.* (1964) working with finishing lambs indicated that dehydrated alfalfa meal was necessary for maximum utilization of high levels of urea. Oltjen *et al.* (1965) and Haskins *et al.* (1967) working with steer calves reported that the substitution of urea for all the supplemental protein (soybean meal) in an all concentrate diet had no effect on weight gain.

Effect of pH in the Rumen on Urea Utilization

In general, ruminal pH values are similar for urea and isolated soy protein fed animals under *ad libitum* feed conditions but, under controlled feeding ruminal pH may be considerably higher (1 to 2 hours after feeding) on the urea diet, due to the rapid hydrolysis of urea to ammonia by the ruminal microorganisms (Oltjen *et al.*, 1968). A low ruminal pH (5.4 to 6.0) may be beneficial to animals fed purified diets in terms of nitrogen retention because the transfer of ammonia through the ruminal wall was much less rapid at a low pH (Hogan, 1961) and more of the free ammonia was trapped for microbial protein synthesis. Ammonia absorption was governed by both concentration gradient and pH. Since ammonia has been shown to be a weak base with a pK_a of 8.8 at 40°C; the increased absorption of ammonia at higher pH was probably the results of an increase in the amount of ammonia in relation to ammonium ion, which may more readily penetrate the lipid

layers of the rumen mucosa (Hogan, 1961; Bloomfield *et al.*, 1963). An elevation of rumen pH occurs during urea feeding as a result of the rapid hydrolysis of urea to carbon dioxide and ammonia. Unfortunately, the alkaline buffering capacity of rumen fluid is not as great as its acid buffering capacity (Bloomfield *et al.*, 1966). Thus conditions in the rumen from urea feeding are conducive not only to rapid production but also to increased absorption of ammonia. High production and absorption of ammonia could lead to a great loss of nitrogen in urine and also cause urea toxicity. The conclusions reported above indicate that a reasonable acidic pH would be more beneficial for efficient utilization of urea nitrogen.

Effects of Diethylstilbestrol (DES)

Bell *et al.* (1957) have shown that diethylstilbestrol (DES) increased the retention of nitrogen when fed to lambs receiving rations containing urea. McLaren *et al.* (1959) designed an experiment to determine the influence of DES and time, and the possible relationship between these factors upon the utilization of nitrogen by lambs fed crude biuret and urea-supplemented rations. Their data indicated that the utilization of absorbed nitrogen was increased by prolonging the preliminary period or supplementing the ration with DES. The replacement of urea nitrogen with biuret depressed the digestibility of organic

matter and crude fiber and apparent digestibility of protein, but did not influence the percentage retention of absorbed nitrogen.

McLaren *et al.* (1960) reported that DES was without effect on the excretion of metabolic fecal nitrogen, endogenous urinary nitrogen, creatine, or allantoin in lambs fed biuret or urea, but they concluded that DES and time influence the retention of absorbed nitrogen through direct action on the tissue to promote better utilization of NPN.

Welch *et al.* (1957) reported that the addition of DES to a diet containing 1.7% nitrogen reduced the period required for the maximum utilization of urea and crude biuret N from 35 to 10 days, but did not change the apparent digestibility of N.

Effects of Sulfur

Some benefits have been demonstrated in ruminants from adding either inorganic or organic sulfur to purified rations low in sulfur and having a part or all of the protein replaced by urea. Goodrich *et al.* (1967) found that both sodium and elemental sulfur improved nitrogen retention of lambs fed urea. Starks *et al.* (1954) found that the addition of different levels of elemental sulfur, sodium sulfate, or DL-methionine to supplement a purified diet containing 92% of its nitrogen from urea improved weight gains and wool growth of lambs significantly.

Thomas *et al.* (1951) demonstrated that sulfur deficiency in purified diets limits NPN utilization. The findings of Oltjen *et al.* (1967) contradict the results of most purified diet studies. When they added DL + methionine to urea-containing purified diets for sheep, no response, as measured by gains and feed efficiency, was observed. Many researchers have been unable to show that animal performance has been affected by adding supplemental sulfur to practical rations. Lack of response to sulfur has been attributed to most commonly used feedstuffs being good sources of sulfur (Conrad *et al.*, 1967; and Davis *et al.*, 1954).

The Effect of Coated or Treated Urea

Several attempts have been made to slow ammonia release from urea by coating it with water insoluble materials. Johnson *et al.* (1962) tested urea prills coated with 20 different fat and waxy type materials. They found that coated prills were utilized by rumen bacteria *in vitro* but were less acceptable than urea in 12% urea supplements for lambs, and there was no indication that the materials were more efficiently utilized by ruminants. Martin *et al.* (1969) observed in sheep that a level of 2% sodium bentonite improved the utilization of nitrogen in urea-containing rations. Ward and Cullison (1970) compared ethyl cellulose coated urea with noncoated urea. The coated urea was less toxic and more palatable

than the noncoated form. In nitrogen balance studies with steers the coated urea-containing ration did not enhance nitrogen retention.

Effect of Time

It is known that urea nitrogen fed intermittently is not utilized by rumen flora as well as nitrogen from less soluble (NPN) compounds which tend to release their nitrogen at a slower rate. However, when the intervals between feed intake are short and the amount of feed consumed at any one time is relatively small, as in continuous or self-feeding, satisfactory performances may be obtained on rations in which urea furnished almost all of the nitrogen in the ration (Starks *et al.*, 1953; Albert *et al.*, 1956).

Repp *et al.* (1955) suggests that longer preliminary periods may be required to permit lambs to adjust to NPN supplemented rations. The length of time allowed for lambs to adjust to the utilization of NPN compounds was 37 days in the work reported by Gaither *et al.* (1955) and 15 days in the work reported by Anderson *et al.* (1959). This factor may account for the differences in the utilization of urea obtained in different experiments.

Welch *et al.* (1957) reported that utilization of urea, biuret, and 2:1 urea and creatine mixture in a ration containing 1.7% total nitrogen two-thirds of which was supplemented by the NPN sources increased as a function of

time during which the NPN sources was fed, reaching maximum values after 35 days. McLaren *et al.* (1959, 1960) and Campbell *et al.* (1963) demonstrated that NPN utilization was increased with time during which they were fed.

Oltjen *et al.* (1968) in studies with urea, biuret, urea-phosphate and uric acid as NPN sources for cattle reported that a period of 21 days was needed for efficient utilization of the NPN source by rumen microorganisms.

Farlin *et al.* (1968) in studies with biuret found that approximately 50% of dietary biuret during the initial 11 - 17 day feeding period was excreted in urine as biuret and decreased to about 30% after feeding for 45 days. Their results supported the hypothesis that the adaptation to biuret utilization was due to changes in the ability of microorganisms to metabolize biuret. The same trend was found for urea.

Schaadt *et al.* (1966) reported that urea utilization was improved by prolonging the feeding period from 1 - 72 days.

Clifford and Tillman (1968) studied urea and soybean protein when they were incorporated in purified diets in the following proportions of total nitrogen: Diet 1, all urea; Diet 2, 3/4 urea + 1/4 soybean protein; Diet 3, 1/2 urea + 1/2 soybean protein; Diet 4, 1/4 urea + 3/4 soybean protein; Diet 5, soybean protein. These isonitrogenous diets were self-fed to 20 lambs over a 60-day growth and balance trial.

Feces and urine were collected for the 60 days and composited into successive 10-day intervals. Their results have shown that growth and performance of lambs fed diets containing urea as the sole source of nitrogen were approximately 70% as great as that obtained with soybean protein. Nitrogen retention in all diets increased with time on diets and reached a maximum value at 50 days which was concurrent with a decreased urinary loss. This was in agreement with the results of McLaren *et al.* (1960), Oltjen and Putnam (1966) and Barth *et al.* (1961) in which they indicated that retention of nitrogen by sheep fed diets containing large amounts of urea increased with time on feed. As a result the present concept of "adaptation" to urea developed.

Measurement of the Adaptation Response to Urea Nitrogen

Indirect evidence from the review of Reid (1953), the work of Repp *et al.* (1955) and Ewan *et al.* (1958) suggested that the utilization of non-protein nitrogen compounds may be improved as the period during which they were fed was extended. Metabolism studies conducted at the West Virginia station have shown that non-protein nitrogen (NPN) utilization by lambs was significantly improved by extending the period during which compounds of this type were fed (Welch *et al.*, 1957; McLaren *et al.*, 1959). This improvement has been termed the "Adaptation Response".

The effects of prolonged feeding of urea containing rations was estimated through regression analyses using data obtained from 63 lambs in 19 digestion and metabolism trials (Welch *et al.*, 1957). The ration fed contained approximately 1.7% nitrogen, about two-thirds of which was provided by urea. Smith *et al.* (1960) as a result of these studies developed the following regression equation:

1) Nitrogen Utilization $\hat{Y}_a = 111.93 - 1.093X_1 - 1.065X_2 + 0.201X_3 - 0.006X_{4a}$

which represents the regression of the percentage of absorbed nitrogen upon the following variables:

X_1, percentage ration urea nitrogen

X_2, percentage ration nitrogen

X_3, length of time urea was fed

X_{4a}, year of trial

By excluding the year effect the equation would be

$$\hat{Y}_a = 111.93 - 1.093X_1 - 1.065X_2 + 0.201X_3$$

without jeopardizing the accuracy of the equation.

2) $\hat{Y}_b = 85.3 - 0.005X_1 - 13.02X_2 + 0.01X_3 - 0.34X_4$

3) $\hat{Y}_c + 81.6 - 0.75X_1 - 14.35X_2 - 0.05X_3 + 1.66X_4$

which represents, respectively, the regression of organic matter digestibility and crude fiber digestibility upon the following variables:

X_1, percentage ration urea nitrogen

X_2, percentage ration nitrogen

X_3, length of time urea was fed

X_4, percentage of crude fiber

Their results indicated that the retention of absorbed nitrogen was significantly improved by approximately two percentage units with each consecutive 10 day feeding period up to 50 days with no measurable change in the digestibility of organic matter or crude fiber. Increasing the percentage of total nitrogen supplied as urea from 54 to 68% significantly depressed the retention of absorbed nitrogen to the extent of approximately 12 percentage units;

but had no significant effect upon the digestibility of organic matter and significantly depressed the digestibility of crude fiber by approximately 8 percentage units. It may be seen that nitrogen utilization improves 0.201% units per day of urea feeding during a 50 day feeding period.

Since it was felt that all-urea supplemented rations might influence the "adaptation response" previously reported Barth (1962) conducted multiple regression analyses in which metabolism data obtained from lambs fed the all-urea supplemented rations were used. The content of readily available energy in several metabolism trials was varied so it was possible to include this as a variable in the analyses. The equation

$$Y = -98.525 + 0.385X_1 + 0.054X_2 + 58.230X_3 + 0.035X_4$$

represents the regression of per cent nitrogen utilization upon the following variables:

X_1 = length of time urea was fed (days)

X_2 = per cent of total nitrogen supplied by urea nitrogen

X_3 = per cent of urea nitrogen in the ration

X_4 = gross energy from readily available carbohydrates

In an effort to simplify this prediction equation, the means of X_2, X_3 and X_4 were inserted and the influence of time on

the per cent of absorbed nitrogen retained was shown to be equal to $37.59 + 0.389X_1$. This indicates an increase of 0.39% in the retention of absorbed nitrogen per day of feeding lambs all-urea supplemented rations.

In a 72-day metabolism trial, adaptation to urea was indicated by increased nitrogen balance and percent biological value (Schaadt *et al.*, 1966). There was an increase with time in nitrogen balance with the urea supplemented lambs. Biological value increased from 55.2% at 18 days to 64.1% at 72 days. This was in agreement with Smith *et al.* (1960), Welch *et al.* (1957) and McLaren *et al.* (1959).

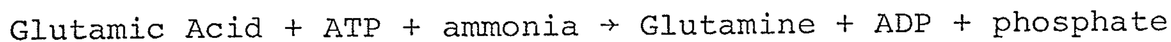
McLaren *et al.* (1965b) summarized the effects of level of readily available carbohydrates and length of time of urea feeding on nitrogen retention by lambs fed semi-purified diets containing 75% of their nitrogen from urea. Regression analysis indicated that the retention of absorbed nitrogen was improved 3 percentage units with each 10-day period of urea feeding and 2% for each 100 kcal of readily available carbohydrates in the diets.

McLaren *et al.* (1961) and Hoshino *et al.* (1966) suggested that the activity of nitrogen metabolizing enzymes could be used as criteria for measuring the adaptation response of the animal to dietary urea.

The Activity of Enzymes in Ruminant Tissues

Glutamine Synthetase

[L-glutamate: ammonia ligase (ADP) EC 6.3.1.2]


Glutamic Acid + ATP + ammonia → Glutamine + ADP + phosphate

Glutamine synthetase catalyzes the above reaction. Glutamine synthetase from sheep brain, pigeon liver, and peas have similar molecular and enzymatic properties (Colowick and Kaplan, 1957). The enzyme system was also present in the kidneys of rats, guinea pigs, rabbits and sheep (Lyon and Pitts, 1969). Glutamine synthetase and glutaminase in the kidney of rat and certain other animals (Krebs, 1935) serve to produce and to utilize ammonia and thus to equate net ammonia production to the need of the body for excretion of hydrogen ions (Kamin and Handler, 1951; and Orloff and Berliner, 1956).

ATP cannot be replaced by ADP or AMP. The enzyme appears to be relatively unspecific with respect to glutamic acid. Although aspartic acid and other commonly occurring amino acids are inactive, recent work using preparations from both peas and sheep brain has shown that D-glutamic acid and α-aminoadipic acid will yield hydroxamic acid (Levintow and Meister, 1953). The latter nevertheless was incapable of reacting with ammonia. α-Methyl-Glutamic acid can also replace glutamic acid in the enzyme system

from sheep brain (Lichtenstein *et al.*, 1953).

Ammonia, hydroxylamine, and hydrazine are all equally active in the pigeon liver and sheep brain enzyme systems when L-glutamic acid was the other substrate present (Colowick and Kaplan, 1957). Mg or Mn ions are essential cofactors. Mg ions activate maximally at 0.02M with the brain and pea enzymes. Mn ions at a concentration of 0.003M give 30% with the pea enzyme and 40% with the brain enzyme of the activity obtained in the presence of 0.02M Mg ions. Higher concentrations of Mn ions cause decreased activity. The brain enzyme was inhibited by Ca ions, the latter apparently being competitive with Mg. The enzyme was completely inhibited by 10^{-3}M fluoride and by the same concentration of p-Chloromercuribenzoate. The sulfoxide derived from methionine was a competitive inhibitor with respect to glutamic acid. Crystal violet inhibits the bacterial and pea enzymes but not those from brain and pigeon liver. ADP was a powerful inhibitor of glutamine synthetase, apparently being competitive with ATP; the sheep brain enzyme was 50% inhibited when the ratio ADP/ATP was 0.3. The enzyme from sheep brain has an optimum at pH 7.2, the activity falling to zero at pH 4.5 and to 50% at pH 8.5.

Glutamine synthetase catalyzes synthetic, transfer and "arsenolytic" reactions (Meister, 1956). The synthetic reaction, namely combination of glutamate with ammonia to form glutamine, was endergonic and requires the cleavage of

ATP and the presence of magnesium ions (Elliott, 1953; and Speck, 1949). The presence of reducing agents permits maximal rates of glutamine synthetase (Speck, 1949).

Studies on ammonia metabolism in rumen microorganisms and rumen mucosa (Chalupa *et al.*, 1970) indicated that glutamine synthetase and the five urea cycle enzymes appeared to be present in trace amounts in rumen mucosa. Glutamine synthetase activity in rumen mucosa was suggested by Hoshino *et al.* (1966).

Chalupa *et al.* (1970) reported that rumen bacterial glutamine synthetase activity was higher with urea-fed sheep in comparison with those fed soy protein, and they suggested that this enzyme plays an important role in the ruminal assimilation of ammonia. Buchall *et al.* (1964) were unable to demonstrate the presence of a glutamine synthetase in *Streptococcus bovis*, but an asparagine synthetase was purified and characterized. Bryant and Robinson (1961) suggested that *Bacteroides succinogenes* might contain glutaminase. Holzer *et al.* (1968) reported that there was two forms of glutamine synthetase in *Escherichia coli.* Wu and Yuan (1968) found that the optimal concentration of ammonium sulfate in an *Escherichia coli* growth medium for glutamine synthetase was 0.4 mM. When glucose was limiting enzyme synthesis became dependent on oxygen and L-Glutamate induced enzyme synthesis.

Mugerwa and Conrad (1971) found that plasma glutamine

concentration increased with increasing levels of urea in the ration. McLaren *et al.* (1961) reported that ruminal mucosa has the ability to synthesize glutamine, while Hoshino *et al.* (1966) confirmed this and observed that urea in the rations of sheep increased plasma glutamine levels. These observations indicated that glutamine might be an important metabolite in nitrogen metabolism, especially where the rations contained high levels of urea. Duda and Handler (1958) also found that glutamine plays a very important role in nitrogen metabolism of rat liver. They also suggested the importance of glutamine synthetase which was the synthesizing agent of glutamine.

<u>Glutamate Dehydrogenase</u>

[L-glutamate: NAD(P) oxidoreductase (deaminating), EC 1.4.1.3]

$$\text{L-Glutamate} + NAD^{+} + H_2O \rightleftarrows \alpha\text{-Ketoglutrate}^{=} + NADH + NH_4^{+} + H^{+}$$

The enzyme which catalyzes the above reaction is glutamate dehydrogenase. It has been confirmed that this enzyme plays a very important role in nitrogen metabolism (Strecker, 1955). In the forward direction the purified enzyme appears to be absolutely specific for L-glutamate having no activity with the substrates γ-ethylglutamate, diethylglutamate, D-glutamate, N-acetyl glutamate, N-carbamyl glutamate, N-phthalyl glutamate, glutamine, γ-ethyl amide, and γ-methyl amide of glutamic acid. NADP

and diamino NAD can be substituted for NAD, although the activity was less and no activators have been reported. In the presence of equimolar concentrations of L-glutamate the following percent inhibitions were noted: aspartate, 10; glutamine, 20; D-glutamate, 50; p-Mercurichlorobenzoate inhibits 50% at a concentration of 1.1×10^{-4}M. Silver, mercury, zinc and ferric ion and high salt concentrations were also inhibitory. Hydroxylamine inhibits 30% in the reverse direction in the presence of equimolar concentrations of NH_4Cl (Strecker, 1955; and Frieden, 1965). The optimum pH for enzymatic activity for the oxidation of glutamate was 8.5 to 8.6. The activity at any particular pH appears to depend somewhat on the buffer used. For example, at pH 7.6 the activity in Tris buffer was about one-third of that obtained in a buffer mixture consisting of phosphate, Tris, and 2-amino-2-methyl-1,3-propanediol, whereas at pH 8.5 the activity in the former buffer was slightly higher than the latter.

The values of K_m obtained for the various reactants at pH 7.6 in 0.5M potassium phosphate buffer by the application of Lineweaver and Burk method were: glutamate, 1.92×10^{-3}; NAD, 2.47×10^{-5}; α-ketoglutarate, 1.23×10^{-4}; NH_4^+, 0.057; NADH, 1.8×10^{-5}. The values for K_m for glutamate, however, have been found to change markedly according to the pH and buffer used. Brdiczka *et al.* (1968) concluded that glutamate dehydrogenase was located within

the mitochondrial matrix and was not present within the outer mitochondrial membrane or the intracristal space. Pavel (1969) confirmed that the most active dehydrogenation enzyme in rumen fluid was glutamate dehydrogenase which was present only in the intracellular form. di Prisco and Strecker (1970) reported that glutamate dehydrogenase activity was present in both the nuclear and mitochondrial fractions of the liver cells. Hogeboom and Schneider (1953), Christie and Judah (1953), and Beaufay *et al.* (1959) had confirmed that glutamate dehydrogenase activity was associated exclusively with the mitochondrial fraction of animal tissue homogenates. Glutamate dehydrogenase has been reported to be localized on the surface of the mitochondria (Jackson, 1968).

Palmquist and Baldwin (1966) studied the effect of roughage/concentrate ratio in the ration on the enzyme activity in rumen microorganisms. From their results, the ratios of NAD-specific glutamate dehydrogenase to the NADP-specific enzyme indicate that the proportion of the type (NADP) found in *Streptococcus bovis* increased as the proportion of dietary concentrates increased. They also reported that extract from ruminal protozoa and bacteria as well as mucosa from rumen reticulum and omasum contained NAD-linked glutamate dehydrogenase.

di Prisco *et al.* (1968) found two glutamate dehydrogenases in rat liver, one associated with nuclei, and the

other with mitochondria. The two enzymes differ in regard to activation by inorganic phosphate, in the effects of pH on activity, in apparent K_m values for some of the substratas and in the phase of curve obtained by a plot of NAD concentration versus activity (Banay-Schwartz and Strecker, 1970).

Francesconi and Villee (1969) reported a marked increase in the activity of L-glutamate dehydrogenase when liver slices from rats were incubated in the presence of malate, lactate, glutamate, or α-ketoglutarate (Strickland, 1969).

Schimke (1962) studied the effect of dietary protein intake on the activity of rat urea cycle enzyme. The diets contain 15, 30 and 60% casein. He found that glutamate dehydrogenase underwent no significant changes with varying protein intake.

Whanger and Church (1970) studied the effect of diets containing urea on the activities of a number of enzymes involved in nitrogen utilization in the liver and rumen epithelium, and compared the activities of several enzymes of the epithelial tissue from the rumen, reticulum, omasom, abomasum and small intestine with that of the liver in steers. They reported that glutamate dehydrogenase was higher in the liver than in the epithelial tissue of the digestive tract. There was no differences in activity of the enzyme in the different parts of the digestive tract. They also confirmed that the level of the enzyme was

slightly higher in the liver and rumen epithelium in animals fed the urea diets. None of these increases, however, were statistically significant. The results do indicate that the rumen epithelium was capable of metabolizing inorganic and organic nitrogen compounds and they suggested that this could be an important feature of non-protein nitrogen metabolism in ruminants. The results tend to support the results of Cooper (1962), who reported no significant increase of glutamate dehydrogenase activity in rumen mucosa of urea-adapted lambs.

Carbamyl Phosphate Synthetase

[ATP: carbamate phosphotransferase (dephosphorylating) EC 2.7.2.5]

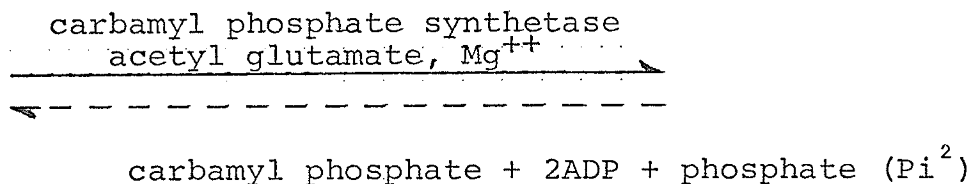
Carbamyl phosphate is the energy-rich phosphate compound that donates its carbamyl group to ornithine, aspartic, and oxamic acid in the enzymatic synthesis of, respectively, citrulline, carbamyl aspartate, and carbamyl oxamic acid.

The synthesis of carbamyl phosphate is catalyzed by the reaction of carbamyl phosphate synthetase in mammals or the reaction of carbamyl phosphokinase in micro-organisms. The differences between these two enzymes is that carbamyl phosphate synthetase utilizes 2 molecules of ATP for the synthesis of 1 molecule of carbamyl phosphate while carbamyl phosphokinase utilizes only one molecule of

ATP. Also, the rate of the reverse reaction of carbamyl phosphate synthetase is slower than the forward reaction.

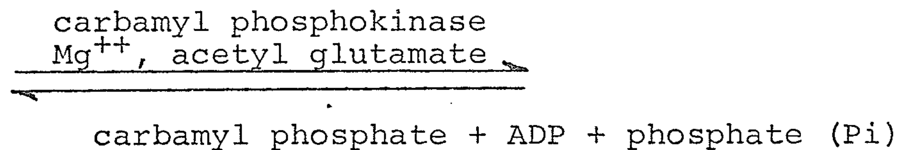
Carbamyl phosphate is a very important compound in the synthesis of urea after it has been produced by the action of either enzymes as follows:

Ammonia + bicarbonate + 2ATP



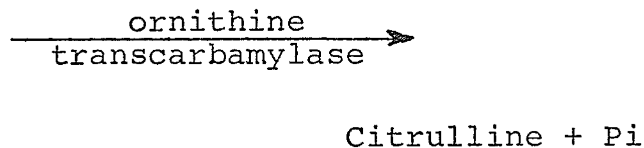
carbamyl phosphate + 2ADP + phosphate (Pi^{2})

Ammonia + bicarbonate + ATP



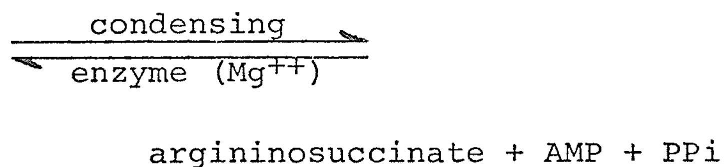
carbamyl phosphate + ADP + phosphate (Pi)

Carbamyl phosphate + ornithine

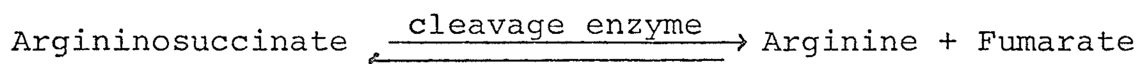


Citrulline + Pi

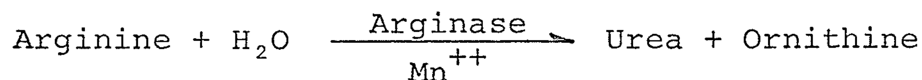
Citrulline + aspartate + ATP



argininosuccinate + AMP + PPi



Argininosuccinate $\xrightleftharpoons{\text{cleavage enzyme}}$ Arginine + Fumarate



Arginine + H_2O $\xrightarrow[Mn^{++}]{\text{Arginase}}$ Urea + Ornithine

The enzyme is specific for ammonium carbamate; the carbamate of hydroxylamine or hydrazine will not react. Neither ATP or ADP can be replaced by other nucleotides.

There is no inhibition by ATP or ADP as long as the magnesium ion concentration is the same as the nucleotide concentration up to 0.03M (Colowick and Kaplan, 1957). Fluoride up to 0.01M has little or no inhibitory effect. P-Chloromercuribenzoate inhibited the enzyme at 10^{-5}M concentration. Acetyl glutamate neither inhibits nor stimulates the enzyme.

The forward reaction, formation of carbamyl-P, and the reverse reaction, formation of ATP, have different pH optima. The optimum pH for the forward reaction is 9.5, with a rather sharp drop toward more-acid pH levels, so that at 8.5 the rate is one-half and at pH 8.0 it is one-quarter that at 9.5. At pH 10.5 the rate is three-quarters of the optimal rate. The reverse reaction has a very broad optimum from pH 4.5 to pH 8. Even at pH 4 and 9.5 the rate of the reverse reaction is 30% of the optimal rate. This broad pH optimum should make this an excellent ATP-generating system for reactions in the acid or alkaline

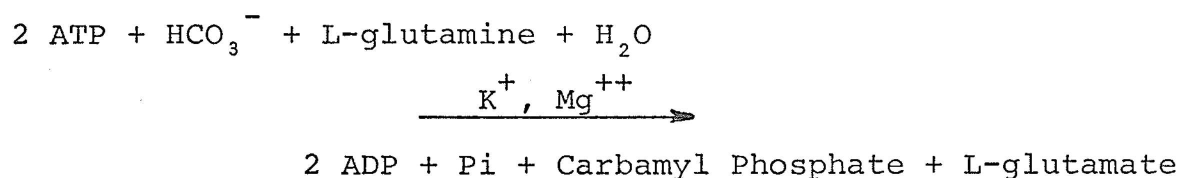
ranges.

At pH 8.5, where both the forward and reverse reactions proceed at about 70% of their optimal rates, the synthesis of ATP occurs at about ten times the rate of the synthesis of carbamyl-P. For both reactions the rate at 30°C is ten times the rate at 0°C. Accurate K_m values are not available but appear to be near the following values: ADP, 1×10^{-3}M; Carbamyl-P, 1×10^{-3}M; Mg^{++}, 1×10^{-3}M; ATP, 1×10^{-3}M and carbamate, 5×10^{-3}M (Colowick and Kaplan, 1957).

Bayer and McMurray (1969) in studies on rat liver found that aspartic acid analogues have a strong inhibitory effect on carbamyl phosphate synthetase and argininosuccinate synthetase. The concentrations required to give 50% inhibition were: N-allylaspartate, 0.248M; α-methylaspartate, 0.140M; β-methylaspartate, 0.078M; and β-hydroxy-β-methylaspartate, 0.038M. They suggested that part of the inhibition was related to the ability of the analogues to complex Mg^{++}, since increased concentrations of Mg^{++} prevented the inhibition of carbamyl phosphate synthetase and reduced the inhibition of argininosuccinate synthetase by α-methylaspartate and N-allylaspartate. In addition, β-methylaspartate was found to depress oxidative and phosphorylative reactions, thus interfering with the energy production required for urea formation. The analogues did not inhibit the other urea cycle enzymes.

Nesheim *et al.* (1967) and Nesheim (1968) found a high level of tissue lysine in chicks when kidney arginase activity was high. These chicks exhibited a low growth performance, but the addition of an excess of arginine improved the performance of the chicks. Jones *et al.* (1967) published data showing that excesses of lysine in diets for chicks caused a reduction in growth rate and an increase in kidney arginase level. The effects of the excess lysine can be counteracted by higher levels of dietary arginine, so that in effect, high levels of lysine increase the arginine requirement.

Carbamyl phosphate synthetase from *Escherichia coli B* catalyzes the following reaction (Anderson and Meister, 1965):



$$2\ ATP + HCO_3^- + L\text{-glutamine} + H_2O \xrightarrow{K^+,\ Mg^{++}} 2\ ADP + Pi + \text{Carbamyl Phosphate} + L\text{-glutamate}$$

The enzyme activity was subjected to feedback inhibition by uridine monophosphate (UMP), and was activated by ornithine and by inosine monophosphate (IMP) and other end products of purine nucleotide biosynthesis (Anderson and Meister, 1966; Anderson and Marvin, 1968). Activation by ornithine provides a mechanism for reversing feedback inhibition by UMP; this assures a continued supply of carbamyl phosphate when it was needed for arginine biosynthesis, since the rate of biosynthesis of ornithine will increase when the supply of

arginine was decreased (Pierard, 1966).

Niels *et al.* (1968) stated that the activity of carbamyl phosphate synthetase was very low in foetal rat liver and reached its maximum at 20 days after birth.

Carbamyl phosphate synthetase has been reported to be extremely unstable after preparation in anything other than the frozen state (Metzenberg *et al.*, 1957; Jones *et al.*, 1961). The preparations of Metzenberg *et al.* (1957), although reasonably stable in 3M ammonium sulfate at -80°C, had a half-life of 1 hr when dissolved in dilute Tris buffer, pH 7.4, at 0°C. More recently Schooler (1964) has reinvestigated this problem with a view to obtaining preparations which could be stored at more generally available temperatures (-20°C) or had a usable half-life at 0°C. He investigated the stabilizing effects of manganous chloride, mercaptoethanol, ammonium sulfate, glycerol and carbowax. The best results were obtained with the last two substances which gave, after 24 hr, percentage residual activities of 7.5 and 14 at 0°C and 15.5 and 39 at 25°C, respectively. Similar results were obtained with 1 mM glutathione (Jones *et al.*, 1961). Watts *et al.* (1969) reported that the activity of the extracted enzyme first increases and then slowly decreases in a solution of sucrose and glucose.

Shimbayashi and Yonemura (1970) found that the activities of the urea cycle enzymes of goat liver were

very similar to those of rats. The activities of the urea cycle enzymes were affected by the protein level of the diet. The addition of 2% urea to the diet increased the activities of carbamyl phosphate synthetase and the other urea cycle enzymes. When crude protein in the diet was increased from 8.9 to 20.9, all urea cycle enzymes activities were increased.

Szepesi and Freedland (1969) reported that urea cycle enzymes in rat liver were the site of adaption when the animals had been shifted from a protein-free diet to a high protein diet. The activities of those enzymes increased significantly by shifting the animals from a protein-free diet to a high protein diet and reached maximum levels within 5 - 7 days. They stated that the more extreme the dietary shift with respect to protein content, the shorter the half-life of these enzymes until steady state values are reached.

Chalupa *et al.* (1970) studied the activity of urea cycle enzymes in sheep liver and found that carbamyl phosphate synthetase activity was decreased in spite of increased urea excretion in urea-fed animals. Their results were not in agreement with the results of Schimke (1962), Schimke (1962), and Schimke (1963), who found an increase in urea excretion associated with an increase in urea cycle enzyme activities when rats were shifted from low protein to high protein diets (15, 30 and 60). They suggested

that these effects could be the results of ammonia *per se*, either by direct or indirect mechanisms, or could be due to a suboptimum amino acids nutritional status. Hepatic urea synthesis depends not only on activities of urea cycle enzymes, but also on liver ornithine content. Katunuma *et al.* (1966) demonstrated *in vitro* that ammonia caused increases in liver ornithine content.

Chalupa *et al.* (1970) in studies on enzymes involved in ammonia metabolism in rumen bacteria and rumen mucosal tissue of sheep, found that carbamyl phosphokinase activities increased more in urea-fed animals than soy protein fed animals, but the activities of this enzyme was very low suggesting that this enzyme system was of minor importance in the ruminal assimilation of ammonia by the mixed rumen microbial population. They did not report the activity of this enzyme in rumen mucosa because of the unmeasurable activities they obtained. Carbamyl phosphokinase was found in many microorganisms, and Niederman and Wolin (1967) indicated that carbamyl phosphokinase was involved in the synthesis of arginine by *Streptococcus bovis*. Beckwith *et al.* (1962) and Thorne and Jones (1963) indicated that the substrate concentration and pH required to satisfy the thermodynamic and kinetic needs of the forward reaction (Carbamyl phosphate synthesis) of this reversible system would almost have to be unphysiological. A glutamine-dependent carbamyl phosphate synthetase has been reported

in *Agaricus bisporus* (Levenberg, 1962) and in *Escherichia coli* (Rerard and Wiame, 1964; and Kalman *et al.*, 1966).

Schneider and Hogeboom (1950) revealed that there was no effect of dietary protein on the distribution of carbamyl phosphate synthetase and ornithine transcarbamylase, both of which were present only in mitochondria.

Martincic and Krvavica (1964) reported the presence of a highly active arginase in ruminal mucosa. The activity in the ruminal mucosa was slightly weaker than it was in the liver and much higher than the activity of this enzyme in the kidney. In another experiment Krvavica *et al.* (1964) tried to measure the activity of carbamyl phosphate synthetase in rumen mucosal tissue and liver of cattle. They reported that the enzyme was not present in the rumen mucosa but it was present in the liver. They were measuring the activity of the enzyme by measuring the amount of carbamyl phosphate formed by the enzyme reaction.

CHAPTER III

MATERIALS AND METHODS

Animals

Two Angus and one Hereford fistulated bull calves were used in these studies. The average calf weight at the beginning of the experiment was 241 Kg. The animals were provided with water *ad libitum* and were fed using a continuous feeder (Ibrahim, 1969) to provide 7 Kg of feed a day. The animals were dehorned before the start of the experiment. Plastic bags were used for fecal collection and urine was collected in stainless steel trays underneath the animals.

Experimental Diets

Three isonitrogenous semi-purified diets containing an average of 2.67% N were fed. Ground alfalfa hay containing 2.42% total N was used as a base line experimental diet (Table 1). The diets were mixed, pelleted and analyzed for total N for each experimental period. The diets were fed according to the standard requirements (NRC) for beef calves. The diet containing 0% N from urea (all N supplemented by soybean meal) and the diet containing 50% N from urea and 50% from soybean meal

Table 1. Composition and nitrogen content of experimental diets.

Ingredient	Percent Composition†			
	100% Urea	50% Urea	0% Urea	Hay
Wheat straw	38.00	25.29	12.68	
Glucose	15.90	16.00	16.00	
Corn starch	27.00	27.00	27.00	
Sunflower oil	2.00	2.00	2.00	
Alphacel	5.00	5.00	5.00	
Soybean meal		14.91	29.82	
*Mineral mixture salts	4.50	4.50	4.50	
**Vitamins A and D	+	+	+	
Molasses	3.00	3.00	3.00	
Urea	4.60	2.30		
Nitrogen	2.72	2.66	2.63	2.42

*Mixture of the following salts as a percentage: $CaHPO_4$, 48.94; K_2CO_3, 31.55; $MgSO_4$, 10.75; NaCl, 7.43; $FeSO_4$, 0.91; $Na_2B_4O_7$, 0.19; $ZnSO_4$, 0.07; $MnSO_4$, 0.10; $CuSO_4$, 0.02; KI, 0.3; MoO_3, 0.0008; and $COCl_3$, 0.0003.

**Vitamins: Supplied per ton (metric) of diet: Vitamin A, 22gm (250,000 IU/gm) and vitamin D, 50 gm (100,000 IU/gm).

†Nitrogen values are the average for the diet mixed and analyzed at 3 times.

produced foamy rumen contents and resulted in some loss of rumen fluid through the fistula. To overcome this problem one-half Kg wheat straw was added to the three diets after the first 10 days on the experiment.

Experimental Design

The experiment was designed to study four factors: periods, animals and treatments as columns and rows in the latin square design, and time effect was examined as a split plot within the latin square for urea adaptation in ruminants (Table 2).

Before each experimental period the three animals were fed the basal ground alfalfa hay diet for 15 days to establish the base line for the experimental diets. During the last 5 days urine and feces were collected for N balance determination. Rumen and blood samples were taken on the 1st, 3rd, and 5th day of the collection period, liver samples on the 4th day of the collection period, and rumen epithelial samples on the 5th day of the collection.

After the baseline experimental period the animals were fed the experimental diets which contained either 100%, 50%, or 0% of the total N as urea. The experimental period was 40 days divided into four 10 day intervals. During the last 5 days of each 10 day period urine and feces were collected for N balance determinations. Rumen and blood samples were taken on the 1st, 3rd, and 5th day of each collection to determine ammonia-N, urea-N and

Table 2. Experimental Design

Period	Animals			
	1, 2, and 3	1	2	3
	Number of days			
	15	40	40	40
	Partition of Experimental Period			
	10 15	10 20 30 40	10 20 30 40	10 20 30 40
	Collection System			
	10 5C†	5 5C 5 5C 5 5C 5 5C	5 5C 5 5C 5 5C 5 5C	5 5C 5 5C 5 5C 5 5C
1	Ground Alfalfa Hay	Diet I 0% Urea	Diet II 00% Urea	Diet III 50% Urea
2	Ground Alfalfa Hay	Diet III 50% Urea	Diet I 0% Urea	Diet II 100% Urea
3	Ground Alfalfa Hay	Diet II 100% Urea	Diet III 50% Urea	Diet I 0% Urea

†5c refers to the 5 days collection and sampling period.

amino acids in the plasma, and ammonia-N in the rumen. Liver samples were obtained on the 4th day of each collection for the determination of glutamate dehydrogenase, glutamine synthetase and carbamyl phosphate synthetase, and liver amino acid levels. Rumen microorganisms and rumen epithelial samples were obtained on the 5th day of each collection for the determination of the above enzyme activities and amino acid levels.

When the first experimental period was ended the animals again were fed the ground alfalfa hay for another base line as previously described, then the animals were switched to the diets for the second experimental period. After the second experimental period was ended, a third base line was conducted and animals were switched to the third and final experimental period.

Sampling Techniques

Liver samples were taken by aspiration biopsy technique (Erwin *et al.*, 1956). Instruments used in the biopsy technique described herein are shown in Figure 1. The cannula was constructed from a stainless steel tube 16 cm long with an inside diameter of 8 mm. The trocar was constructed from a solid stainless steel rod, which fits with a minimum clearance inside the cannula so that the four levels at the point match with the serrated edges of the cannula. Sharpening of the instruments was facilitated by placing the trocar inside the cannula.

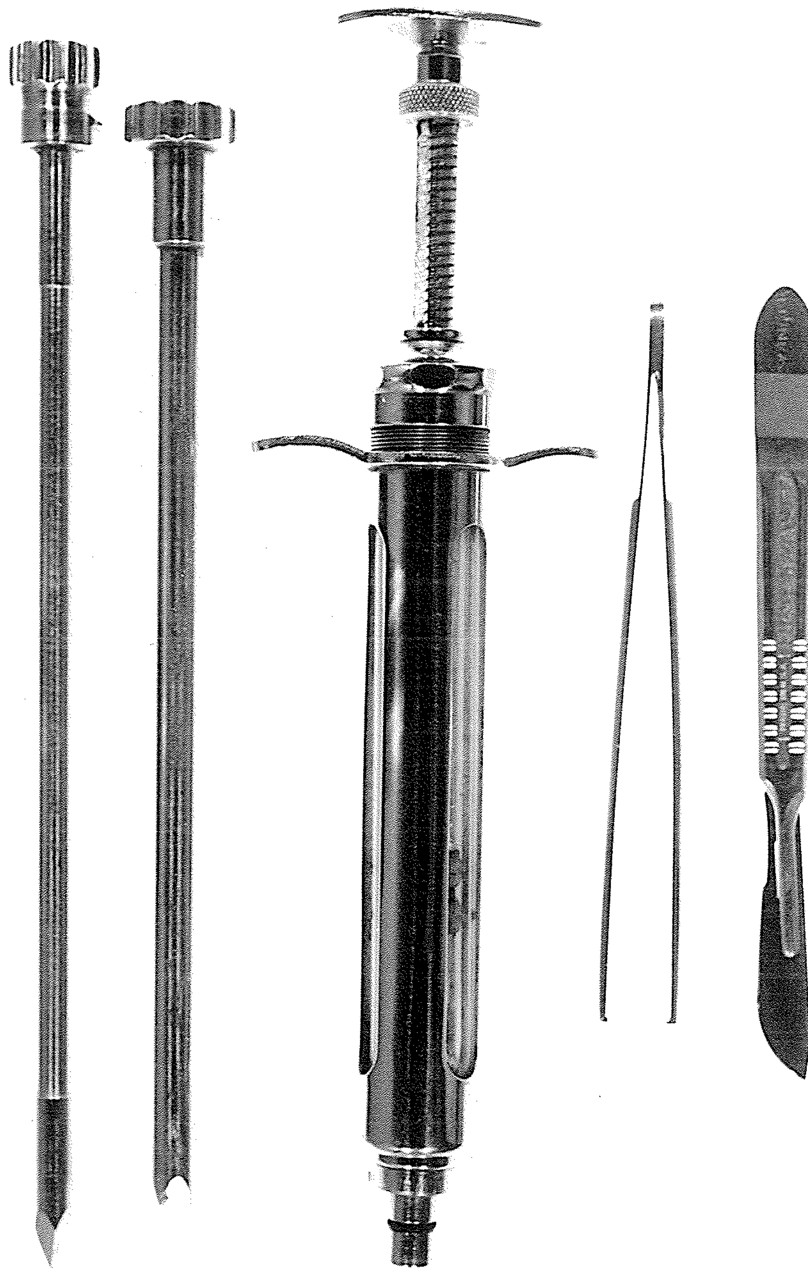


Fig. 1. Instruments used in biopsy techniques.

A 20 ml modified Record syringe was used (Figure 1). The original needle attachment was removed and replaced with a brass tooled lock-type cannula attachment. This attachment was constructed with a rubber gasket and a lock mechanism so that when the cannula was attached by a quarter turn to the syringe, no pressure was lost from the syringe. Other workers who have reported instruments used for liver biopsy techniques (Dick, 1944; Seghitti and Marsh, 1953; and Bone, 1954) reported the use of slip-type-cannula adaptors.

To obtain the liver samples, the area of operation was shaved free from hair and was disinfected. An incision approximately one-half inch long and parallel to the ribs was made by a quick thrust with a scalpel in the twelfth intercostal space, about six inches from the dorsal median plane on the right side of the animal. A simple and rapid method for determining the point of incision was for the operator to locate the last rib with his left hand. Then, proceeding anteriorly, locating the twelfth intercostal space at a span equal to the distance from the thumb to the index finger from the backbone, arriving at the point of incision. The scalpel was replaced in 70% ethyl alcohol or germicide solution and the sterile trocar and cannula were removed and rapidly inserted through the incision. After the trocar and cannula were through the skin and musculature, the liver was located by pointing the instru-

ment ventrally at an angle of 45 degrees with the horizontal plane and anteriorly 45 degrees with the saggital plane. The location of the liver appeared to vary between animals; however, as the operator became more skilled the liver was located with the trocar cannula by texture.

The trocar was then removed rapidly, care being taken not to lose the position with the cannula. The syringe was rapidly attached to the cannula and inserted into the liver about two to three inches further with a rotating motion. The plunger was withdrawn, causing negative pressure, and the syringe and cannula were removed rapidly.

The sample was then removed from the cannula by releasing pressure of the syringe into a 50 ml beaker. The sample was then prepared for the different analyses.

Rumen Epithelial Tissue Sampling

Rumen epithelial samples were taken from the fistulated animals by removing the cannula from the rumen. The rumen was then emptied to one-half of its capacity. The dorsal sac was pulled out and washed with warm water. A curved scissor was used to cut the rumen papilla as close as possible to the rumen wall. A sample of one to two grams was added to 20 ml of Krebs Ringer bicarbonate buffer (2 - 5°C) and kept on ice. Rumen contents were then placed back in the rumen. The cannula was replaced in the rumen fistula and plugged with an air containing rubber plug.

More air was injected into the plug to assure the tightness of the cannula. The area of the fistula was then washed with warm water and 70% ethyl alcohol to prevent infection. The samples were then prepared for analyses.

Rumen Samples

Rumen ingesta was obtained through the cannula and squeezed by hand through cheesecloth to obtain the desired amount of fluid. The fluid was then mixed with the same volume of 0.05M $KHPO_4$ buffer (pH 7.5). The sample was kept in a thermos container until preparation for analyses.

Blood Samples

Blood samples were taken from the jugular vein in 25 ml tubes containing potassium oxalate as an anticoagulant (0.1 gm potassium oxalate for 20 ml of blood). The samples were kept on ice and centrifuged at 4000x g for 10 minutes. Fresh plasma samples were used directly for ammonia-N determination. Samples were frozen for amino acid and urea-N determinations.

Feces and Urine Samples

Feces were collected for 5 days for each metabolism trial. Daily feces collections were weighed and 10% aliquots were kept frozen until the end of the collection period. At the end of each period feces samples from each animal were mixed and a composite sample was taken for N determination.

Daily urine volumes were measured during the 5 day collection period and 10% samples retained. Samples were kept in the refrigerator until the 5th day. On the 5th day samples from each animal were mixed and a sample was taken for N determination. Toluene and sulfuric acid were used to prevent the loss of ammonia during the collection of urine.

Sample Preparations

Liver Samples

The preparation of liver samples for enzymatic assay was that described by Brown and Cohen (1959). Fresh liver sample was gently blotted on filter paper, weighed, and homogenized at 5°C (Polytron, Brinkmann) for 40 sec at full speed in a 0.1% solution of cetyltrimethyl ammonium bromide (CTB) in the ratio: 1.0 gm of liver to 9.0 ml of CTB. The CTB solution was added at room temperature because of its low solubility at low temperatures. The homogenate was transferred to a chilled plastic tube and was centrifuged for 15 min at 4000 x g at 5°C. The supernatant was decanted, filtered to remove any turbidity and kept on ice. The pellet was rehomogenized with a volume of CTB equivalent to that employed in the original homogenation and recentrifuged. The supernatant was decanted into a second tube. A mixture of both fractions was used for the enzymatic assay.

The method of Wannemacher *et al.* (1965) was employed for amino acid analysis. One ml of 10% sulfosalicylic acid

was added to 2 ml of the above supernatant to precipitate the soluble protein. The precipitate was removed by centrifugation at 39,000 x g for 20 min and the supernatant was brought to a volume of 6 ml with sodium citrate buffer at pH 1.8 and an aliquot was injected into a Beckman Model 116 amino acid analyzer.

Rumen Epithelium

The samples were washed in 5°C Krebs Ringer bicarbonate buffer to remove contaminating bacteria. Homogenates were prepared in a Polytron homogenizer using 1 gm of the tissue and 9 ml of potassium phosphate buffer (0.05M, pH 7.5). No difference was observed between this method and the method of Chalupa *et al.* (1970) using 5°C distilled water as the homogenizing medium or of Whanger and Church (1970) using 0.25M sucrose. The homogenate was centrifuged at 5°C and 20,000 x g for 10 min. The supernatant was used immediately for enzymatic assay.

The amino acid content of the epithelium was estimated by the method described for liver (Wannemacher *et al.*, 1965).

Krebs-Ringer bicarbonate buffer was described by Umbreit *et al.* (1959) and included sodium chloride 0.90% (w/v), potassium chloride 1.15% (w/v), calcium chloride 1.22% (w/v), magnesium sulfate 3.82% (w/v), and potassium dihydrogen phosphate 2.11% (w/v), which were mixed in the

ratio 100:4:3:1:1, and were diluted 1:4 with water. This solution was diluted 84:16 with sodium bicarbonate 1.30% (w/v) and gassed thoroughly with carbon dioxide to pH 7.0.

Rumen Microorganisms

Extracts of rumen microorganisms were prepared in a manner similar to that reported by Baldwin and Palmquist (1965) and Chalupa *et al.* (1970). Forty milliliters of 1:1 rumen fluid and 0.05M $KHPO_4$ buffer (pH 7.5) were centrifuged at 320 x g for 5 min to remove large particulate material. The supernatant was centrifuged at 29,000 x g for 30 min at 0°C. The supernatant fluid was discarded and the microorganism cells were washed once in 20 ml of 0.05M $KHPO_4$ buffer (pH 7.5). The washed cells were suspended in 10 ml of 0.05M $KHPO_4$ buffer (pH 7.5) and disrupted under a hydrogen atmosphere with a Mullard ultrasonic drill (James H. Wilson Ltd., Montreal, Toronto). The debris was removed by centrifugation at 29,000 x g for 30 min at 0°C. Enzyme assays were conducted on the supernatant fluid.

Amino acids were analyzed by the method of Wannemacher *et al.* (1965) as described previously.

Analytical Methods

Glutamate Dehydrogenase Assay Method

Principle: The oxidation of glutamate was measured by following the increase of optical density at 340 nm caused by the reduction of NAD (Strecker, 1955).

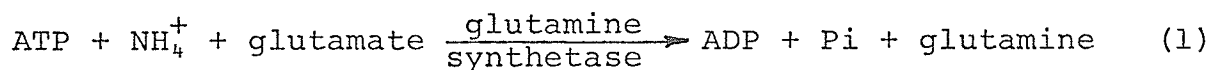
Reagents:

1) potassium glutamate (0.5M)
2) NAD solution; 3μM/ml in water
3) 0.05M potassium phosphate buffer, 5mM EDTA and 2mM 2-mercaptoethanol (pH 7.5)

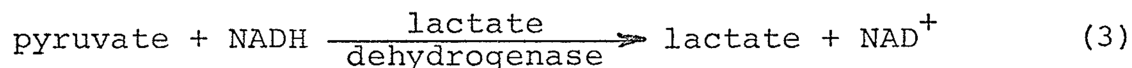
Procedure: The assay was similar to that of Strecker (1955) and included 2.6 ml of buffer, 0.1 ml of the NAD solution and 0.2 ml of the potassium glutamate solution. After 10 min incubation at 30°C the reaction was started by the addition of 0.1 ml of tissue extract. The change in absorbance at 340 nm was recorded on a Gilford 2400 spectrophotometer. A blank cuvette was prepared by adding 0.1 ml of distilled water to the cuvette to replace the extract.

Glutamine Synthetase Assay Method

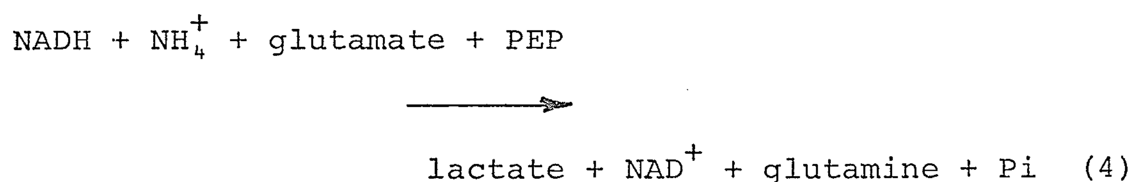
Principle: A coupled glutamine synthetase assay was used. In this assay the ADP produced during glutamine synthesis (reaction 1) was converted into ATP by reaction with PEP catalyzed by pyruvate kinase (reaction 2), and the pyruvate thus produced was reduced by NADH in the presence of lactate dehydrogenase (reaction 3). The overall reaction (4) was measured spectrophotometrically by following the change in absorbance at 340 nm due to the oxidation of NADH (Kornberg and Pricer, 1951; and Kingdon *et al.*, 1968). A summary of the overall reaction is given below:


$$ATP + NH_4^+ + glutamate \xrightarrow[synthetase]{glutamine} ADP + Pi + glutamine \quad (1)$$

$$ADP + PEP \xrightarrow[kinase]{pyruvate} ATP + pyruvate \quad (2)$$


$$pyruvate + NADH \xrightarrow[dehydrogenase]{lactate} lactate + NAD^+ \quad (3)$$

Summary:


$$NADH + NH_4^+ + glutamate + PEP \longrightarrow lactate + NAD^+ + glutamine + Pi \quad (4)$$

Pyruvate kinase was added in about 10-fold excess and lactate dehydrogenase in about 100-fold excess, so that the reaction rate was dependent solely on the amount of glutamine synthetase present in the sample. The reaction mixture contained 50mM imidazole-HCl buffer (pH 7.1), 7.6mM ATP, 1.0mM PEP, 50mM $MgCl_2$, 10mM KCl, 40mM NH_4Cl, 0.35mM NADH, 0.1M monosodium glutamate, 25 μg of pyruvate kinase and 50 μg of lactate dehydrogenase in a volume of 1.0 ml.

PEP and ATP stock solution was mixed together as one solution after neutralization of the ATP solution to pH 7.0. All the mineral solutions were made as one stock solution. This solution was kept at 4°C and was tightly closed to prevent loss of ammonia.

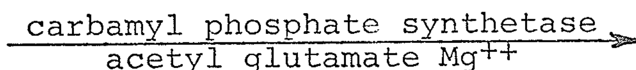
NADH stock solution and the pyruvate kinase - lactate

dehydrogenase solution were prepared fresh every day. The assay mixture was incubated at 30°C for 10 min and the change in absorbance due to the oxidation of NADH was measured at 340 nm in 1.0 ml assay volume using a 1.5 ml capacity cuvette.

Carbamyl Phosphate Synthetase (Carbamyl Phosphokinase) Assay

Principle:

ammonia + bicarbonate + 2ATP



carbamyl phosphate + 2ADP + Pi[2]

Carbamyl phosphate, synthesized by the action of the enzyme, was converted in the presence of excess ornithine transcarbamylase to citrulline. Citrulline was then determined by the method of Archibald (1944). Carbamyl phosphate - ornithine transcarbamylase was prepared by the method described by Burnett and Cohen (1957). Fresh beef liver was obtained from the slaughterhouse and kept chilled on ice and used within an hour. Quantities of about 300 g were diced and homogenized with 900 ml of chilled isotonic KCl solution in a Waring blender; blending time was standardized at 45 sec per batch. After this treatment the 25% homogenate was strained through one layer of

cheesecloth and centrifuged at 3,000 x g for 45 min at 2°C. The supernatant fluid was discarded.

The particulate fraction was suspended in 1 liter of chilled isotonic KCl and again centrifuged at 3,000 x g for 20 min. This step was then repeated. Finally, the particulate fraction was blended with cold acetone (-20°C) for 45 sec. The resulting powder was filtered and dried on a chilled suction funnel under a rubber dental dam to prevent denaturation by moisture. The acetone powder then was used to produce the necessary amount of ornithine transcarbamylase for the assay of carbamyl phosphate synthetase. The isotonic KCl solution contained 0.0128M $NaPO_4$, pH 7.4; 0.123M NaCl; 0.005M KCl; and 0.0033M $MgSO_4$ (Cohen and Hayano, 1946).

The Assay System: The assay system was similar to that of Marshall *et al.* (1958) and consisted of 50 μmoles of NH_4HCO_3, 5 μmoles of ATP, 5 μmoles of L-ornithine, 5 μmoles of N-acetyl-L-glutamate, 10 μmoles of $MgCl_2$, approximately 150 units of partially purified beef liver ornithine transcarbamylase, plus extract in a final volume of 1.0 ml. For convenience the first five reagents were added together in a volume of 0.3 ml after gassing a much larger volume of this mixture (previously adjusted to pH 7.0) with CO_2 gas to pH 6.8 in the cold. This mixture of reagents was stored at -18°C and was stable for months, but it had to be gassed with CO_2 gas to pH 6.8 just before use. Incubation was for

15 min. Incubation periods longer than 30 min were avoided because of loss of CO_2 from the reaction mixture with a consequent increase in pH.

Reactions were started with 0.3 ml of the described reagent mixture after equilibration of tubes and contents (0.1 ml of ornithine transcarbamylase preparation, 0.2 ml of extract and 0.4 ml of water) in a water bath at 38°C. A control tube (zero time) received 5 ml of 0.5M perchloric acid solution before addition of the reagent mixture.

After 15 min incubation the reaction was stopped by the addition of 5 ml of 0.5M perchloric acid to each tube. The tubes were then centrifuged at 10,000 x g for 10 min to precipitate the protein and 4 ml of the clear supernatant was taken from each tube for citrulline determination.

Citrulline Determination: Four milliliters of the above supernatant, 2 ml of a mixture of 1:3 concentrated sulfuric acid and syrupy phosphoric acid, and 0.25 ml of a 3% aqueous solution of diacetylmonoxine were mixed and placed in a boiling water bath for 10 min. The tubes were covered by marbles and protected from direct light during the boiling process. After boiling they were cooled with cold running water and protected from light until measured. A standard solution of citrulline was prepared and treated in the same manner for comparison. The optical density was read at 490 nm and the values for the enzyme were compared to that of the standard.

Urea in plasma was analyzed using a Technicon autoanalyzer II (Model 7-70-140A). The methodology employed was that according to the manual supplied by the manufacturer (Technicon Method No. AAII-1). Blood ammonia and rumen ammonia were determined by a microdiffusion method (Conway, 1957). Feces and urine N were determined by the A.O.A.C. method (1960). Tissue N content was determined by the micro-Kjeldahl method (Chibnall *et al.*, 1943) using the Markham micro-distillation apparatus (Markham, 1942).

CHAPTER IV

RESULTS AND DISCUSSION

Results A

Dry matter digestibility was depressed (P<0.05) when urea supplied 100% of the dietary N (Table 3). Dry matter digestibility increased with time for the 0 and 50% urea-N diets and peaked after 30 days. There was no improvement in dry matter digestibility with time for the 100% urea-N diet.

Protein digestibility tended to improve during the time the diets were fed (P<0.25). Urea containing diets showed greater improvements in protein digestibility than the 0 % urea diet.

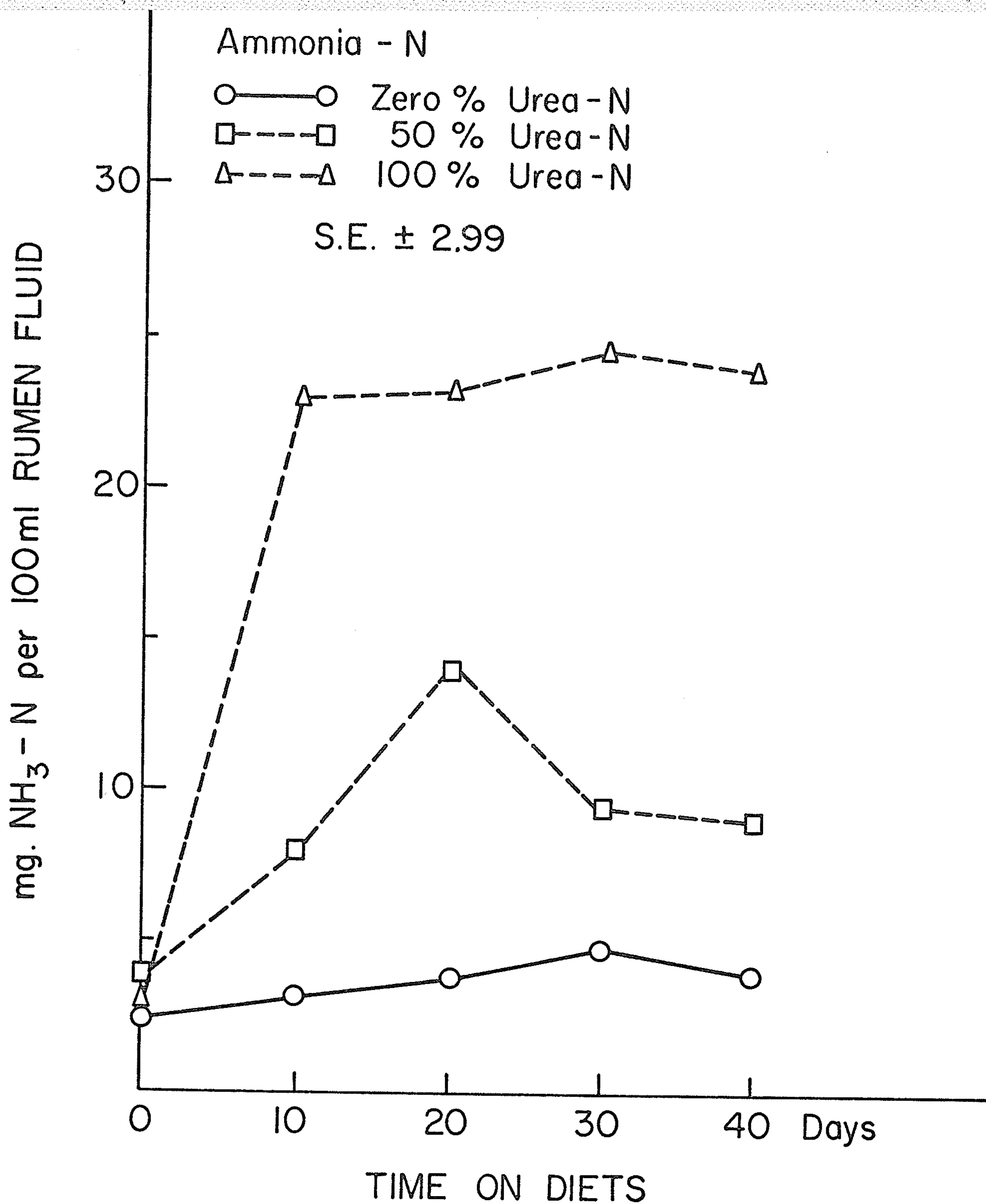
N balance data for the three diets at the five collection periods is shown in Table 3. Calves fed the diet containing 100% urea had lower N retention than calves fed the diets which contained 0 or 50% urea.

Ammonina-N concentration (Fig. 2) in the rumen was significantly higher (P<0.01) for the urea supplemented diets than the soybean meal diet. The values were also significantly higher (P<0.01) for the diet which contained 100% of the N from urea than the diet which contained 50% of the N from urea.

Table 3: Effect of urea supplemented diets fed to calves on D.M., protein digestibility and nitrogen balance.

% N_2 from Urea	Days on the Exp. Diets	D.M. Digestibility	Protein Digestibility	N_2 Retention as % of the Intake
0	0	67.66	70.66	31.50
	10	80.23	78.10	42.43
	20	76.25	73.91	38.29
	30	75.85	73.84	40.17
	40	73.20	76.35	31.92
50	0	68.92	72.48	29.03
	10	75.35	80.97	35.08
	20	75.84	77.26	31.37
	30	77.00	78.79	36.00
	40	72.99	82.35	31.37
100	0	68.09	72.10	28.40
	10	69.99	75.64	17.03
	20	69.72	83.34	39.82
	30	69.80	84.17	26.41
	40	69.44	85.00	22.88
	S.E.[1]	±2.15	±3.48	±4.52

[1] Standard error among treatments



Each value is an average of 9 determinations, 3 animals on each diet at first, third and fifth day of collection.

Fig. 2. Effect of urea supplemented diets fed to calves on rumen ammonia-N (mg/100 ml).

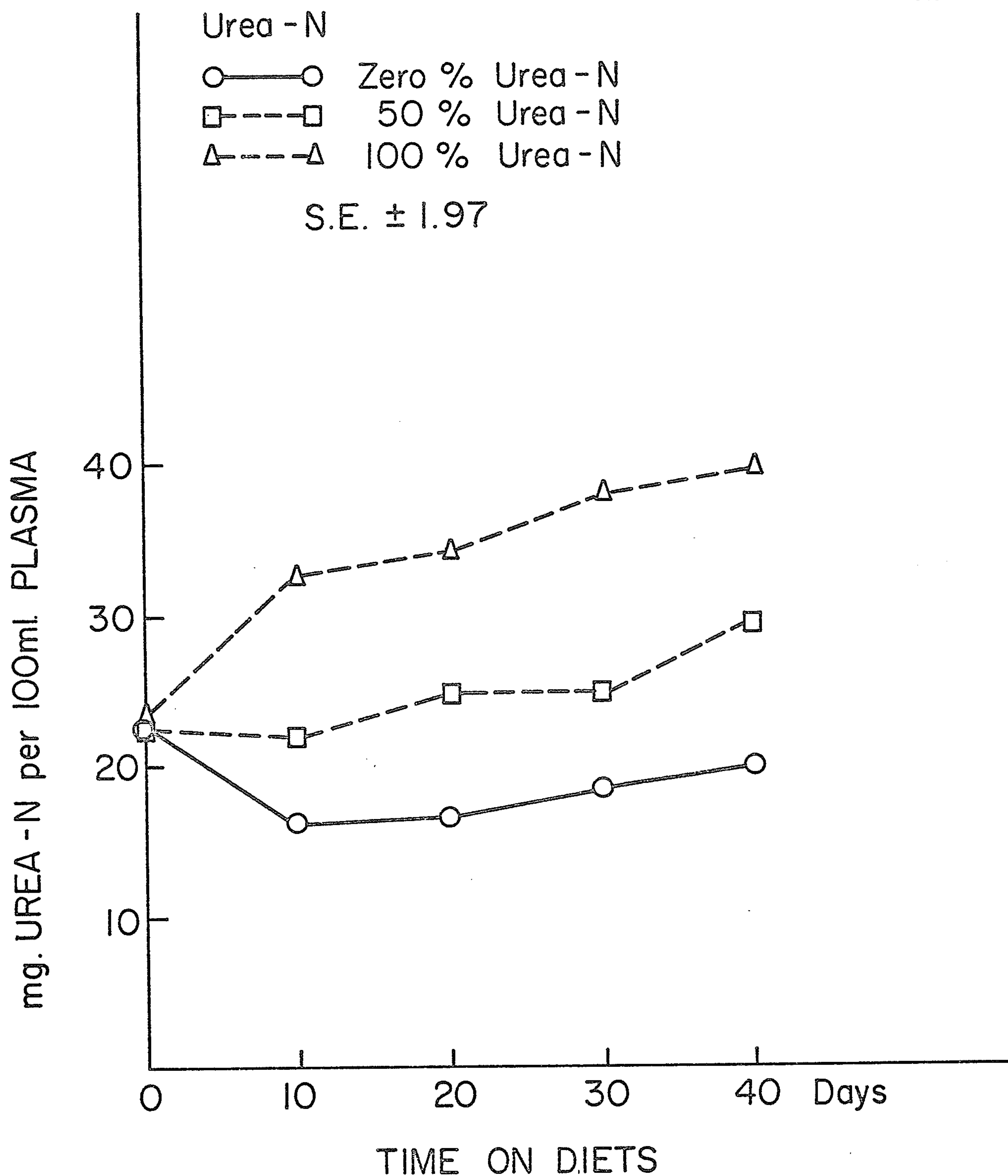
Blood ammonia-N was slightly higher for the urea-supplemented diets than for the soybean diet. There was no change in the level of blood ammonia-N with time on the diets (Table 4).

Blood urea-N concentration was significantly higher ($P<0.01$) for the urea-supplemented diets than for the soybean diet (Fig. 3).

Table 4. Effect of urea supplemented diets fed to calves on blood ammonia-N (mg/100 ml plasma).

% N_2 from Urea	Days on Diets	1st Day of Collection Period	3rd Day of Collection Period	5th Day of Collection Period	Average of Three Determinations
0	0	1.06	1.06	1.06	1.06
	10	0.86	0.72	0.82	0.80
	20	0.87	0.82	0.96	0.88
	30	0.86	0.67	0.91	0.82
	40	0.72	1.01	0.81	0.85
50	0	1.34	1.34	1.34	1.34
	10	1.05	1.15	1.06	1.08
	20	1.06	0.96	1.20	1.07
	30	1.15	1.11	1.25	1.17
	40	1.15	1.01	0.96	1.04
100	0	0.87	0.87	0.87	0.87
	10	1.25	1.63	1.68	1.49
	20	1.54	1.34	1.44	1.44
	30	1.15	1.59	1.54	1.42
	40	1.59	1.35	1.30	1.41
	S.E.[1]	±0.16	±0.13	±0.14	±0.11

[1] Standard error among treatments.



Each value is an average of 9 determinations, 3 animals on each diet at first, third and fifth day of collection.

Fig. 3. Effect urea supplemented diets fed to calves on blood urea-N (mg/100 ml).

Discussion A

It has been established that N digestibility was improved when diets for steers were supplemented with urea when compared to soybean meal or biuret (Hatfield *et al.*, 1959). This improvement was directly correlated with the length of time urea was fed to the animals. Repp *et al.* (1955) suggest that longer preliminary periods may be required to permit the animals to adjust to the NPN supplemented diets. The length of time required for lambs to adjust to the utilization of NPN compound was 37 days as reported by Gaither *et al.* (1955) and 15 days as reported by Anderson *et al.* (1959). Nitrogen retained as a percent of intake increased with time on the NPN supplemented diet (McLaren *et al.*, 1959; Smith *et al.*, 1960; Schaadt *et al.*, 1966). The results in this study indicate that N digestibility was improved by time on the experimental diet. The improvement was relatively higher for urea-supplemented diets. Although there was some improvement for N digestibility with calves fed the soybean diet, the improvement was less than that observed for the urea-supplemented diet. The magnitude of improvement was higher for the diet which contained 100% N from urea than the diet which contained 50% of the N from urea. It appears from these results that N digestibility was increased by time on the urea diets (Table 3).

For N retention there appeared to be some improvement

with time but the trend was not consistent. Johnson and McClure (1964) found no improvement in percent N retained when urea or biuret was fed over a 94-day period. In this study N balance for the urea-supplemented diets was inferior to the 0% urea diet, however, the calves fed the 50% urea diet had a higher N retention than the calves fed the 100% urea diet, Oltjen and Putnam (1966) observed that soybean meal was superior to urea for N retention.

Dry matter digestibility was depressed when 100% of the total N in the diet came from urea. When 50% of the dietary N came from urea, dry matter digestibility was almost the same as that for the soybean diet. Dry matter digestibility has been reprted to decrease on a urea diet (Hatfield *et al.*, 1955, 1959; Ewan *et al.*, 1958; Oltjen *et al.*, 1968), however, Schaadt *et al.* (1966) found no differences between a soybean meal basal diet and a urea supplemented diet in dry matter digestibility.

Ammonia-N concentration (Fig. 2) in the rumen was significantly higher ($P<0.01$) for the urea-supplemented diet than the soybean meal diet. The values were also significantly higher for the diet which contained 100% of the N from urea than the diet that contained 50% of the N from urea which was in agreement with Chalupa *et al.* (1964) for steers fed either 92% urea-N or 46% urea-N in comparison with a corn gluten diet. They found that a 92% urea diet resulted in a higher level of ammonia concentration in the

rumen than a diet of corn gluten or a 46% urea-N diet. The average peak values were 57 mg NH_3-N/100 ml rumen fluid for 92% urea-N diet, 35 mg NH_3-N/100 ml of rumen fluid for the 50% urea diet and 5 mg NH_3-N/100 ml for the 0% urea diet.

Oltjen *et al.* (1968) found that a 4.7% urea diet resulted in a higher rumen ammonia concentration than with uric acid or biuret, but was about the same when compared to urea phosphate. Also Caffrey *et al.* (1967) observed that a high-urea diet yielded a greater ammonia-N concentration in the rumen than a high-corn diet. From our data and the data reported above the relationship of increasing ruminal ammonia N concentration and decreasing N utilization existed, supporting the concept of ammonia absorption into the portal blood system with subsequent synthesis back to urea in the liver and excretion in the urine.

Blood ammonia N was slightly higher for the urea-supplemented diet than for the soybean diet. There was no change in the level of blood ammonia-N with time on the diets (Table 4). Kromann *et al.* (1971) did not find any differences in blood ammonia concentration with different levels of urea in the diet.

Blood urea N concentration was significantly higher ($P<0.01$) for the urea supplemented diets than for the soybean diet. The elevation of blood urea along with the consistent low level of blood ammonia would suggest the effective synthesis of urea from ammonia in the liver and thus prevention of possible ammonia toxicity.

Results B

The protein content of the three tissues studied in this experiment are shown in Tables 5 and 6. The total tissue homogenates had from 2 to 4 fold as much protein as that of the supernatant. There was no effect of treatment or time on the protein concentration of the different tissues. Rumen epithelium had the lowest concentration of soluble protein compared to the other tissues. For the rumen microorganisms, soluble protein concentration was somewhat variable which could be the result of variable concentrations of the rumen microorganisms during the course of the experiment, and possibly to sampling techniques. The concentration of soluble or total protein in rumen epithelium was relatively constant. However, the soluble protein concentration in the liver for the 0% urea diet varied considerably during the experiment. For the 50% urea-N and 100% urea-N diets, the concentration of liver protein was relatively constant.

Glutamate dehydrogenase activity in the liver (Table 7) was consistent for all three diets fed and was not affected by either treatment or time on the experiment.

Although there were small differences in liver glutamine synthetase (Table 7) among treatments , these differences were not significant. The mean value for diets containing urea was slightly higher than the value for the 0% urea diet. The mean values were 3.31 μM of NADH oxidized/

Table 5. Effect of urea supplemented diets fed to calves on liver, rumen epithelial and rumen microorganisms protein content.

% N_2 from Urea	Days on Diet	Liver	Rumen Epithelium	Rumen Microorganism
		(- -	mg protein/g tissue, H	- -)
0	0	105.1 [1]	134.3	142.6
	10	138.1	138.1	196.6
	20	122.8	110.9	188.0
	30	131.9	115.6	166.5
	40	147.6	138.1	110.6
50	0	139.5	135.7	153.6
	10	140.0	130.9	178.2
	20	122.0	147.6	149.9
	30	123.8	147.4	151.8
	40	134.9	149.1	142.6
100	0	160.6	69.3	152.4
	10	161.5	125.8	83.6
	20	128.1	155.3	120.4
	30	121.4	124.7	136.4
	40	131.9	117.7	153.6
	S.E. of means	±14.2	±15.1	±42.7

[1] Each value is an average of three animals and six determinations.

(H) values obtained by analyzing the homogenate of the tissue.

Table 6. Effect of urea supplemented diets fed to calves on liver, rumen epithelial and rumen microorganisms soluble protein content.

% N_2 from Urea	Days on Diet	Liver	Rumen Epithelium	Rumen Microorganism
		(---	mg/g tissue, S	----)
0	0	49.5 [1]	29.6	60.3
	10	55.4	37.3	63.4
	20	58.8	30.5	52.1
	30	80.6	25.5	37.4
	40	85.6	35.5	24.9
50	0	67.5	28.0	92.2
	10	74.7	29.9	94.9
	20	70.3	28.5	66.9
	30	62.9	30.9	69.3
	40	70.0	28.9	40.5
100	0	73.5	32.2	58.4
	10	59.1	34.6	47.1
	20	62.6	26.5	56.8
	30	67.2	27.1	32.3
	40	78.7	29.3	19.5
	S.E. of means	±7.8	±4.2	±18.6

[1] Each value is an average of three animals and six determinations.

(S) values obtained by analyzing the supernatant of the tissue.

Table 7: Effect of urea supplemented diets fed to calves and time on the activities of liver enzymes.

% N_2 from Urea	Days on Diet	Glutamate Dehydrogenase	Glutamine Synthetase	Carbamyl Phosphate Synthetase
0	0	1.96[1]	1.79[2]	7.18[3]
	10	2.81	4.63	20.48
	20	2.64	3.29	26.22
	30	2.67	3.56	49.13
	40	2.63	3.27	59.60
50	0	1.38	2.36	15.36
	10	2.30	5.24	21.39
	20	2.35	3.54	28.42
	30	2.33	4.58	46.37
	40	2.29	2.87	44.87
100	0	2.44	2.45	14.43
	10	2.13	6.04	18.82
	20	2.05	4.14	31.48
	30	2.27	3.92	36.57
	40	2.02	2.38	43.00
	S.E. of means	±0.58	±1.19	±6.59

[1]Values for glutamate dehydrogenase are expressed as micromoles of NAD reduced/min/g of wet liver.

[2]Values for glutamine synthetase are expressed as micromoles of NADH oxidized/min/g of wet liver.

[3]Values for carbamyl phosphate synthetase are expressed as micromoles of citrulline produced/min/g of wet liver.

min/ gm wet liver for the 0% urea diet, for the 50% and 100% urea-N diets the values were 3.72 and 3.79 respectively. The peak values for all diets were reached after the animals were fed the experimental diets for 10 days. After reaching a peak the enzyme activity appeared to decrease to a steady state level.

Liver carbamyl phosphate synthetase activity (Table 7) increased linearly with the time the diets were fed. All treatments followed the same trend. The carbamyl phosphate synthetase reached a maximum activity for all treatments after the diets had been fed for 30 - 40 days. There was no significant difference among diets in enzyme activity. The mean values for the three diets were 32.52, 31.86 and 28.86 μM citrulline produced/min/gm of wet liver for 0%, 50% and 100% urea-N diets, respectively.

There was no significant difference among treatments for the activity of glutamate dehydrogenase in the rumen epithelium (Table 8). The activity reached a maximum value after the animals were fed the experimental diets for 20 - 30 days. The mean values for the 0%, 50% and 100% urea-N diets were 0.37, 0.33 and 0.32 μM NAD reduced/min/gm of fresh tissue, respectively.

Glutamine synthetase activities of rumen epithelium (Table 8) were not significantly different. The mean values for the 0%, 50% and 100% urea-N diets were 2.97, 3.18 and 2.44 μM NADH oxidized/min/gm of fresh tissue, respectively.

Glutamine synthetase activity reached maximum value with all diets after 20 days on the experiment and then decreased. At the peak values, the diet containing 50% of the total N as urea had higher activity than the 100% or 0% urea-N diet.

Carbamyl phosphate synthetase activity of the rumen epithelium (Table 8) was very low and there were no significant differences among treatments. The maximum activity for all treatments was reached after 30 days on the experimental diets. The 100% urea-N diet was slightly higher than either the 0 or 50% urea-N diets which were similar.

Glutamate dehydrogenase activity of rumen microorganism (Table 9) was the highest for the 50% urea-N diet. The mean values for the 0, 50 and 100% urea-N diet were 3.29, 5.68 and 1.29 μM NAD reduced/min/100 of rumen fluid, respectively. The maximum activity of this enzyme was reached for diets which contained 0 or 50% urea-N at 30 days on the experiment. The activity of the enzyme with the diet which contained 50% of the N from urea tended to follow a cubic type of curve with time on the diet.

Glutamine synthetase activity of the rumen micro organisms (Table 9) was significantly different among treatments ($P<0.05$). The diet which contained 50% of the N from urea resulted in a higher glutamine synthetase activity of the rumen microorganisms than diets which contained either 0 or 100% urea-N. The mean values for the 0, 50, and 100% urea-N diets were 9.75, 16.73, and 10.10μM NADH oxidized/

Table 8: Effect of urea supplemented diets fed to calves and time on the activities of rumen epithelial tissue enzymes.

% N_2 from Urea	Days on Diet	Glutamate Dehydrogenase	Glutamine Synthetase	Carbamyl Phosphate Synthetase
0	0	0.18 [1]	1.43 [2]	0.34 [3]
	10	0.28	2.82	0.11
	20	0.55	5.56	0.50
	30	0.51	2.57	0.63
	40	0.35	2.48	0.60
50	0	0.15	1.25	0.34
	10	0.26	1.95	0.35
	20	0.32	7.41	0.28
	30	0.71	2.87	0.63
	40	0.22	2.42	0.49
100	0	0.08	1.00	0.28
	10	0.33	2.00	0.15
	20	0.37	4.66	0.28
	30	0.51	2.63	0.83
	40	0.31	1.88	0.36
	S.E. of means	±0.15	±0.85	±0.28

[1] Values for glutamate dehydrogenase are expressed as micromoles of NAD reduced/min/g of tissue.

[2] Values for glutamine synthetase are expressed as micromoles of NADH oxidized/min/g of tissue.

[3] Values for carbamyl phosphate synthetase are expressed as micromoles of citrulline produced/min/g of tissue.

Table 9: Effect of urea supplemented diets fed to calves and time on the activities of rumen microorganisms enzymes.

% N_2 from Urea	Days on Diet	Glutamate Dehydrogenase	Glutamine Synthetase	Carbamyl Phosphate Synthetase
0	0	1.92 [1]	6.77 [2]	4.78 [3]
	10	4.17	12.97	4.10
	20	3.01	17.00	2.92
	30	4.54	7.76	10.48
	40	2.79	4.23	1.35
50	0	1.94	12.56	3.71
	10	3.01	29.50	8.72
	20	4.61	21.29	4.48
	30	16.69	14.49	10.63
	40	2.16	5.81	3.90
100	0	1.30	7.01	4.30
	10	1.17	13.12	5.58
	20	1.23	22.23	3.70
	30	1.24	5.28	9.40
	40	1.50	2.67	2.81
	S.E. of means	±3.89	±5.62	±2.60

[1]Values for glutamate dehydrogenase are expressed as micromoles of NAD reduced/min/100 ml of rumen fluid.

[2]Values of glutamine synthetase are expressed as micromoles of NADH oxidized/min/100 ml of rumen fluid.

[3]Values for carbamyl phosphate synthetase are expressed as micromoles of citrulline produced/min/100 ml of rumen fluid.

min/100 of the rumen fluid, respectively. The activity of the enzyme on the urea-containing diets (50 or 100%) followed a quadratic type of curve as a function of time during which the diets were fed. The maximum activity of this enzyme was reached after the animals were fed for 10 - 20 days on the experimental diets.

There were no significant differences among treatments for carbamyl phosphokinase activity of rumen microorganisms (Table 9). The mean values for 0, 50 and 100% urea-N diets were 4.73, 6.29 and 5.09 μM citrulline produced/ min/100 ml of rumen fluid for the three diets, respectively. The maximum activity for all treatments was reached after the animals were fed the experimental diets for 30 days.

The percent activity of glutamate dehydrogenase, glutamine synthetase and carbamyl phosphate synthetase as affected by time and freezing on assay repeatability are given in Tables 10, 11 and 12. Liver glutamate dehydrogenase activity decreased by 20% of the original activity 1.5 hr after sampling and remained constant to 12 hr after sampling. There was no difference between rapid freezing in a dry ice-methanol mixture(-80°C) or ordinary freezing at -20°C. The reduction in activity did not exceed 20% after the samples had been frozen for one week. When the samples were frozen directly after obtaining the extract by either the ordinary or the rapid freezing method the decrease in activity was less than when the samples were frozen 12 hr

Table 10: Effects of time and freezing after sampling on liver enzyme activities.

	% Activity		
	Glutamate Dehydrogenase	Glutamine Synthetase	Carbamyl Phosphate Synthetase
1.5 hr after sampling	100.0	100.0	100.0
2.5 hr after sampling	97.4	100.0	-
3.5 hr after sampling	96.7	109.0	89.6
4.5 hr after sampling	84.7	101.1	62.2
5.5 hr after sampling	78.1	90.0	62.1
6.5 hr after sampling	81.5	88.0	60.8
7.5 hr after sampling	81.7	88.0	53.1
8.5 hr after sampling	80.1	88.0	44.0
12.0 hr after sampling	81.2	79.0	24.0
†24.0 hr after sampling	81.9	83.0	11.5
*24.0 hr after sampling	80.2	88.8	14.8
††24.0 hr after sampling	90.1	93.0	60.9
**24.0 hr after sampling	90.1	92.6	69.5
1 wk fast freezing directly	83.3	100.0	32.0
1 wk ordinary freezing directly	86.1	100.0	15.3

†Samples were frozen (-20°C) 13 hr after sampling and reassayed 24 hr after sampling.

*Samples were frozen by rapid freezing (dry ice - methanol mixture, -80°C) 13 hr after sampling and assayed 24 hr after sampling.

††Samples were frozen directly (-20°C) after sampling and were assayed the next day.

**Samples were frozen directly after sampling by rapid freezing (dry ice - methanol mixture, -80°C) and were assayed the next day.

Table 11: Effects of time and freezing after sampling on rumen epithelium enzyme activities.

	% Activity		
	Glutamate Dehydrogenase	Glutamine Synthetase	Carbamyl Phosphate Synthetase
2 hr after sampling	100.0	100.0	100.0
4 hr after sampling	100.0	99.2	80.2
6 hr after sampling	100.0	72.0	60.3
8 hr after sampling	85.6	72.5	40.0
†24 hr after sampling	62.9	72.9	11.2
††24 hr after sampling	74.3	75.4	15.3
*24 hr after sampling	71.4	78.0	15.2
** 4 hr after sampling	71.4	77.8	11.1

†Samples were frozen ($-20^{\circ}C$) 8 hr after sampling and assayed the next day.

††Samples were frozen by rapid freezing (dry ice - methanol mixture, $-80^{\circ}C$) 8 hrs after sampling and assayed the next day.

*Samples were frozen ($-20^{\circ}C$) directly after sampling and assayed the next day.

**Samples were frozen by rapid freezing directly after sampling (dry ice - methanol mixture, $-80^{\circ}C$) and assayed the next day.

Table 12: Effects of time and freezing after sampling on rumen microorganisms enzyme activities.

	% Activity		
	Glutamate Dehydrogenase	Glutamine Synthetase	Carbamyl Phosphate Synthetase
2 hr after sampling	100.0	100.0	100.0
4 hr after sampling	100.0	100.0	90.0
6 hr after sampling	100.0	85.0	40.0
8 hr after sampling	85.0	83.2	26.1
†24 hr after sampling	82.3	81.1	20.0
††24 hr after sampling	81.9	79.3	18.3
*24 hr after sampling	91.0	94.2	18.4
**24 hr after sampling	91.4	94.4	15.3

†Samples were frozen (-20°C) 8 hr after sampling and assayed the next day.

††Samples were frozen by rapid freezing (dry ice - methanol mixture, -80°C) 8 hr after sampling and assayed the next day.

*Samples were frozen (-20°C) directly after sampling and assayed the next day.

**Samples were frozen by rapid freezing directly after sampling (dry ice - methanol mixture, -80°C) and assayed the next day.

after the extracts were prepared.

Liver glutamine synthetase was stable for a longer period of time than glutamate dehydrogenase. After 8.5 hr the samples still contained about 90% of the original activity of glutamine synthetase. Glutamine synthetase activity after freezing for one week was 100%.

Liver carbamyl phosphate synthetase was highly unstable 3.5 hr after sampling. The activity of this enzyme decreased rapidly to 24% 12 hr after sampling. The activity decreased to 11 - 14% of the original when samples were frozen 12 hr after sampling and assayed 24 hr later. Fast freezing showed little advantage over ordinary freezing. One week after direct freezing of the extracts 15 - 32% of the original activity was found.

Rumen epithelial tissue glutamate dehydrogenase was stable up to 8 hr after sampling. Fast or ordinary freezing results were similar. The activity of the enzyme decreased to about 30 - 40% of the original 24 hr after sampling. Glutamine synthetase in rumen epithelium was less stable than in the liver. The activity was stable up to 4 hr after sampling but decreased to 70% when the assay was done 6 hr after sampling. Rapid or ordinary freezing gave the same results. Carbamyl phosphate synthetase in rumen epithelium had the same characteristics as the liver enzyme.

Rumen microbial enzymes (glutamate dehydrogenase and glutamine synthetase) were highly stable up to 6 hr after sampling. After 6 hr of sampling the reduction in enzyme

activities was 20% of the original. Carbamyl phosphate synthetase was unstable after 4 hr of sampling and decreased rapidly to 15 - 20% of the original value 24 hr after sampling. There was no difference between rapid or ordinary freezing.

Since all the enzyme assays were done within 2 - 4 hr after sampling the activities obtained in this experiment would be similar to the original activities at sampling.

Discussion B

Generally, there are three major enzyme reactions responsible for the incorporation of ammonia into organic N compounds.

1) Glutamate Dehydrogenase

The most important feature of the enzyme is that the reaction it catalyzes is readily reversible. The equilibrium constant for this reaction strongly favors the synthesis of glutamate.

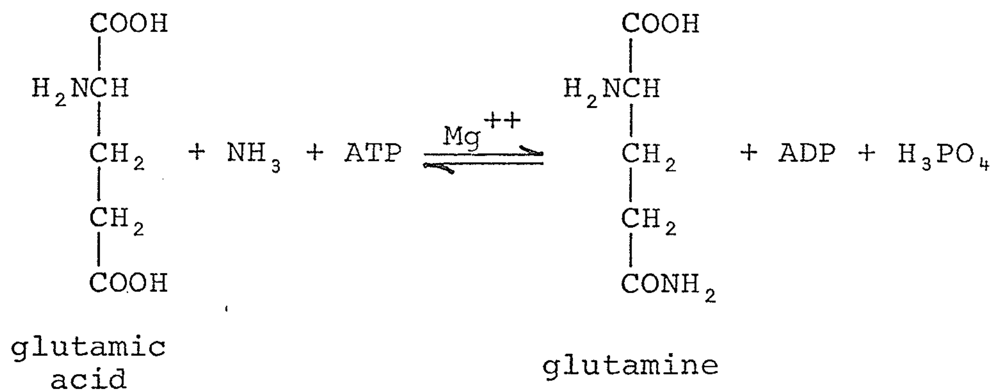
$$\begin{array}{c} COOH \\ | \\ C{=}O \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ COOH \end{array} + NH_3 + NADH + H^+ \rightleftharpoons \begin{array}{c} COOH \\ | \\ H_2NCH \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ COOH \end{array} + NAD^+ + H_2O$$

α-ketoglutaric acid — L-glutamic acid

The N atom in the glutamic acid can be passed on by transamination reactions to make all the other amino acids of the cell, provided the necessary α-keto acids are available.

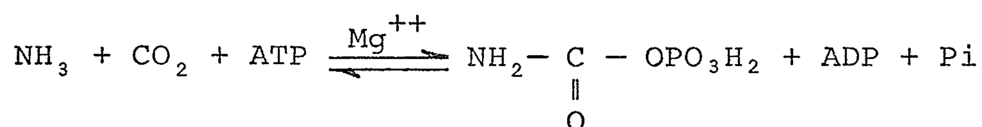
2) Glutamine Synthetase

The enzyme catalyzes the synthesis of the amide of glutamic acid.

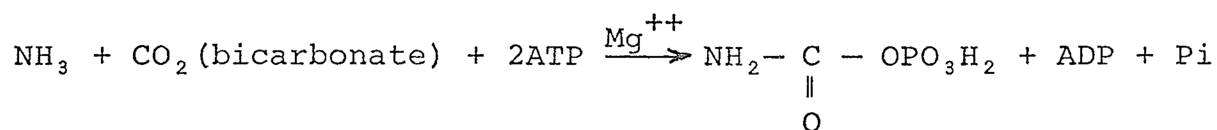


The amide N of glutamine in turn can be donated in specific reactions to appropriate acceptors to form a variety of nitrogenous compounds (Purines, pyrimidines, glucosamine and NAD^+).

The third major route for the uptake of NH_3 into organic compounds is catalyzed by the enzyme carbamyl phosphokinase in bacteria or carbamyl phosphate synthetase in animal tissue. The bacterial enzyme catalyzes the following reaction:



The mammalian enzyme catalyzes the following reaction:



Carbamyl phosphate made by either of these processes serves

as a donor of the carbamyl group in citrulline biosynthesis and in pyrimidine biosynthesis.

Because of the importance of these three enzyme systems the activities and the response of animals fed a high urea diet were investigated. The role of these enzymes in urea metabolism was also followed since urea hydrolyzes to ammonia in the rumen before being utilized.

Since urea is rapidly hydrolyzed to ammonia in the rumen, the urea fed must be synthesized into protein by the rumen microorganisms for maximum utilization of dietary N by the animal. McLaren *et al.* (1960) postulated that the adaptation response of lambs to urea-rich diets may be an increased utilization of ammonia at the tissue level. Hoshino *et al.* (1966) reported that at high rumen ammonia concentrations, ammonia was incorporated into glutamic acid by reduction of alpha-ketoglutarate in the rumen mucosa; furthermore, a part of the glutamate entered into the circulation through the rumen wall after it was converted to glutamine. These synthetic reactions should be carried out in the stomach mucosa and the rumen microorganisms.

In this study there was no significant differences in the activity of glutamate dehydrogenase among diets, although the 0% urea diet (soybean meal) had a higher liver and epithelium activity than the urea-containing diets. Chalupa *et al.* (1970) found that 4.3% urea in the diet increased glutamate dehydrogenase activity in the liver of

urea-fed animals more than soybean meal fed animals. Whanger and Church (1970) had also observed that glutamate dehydrogenase activity for urea-fed steers was higher than steers fed cottonseed meal (control) in both liver and rumen epithelium. However, this experiment and studies by Chalupa *et al.* (1970), Whanger and Church (1970), and Cooper (1962) were in agreement in that there were no significant differences in glutamate dehydrogenase activity in animal tissues when urea was compared to either soybean meal or cottonseed meal.

The lower activities obtained in the liver and rumen epithelium of the urea-fed animals may suggest that the adaptation response of these tissues to the urea-containing diet occurred within 1 to 10 days, a period which was not measured. Another possibility is that the adaptation response may have taken place after 40 days on the urea diet, since the increased activity of liver glutamate dehydrogenase obtained by Chalupa *et al.* (1970) was a result of measurement taken 70 days after urea had been fed to the animals.

The diet which contained 50% urea-N resulted in a higher activity of glutamate dehydrogenase in the rumen microorganisms when compared to the 0% or 100% urea-N diets. This may suggest that amination reactions were important pathways in the assimilation of ammonia by rumen microorganisms. This was also suggested by the presence of

many keto acids, including -ketoglutaric and pyruvic, in rumen liquor (Vander Horst, 1961). In tracer studies with ^{15}N, Richardson and Tsein (1963) found that ^{15}N activity appeared rapidly in glutamate and aspartate, and glutamate was found in large amounts in rumen microbial protein. This may indicate that glutamate dehydrogenase is probably the major system in the fixation of ammonia into cellular protein by ruminal microorganisms. However, other dehydrogenases and transaminases may be important as well. Otagaki *et al.* (1955), on the basis of degradation of ^{14}C-labelled amino acids, postulated general transaminase activity in rumen microorganisms. In microorganisms in which glutamate synthesis represents the primary means of ammonia assimilation, transaminases would be extremely important in the synthesis of other amino acids. Glutamate-pyruvate transaminase had previously been reported to be present in extracts of mixed ruminal microorganisms (Palmquist and Baldwin, 1966) and in extracts of seven of nine pure cultures of rumen bacteria (Joyner and Baldwin, 1966).

Liver glutamine synthetase activities were slightly higher for urea-fed animals than animals fed the soybean diet (0% urea). The maximum activity of this enzyme in the liver tissue was reached within 10 days on the experimental diets and this could be due to rapid response by the liver to the change in N source in the diet. Although increased activities of liver glutamine synthetase occurred

there were no significant differences among the three diets. This was in agreement with the results obtained by Chalupa *et al.* (1970) who found that glutamine synthetase activity was not affected by the dietary N source. In contrast to the results we obtained, they found that the activity of liver glutamine synthetase was slightly higher for the soybean-fed animals than the urea-fed animals.

Duda and Handler (1958) in tracer studies administered ^{15}N ammonia, glutamine, L and D-leucine to rats, and measured the ^{15}N of liver urea, glutamine, glutamate, aspartate, alanine, and glycine as well as total body glutamine and urea at various time intervals. Glutamine synthesis was found to be the major fate of ammonia. They found that 5 min after administration of NH_3-^{15}N, the specific activity of glutamate was one-third that of the amide-N of glutamine. Thereafter, ammonia was incorporated into the amide position of glutamine considerably more rapidly than into any of the other nitrogenous compounds of liver. When peak activity was attained, the specific activity of glutamine was approximately 7 times that of either urea or glutamic acid. This could be an indication of the glutamine synthesis system (glutamine synthetase enzyme) in the assimilation of ammonia in the liver. In this study there were no significant differences in the activity of this enzyme in the liver which may be explained on the

basis that when the animals were fed the 100% urea-N diet enzyme synthesis was reduced as a result of ammonia *per se* or as a result of certain other factors which caused inhibition of enzyme synthesis. Katunuma *et al.* (1966) have suggested that ammonia inhibits the conversion of isocitrate to α-ketoglutarate. These workers have shown that ammonia *in vitro* activates pyridine nucleotide oxidase which oxidizes NADH and NMNH to NAD and NMH, respectively. Matsuda and Katunuma (1969) have shown that ammonia also activates a pyridine nucleotide pyrophosphatase in rat liver mitochondria or microsomes which cleave NADH and NADPH to NMNH. Thus, if ammonia activates the pyrophosphatase pathway of NADPH breakdown, a decrease in the total NADP + NADPH would be expected and this was observed in the liver of urea-fed lambs by Prior *et al.* (1970).

The same type of discussion could be applied for rumen epithelium activities of glutamine synthetase. In this study there was no significant difference among the three diets, but the 50% urea-N diet had the highest value and the 100% urea-N diet had the lowest value. This would indicate the importance of this enzyme system in the assimilation of ammonia. Hoshino *et al.* (1966) reported an increase in glutamine content of the rumen and the plasma and they suggested that initially glutamate may be synthesized in the rumen and the rumen wall, then converted to glutamine. The limited data supports the evidence that

glutamine synthesis occurs in the rumen microorganisms as well as in the rumen mucosa. Bryant and Robinson (1961) suggested that *Bacteroides succinogenes* might contain a glutaminase since the organism could utilize glutamine for growth. Warner (1964) found that a large part of the glutaminase activity was associated with the protozoal fraction and medium-sized bacteria. Cook *et al.* (1965) observed that there was a fourfold increase in concentration of asparagine and glutamine in the rumen vein blood 20 min after addition of amino acid mixture to the rumen. They suggested that asparagine and glutamine were rapidly synthesized in the rumen wall and absorbed into the blood stream via the rumen wall. Chalupa *et al.* (1970) observed that glutamine synthetase and the five urea cycle enzyme systems were present in trace amounts in rumen mucosa. They suggested that the amounts of these enzymes present in rumen mucosa could have been of bacterial origin. The data in this study was in contrast with Chalupa *et al.* (1970). Glutamine synthetase activity of rumen mucosa in the present study was quite high which indicated that this enzyme system was very important for the assimilation of ammonia in the rumen epithelium.

In the present study, rumen microbial glutamine synthetase activity was significantly higher ($P<0.05$) for animals fed 50% urea-N in the diet than those fed 0% or 100% urea-N diets. As previously discussed, glutamine

synthetase is present in large amounts in rumen microorganisms. This and other data obtained by Chalupa *et al.* (1970) in which they found increased activity of this enzyme with urea-fed sheep when compared to soybean-fed sheep, indicated that this enzyme system is a very important pathway for ammonia metabolism in the rumen microorganisms of urea-fed animals. The reason for the lower activity of this enzyme when 100% urea-N diet was fed in comparison with the 50% urea-N diet may be explained by the inhibition of enzyme synthesis by ammonia on the 100% urea-N diet; as a result, the amount of enzyme was reduced.

The data obtained in the present experiment would indicate that liver was the most important site for the urea cycle enzymes. Liver carbamyl phosphate synthetase was significantly higher ($P<0.01$) than that of rumen epithelium or rumen microorganisms. There were no significant differences among diets in the activity of liver carbamyl phosphate synthetase although the 0% urea diet had higher enzyme activity when compared to 50% or 100% urea-N diets. The same trend was found for the rumen epithelium. This could be possible since Chalupa *et al.* (1970) found that carbamyl phosphate synthetase, ornithine transcarbamylase and arginase activities were decreased in the liver of urea-fed animals when compared to soybean-fed animals in spite of increased urea excretion. However, Whanger

and Church (1970) reported that rumen epithelium ornithine transcarbamylase and arginase were increased for steers fed urea when compared to cottonseed. Although an increase in urea excretion was usually associated with an increase in urea cycle enzyme activities (Schimke, 1962 and 1963), the results obtained herein could be the result of ammonia *per se*, either by direct or indirect mechanisms, or could be due to a suboptimum amino acid nutritional status. Since ammonia can cause changes in cellular energy metabolism including depletions in α-ketoglutarate (Bessman and Bessman, 1955; and Katunuma *et al.*, 1966), impaired decarboxylation of pyruvate and α-ketoglutarate (McKhann and Tower, 1961), depletion of creatine phosphate and ATP (Schenker *et al.*, 1967, and Katunuma *et al.*, 1966), the decreases in enzyme activities could be the indirect results of the biochemical aberrations due to decreases in enzyme synthesis produced by ammonia.

In contrast to liver and rumen epithelium, rumen microorganisms carbamyl phosphokinase activity for the urea-fed animals was slightly higher than that of soybean fed animals. Carbamyl phosphokinase was found in many microorganisms, and Niederman and Wolin (1967) indicated that the enzyme was involved in the synthesis of arginine by *Streptococcus bovis*. Chalupa *et al.* (1970) reported that urea-fed sheep had more carbamyl phosphokinase activity in rumen microorganisms than soybean-fed animals.

However, they postulated that this enzyme system was of minor importance in the ruminal assimilation of ammonia by the mixed rumen microbial population. The data of Hill *et al.* (1962) indirectly indicated that the urea cycle in ruminal microorganisms was reversible which, according to Cohen and Brown (1960), was thermodynamically possible. However, Beckwith *et al.* (1962) and Thorne and Jones (1963) indicated that the substrate concentration and pH required to satisfy the thermodynamic and kinetic needs of the forward reaction (carbamyl phosphate synthesis) of the carbamyl phosphokinase reversible system would almost have to be unphysiological. A glutamine-dependent carbamyl phosphate synthetase has been reported in *Escherichia coli* by Rerard and Wiame (1964) and Kalman *et al.* (1966). This could be an important pathway for glutamine synthesis by rumen microorganisms. The increased activity of carbamyl phosphokinase in rumen microorganisms of the urea-fed animals in this experiment was an indication for an increase in urea synthesis to maintain N economy for the urea-fed animals. This could be a result of rumen populations changing to those which have increased ability for ammonia fixation.

On the basis of the arguments presented above, the most reasonable conclusion would be that a part of ammonia was incorporated into glutamate by the reaction of glutamate dehydrogenase of rumen microorganisms and rumen mucosa.

The adaptation of the animals to the urea diet was more clearly indicated by the increased activity of glutamate dehydrogenase of rumen microorganisms obtained from the urea-fed animals. The higher activity for this enzyme obtained for the 50% urea-N diet than for the 100% urea-N diet could have occurred as a result of enzyme inhibition by ammonia after it had reached a saturation level, or the depletion of α-ketoglutarate and pyridine nucleotide by alterations of carbohydrate metabolism. The oxidation of isocitrate to α-ketoglutarate may be the step of the tricarboxylic acid cycle which was most sensitive to ammonia. Thus, a portion of the glutamate was absorbed into circulation after it was converted to glutamine in the rumen microorganisms and rumen mucosa. The adaptation response for the urea diet was by a greater increase in the activity of glutamine synthetase for the urea-fed animals as compared to the soybean fed animals. This response was more effective for the rumen microorganisms obtained from the urea-fed animals. Liver and rumen epithelium glutamine synthetase activities obtained from these animals were also higher than those of the soybean fed animals. This indicated that glutamine synthetase plays a very important role in ammonia assimilation, particularly for the urea-fed animals. Anabolic effects of ammonia concerning glutamine synthesis seem to have physiological significance in N metabolism in ruminants, since not only enzymes but also sufficient

substrates for their actions were present in the rumen. On a high urea siet, it was probably that an animal increased N economy to preserve ammonia in the form of glutamine. The other portion of ammonia was synthesized to urea in the rumen microorganisms, rumen epithelium, and liver. The urea thus synthesized could diffuse through the rumen wall to the rumen for reutilization. Also, part of the synthesized urea could be excreted in the urine. The results of the present study indicated that carbamyl phosphate synthetase activity in liver and rumen epithelium from urea-fed animals was slightly lower than in soybean-fed animals. This may be an attempt by the animal to conserve tissue amino acids especially with urea-fed animals, since these animals were reported to have a suboptimal amino acid nutritional status (Chalupa, 1968; Oltjen, 1969; Schelling *et al.*, 1967; and Schelling and Hatfield, 1968), and also this enzyme was not rate limiting for urea synthesis. In support of this theory, enzyme systems which normally exhibit the least activity and have the least reserve (arginine synthetase and argininosuccinase) were not decreased by the addition of 4.3% urea to the diet in the work of Chalupa *et al.* (1970). Also, when Schimke (1963) added 7.5% urea to a 15% casein diet, rats excreted about four times as much urea without a concomitant increase in activities of urea cycle enzyme systems. With rumen microorganisms the situation was different, the activity of

carbamyl phosphokinase was increased more for urea-fed animals than that of soybean-fed animals. This could be a result of changing rumen microorganisms toward ammonia utilization. In all cases the 50% urea-N diet was more efficiently utilized than the 100% urea-N diet. The lack of statistically significant results in this experiment for the activities of the individual enzymes could be normal since the adaptation response to urea is a general change in the animal involving many enzymes concerned with N metabolism as well as possibly many tissues of the body. That is, the combined increase of the enzymes involved in N metabolism may be significant, but the increase of one of these enzymes was not enough to be statistically significant. This was also suggested by Whanger and Church (1970) in a study with steers fed either urea or cottonseed meal.

The stability studies of the three enzymes for the three tissues are shown in Tables 10, 11 and 12. All three enzymes in all three tissues were relatively stable within 4 - 6 hr of sampling time and decreased slowly with the exception of carbamyl phosphate synthetase activity which decreased sharply 4 hr after sampling time. Since all the enzyme assays were done within 2 - 3 hr of sampling time, the activities obtained were considered to be closely associated to the original activities of the enzyme. Also all enzyme assays were done at the same time so as to obtain comparative results.

Results C

Plasma Amino Acids

The blood plasma amino acid patterns indicate that when urea-containing diets were fed, the level of arginine aspartic acid, citrulline, glutamic acid, glycine, methionine, ornithine and proline were increased while valine alanine, cystine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan and tyrosine were decreased. All the essential amino acids with the exception of methionine were decreased on the urea-containing diets. All the amino acids related to urea synthesis such as arginine, aspartic acid, glutamic acid, citrulline and ornithine were increased on the urea containing diets.

Amino Acid in Rumen Microorganisms

All the amino acids of rumen microorganisms were increased on the 50% urea-N diet with the exception of arginine and threonine, which decreased on the urea diets. The reduction of both arginine and threonine was greater for the 100% urea diet than for the 50% urea diet. The amino acids related to urea synthesis, aspartic acid, citrulline, glutamic acid, and ornithine were higher for the diet which contained 50% of the N from urea than the diet which contained 100% of the N from urea. The essential amino acids levels were also higher for the diet which contained 50% of the N from urea than the diet which contained 100%.

Isoleucine, leucine, valine and phenylalanine were reduced to a much greater extent for the diet which contained 100% of the N from urea. For all amino acids, the diet which contained 100% of N from urea was inferior to the diet which contained 50% of the N from urea or the 0% urea diet.

Liver Amino Acids

All the essential amino acid in the liver were reduced on the urea-containing diets with the exception of histidine and phenylalanine. The diet which contained 50% of the N from urea produced higher levels of liver amino acid than the diet which contained 100% of the N from urea. Glutamic acid, cystine and ornithine were at a higher level for the 100% urea-N diet than the 50% urea-N diet. It appeared that for all amino acids in the liver, the 50% urea-N diet was similar to the soybean diet (0% urea).

The presence of some amino-N compounds such as orthophosphoserine, orthophosphoethenolamine and orthophosphothionine were observed in the liver. These compounds were not detected in the blood plasma or rumen microorganisms. Glutamic acid was present in the liver at a much higher level than in any other tissue and represented approximately 44% of the total amino acids in the liver. Glycine represented about 14%, alanine 9%, and aspartic about 6.5% of total liver amino acids.

Amino Acid in Rumen Epithelium

In the rumen epithelium tissue there was a reduction of the essential amino acids such as valine, methionine, leucine, isoleucine, lysine and histidine. Tryptophan was not present and could be due to analysis technique. Glutamic acid was present as a high percentage of the total amino acids in the rumen epithelium. Glycine, alanine and aspartic acid were also present at a high percentage of the total amino acids. Aspartic acid, citrulline and ornithine were increased on the urean-containing diets and would indicate an increase in urea synthesis under these conditions.

The diet which contained 50% of the N from urea was generally superior to the diet which contained 100% of the N from urea.

Table 13: Effect of urea supplemented diets fed to calves on plasma amino acids concentrations.

(compounds increased on 100% urea diet)

Amino Acid	Concentrations in mg/100 ml plasma			S.E.
	0% N from urea	50% N from urea	100% N from urea	
Arginine	1.693[1]	1.642[1]	1.786[1]	±0.097
Aspartic Acid	0.238	0.229	0.331	±0.066
Citrulline	0.834	0.862	1.062	±0.079
Glutamic Acid	2.399	2.361	2.585	±0.367
Glycine	1.912	1.903	2.121	±0.139
Methionine	0.301	0.401	0.228	±0.067
Ornithine	0.944	0.844	1.135	±0.104
Proline	0.426	0.382	0.521	±0.218

[1]Values are means of five determinations obtained at 0, 10, 20, 30, and 40 days on the experimental diets.

Each value is a mean for three replicates of the three animals.

Table 14: Effect of urea supplemented diets fed to calves on plasma amino acids concentrations.

(compounds decreased on urea diets)

Amino Acid	Concentrations in mg/100 ml plasma			S.E.
	0% N from urea	50% N from urea	100% N from urea	
Alanine	2.015 [1]	1.975 [1]	1.866 [1]	±0.222
Cystine	0.245	0.224	0.121	±0.027
Histidine	1.102	0.904	1.015	±0.162
Isoleucine	1.751	1.437	1.273	±0.222
Leucine	1.791	1.360	1.279	±0.214
Lysine	1.538	1.343	1.285	±0.154
Phenylalanine	0.779	0.673	0.605	±0.068
Threonine	0.934	0.893	0.887	±0.053
Tryptophan	0.756	0.536	0.559	±0.215
Tyrosine	1.027	0.878	0.800	±0.118
Valine	3.452	2.792	2.500	±0.433

[1] Values are means of five determinations obtained at 0, 10, 20, 30, and 40 days on the experimental diets.

Each value is a mean for three replicates of the three animals.

Table 15: Effect of urea supplemented diets fed to calves on rumen microorganisms amino acids concentrations

(compounds increased on 50% urea diet)

Amino Acid	Concentrations in mg/100 ml rumen fluid			S.E.
	0% N from urea	50% N from urea	100% N from urea	
Alanine	0.262 [1]	0.440 [1]	0.174 [1]	±0.198
Aspartic Acid	0.103	0.162	0.097	±0.054
Citrulline	0.033	0.087	0.024	±0.025
Cystine	0.013	0.023	0.029	±0.015
Glutamic Acid	0.372	0.447	0.223	±0.192
Glycine	0.100	0.124	0.059	±0.054
Histidine	0.023	0.064	0.022	±0.033
Isoleucine	0.070	0.100	0.041	±0.051
Leucine	0.109	0.141	0.060	±0.079
Lysine	0.222	0.226	0.138	±0.109
Methionine	0.051	0.068	0.036	±0.035
Ornithine	0.058	0.106	0.087	±0.044
Phenylalanine	0.050	0.068	0.022	±0.042
Tyrosine	0.058	0.060	0.023	±0.044
Valine	0.106	0.145	0.048	±0.077

[1]Values are the means of five determinations obtained at 0, 10, 20, 30, and 40 days on the experimental diets.

Each value is a mean for three replicates of the three animals.

Table 16: Effect of urea supplemented diets fed to calves on rumen microorganisms amino acids concentrations.

Amino Acid	Concentrations in mg/100 ml rumen fluid			S.E.
	0% N from urea	50% N from urea	100% N from urea	
Arginine	0.081 [1]	0.069 [1]	0.045 [1]	±0.041
Threonine	0.115	0.103	0.066	±0.055

[1] Values are the means of five determinations obtained at 0, 10, 20, 30, and 40 days on the experimental diets.

Each value is a mean for three replicates of the three animals.

Table 17: Effect of urea supplemented diets fed to calves on liver amino acids concentrations.

Amino Acid	Concentrations in mg/100 gm wet Liver			S.E.
	0% N from urea	50% N from urea	100% N from urea	
Alanine	18.750[1]	19.407[1]	16.268[1]	±3.079
Aspartic Acid	13.258	14.013	12.361	±4.484
Citrulline	2.036	5.052	3.320	±0.938
Cystine	3.537	2.701	4.056	±0.897
Glutamic Acid	92.455	85.901	101.298	±14.694
Glycine	28.103	28.203	27.210	±3.170
Histidine	6.458	6.777	5.517	±1.112
Ornithine	9.711	10.021	10.716	±1.710
O-Phosphoethanol-amine	5.489	9.013	7.658	±4.427
Phenyl alanine	2.101	2.660	1.889	±0.704

[1]Values are the means of five determinations obtained at 0, 10, 20, 30, and 40 days on the experimental diets.

Each value is a mean for three replicates of the three animals.

Arginine was not detected in the liver by the method used.

Table 18: Effect of urea supplemented diets fed to calves on liver amino acids concentrations.

Amino Acid	Concentrations in mg/100 gm wet liver			S.E.
	0% N from urea	50% N from urea	100% N from urea	
Isoleucine	2.151 [1]	1.896 [1]	1.466 [1]	±0.445
Leucine	4.448	4.362	3.678	±0.548
Lysine	5.083	4.953	4.521	±1.132
Methionine	0.740	0.737	0.600	±0.115
Threonine	4.055	3.693	3.271	±0.517
Tryptophan	3.711	2.055	1.859	±1.478
Tyrosine	2.936	2.874	2.764	±0.409
O-Phosphoserine	1.684	1.516	1.365	±1.083
O-Phosphothionine	0.436	0.216	0.188	±0.285
Valine	4.501	4.427	3.585	±0.987

[1]Values are the means of five determinations obtained at 0, 10, 20, 30, and 40 days on the experimental diets.

Each value is a mean for three replicates of the three animals.

Table 19: Effect of urea supplemented diets fed to calves on rumen epithelium amino acids concentrations.

Amino Acid	Concentrations in mg/100 gm wet tissue			S.E.
	0% N from urea	50% N from urea	100% N from urea	
Aspartic Acid	4.448 [1]	3.990 [1]	4.615 [1]	±0.999
Citrulline	0.151	0.158	0.123	±0.116
Cystine	0.776	0.891	0.531	±0.384
Ornithine	1.690	1.469	1.913	±0.182
Phenylalanine	0.049	.016	0.080	±0.033
Threonine	1.289	1.262	1.739	±0.301
Tyrosine	0.070	0.042	0.109	±0.055

[1] Values are the means of five determinations obtained at 0, 10, 20, 30, and 40 days on the experimental diets.

Each value is a mean for three replicates of the three animals.

Arginine was not detected by the method used.

Table 20: Effect of urea supplemented diets fed to calves on rumen epithelium amino acids concentrations.

Amino Acid	Concentrations in mg/100 gm wet tissue			S.E.
	0% N from urea	50% N from urea	100% N from urea	
Alanine	3.478 [1]	3.342 [1]	3.172 [1]	±0.203
Glutamic Acid	17.175	16.268	13.410	±1.963
Glycine	6.088	5.363	4.921	±0.414
Histidine	0.458	0.305	0.351	±0.050
Isoleucine	0.497	0.228	0.227	±0.051
Leucine	1.000	0.566	0.735	±0.104
Lysine	1.779	1.267	1.449	±0.140
Methionine	0.237	0.078	0.096	±0.056
O-Phosphoserine	0.113	0.097	0.066	±0.067
O-Phosphoethanol-amine	0.407	0.189	0.339	±0.209
Valine	0.967	0.709	0.721	±0.166

[1]Values are means of five determinations obtained at 0, 10, 20, 30, and 40 days on the experimental diets.

Each value is a mean for three replicates of the three animals.

Discussion C

It has been shown that when urea was substituted for isolated doy protein in diets fed to cattle on an isocaloric and isonitrogenous basis, significantly reduced blood plasma levels of leucine, isoleucine, valine and phenylalanine and significantly increased levels of serine and glycine were found (Oltjen and Putnam, 1966). In this study blood plasma levels of valine, alanine, cystine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan and tyrosine were decreased on the urea containing diets and arginine, aspartic acid, citrulline, glutamic acid, glycine methionine, ornithine and proline were increased when urea provided either 50% or 100% of the total N in the diet. Slyter *et al.* (1971) found that on a urea-containing diet fed to steer claves, blood plasma levels of threonine, valine, methionine, isoleucine, leucine, phenylalanine, and lysine were decreased while serine and glycine were increased. High plasma serine (Oltjen *et al.*, 1968) and glycine (Virtanen, 1966; Oltjen and Bond, 1967; Schelling *et al.*, 1967) have been reported in ruminants fed urea-purified diets compared to control animals fed natural diets. The method used for the estimation of the amino acids in this study did not allow for the detection of serine, but with this exception, the results are ine agreement with the previous reports. The concentrations of the essential amino

acids increased and nonessential amino acids decreased in plasma when urea-fed animals were compared to soybean-fed animals and may have been a result of an amino acid imbalance which occurred for urea-fed animals resulting in a reduced N retention.

The amino acid concentrations in plasma found in this study were somewhat lower than those reported for lactating cows fed a natural diet (Verbeke and Peeters, 1965). However, the cows were given a large amount of feed and the blood samples were taken shortly after feeding. In our experiment the animals were fed on a continuous feeder to produce a steady state metabolism. The amino acid levels obtained were the average level of the steady state condition while the samples obtained by Verbeke and Peeters were probably nearer the peak concentration. The total amino acid concentrations of blood plasma in the present study were also lower than those obtained for sheep fed natural diets when sampled just prior to feeding (Leibholtz,1965) and Oltjen *et al.* (1968) for steers fed urea, biuret, urea-phosphate or uric acid containing diets. It has been reported by Oltjen *et al.* (1969b) that plasma amino acid patterns of calves fed a urea-purified diet and an isolated soy protein diet were similar at 42 days of age while the calves were still receiving milk, but at 84 (after weaning) and 189 days of age the concentrations of threonine, phenylalanine, valine, leucine, isoleucine, cystine, hydroxyproline and tyrosine

were especially low, whereas urea and serine concentrations were especially high in urea-fed calves.

Oltjen *et al.* (1968) found that blood plasma amino acid levels were significantly affected by NPN source, time after feeding or an interaction between these when steers were fed urea, biuret, urea phosphate or uric acid. Blood plasma concentrations of threonine, serine, proline, glycine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine and lysine were all found to be significantly lower 4 hr after feeding than before feeding. The concentration of glutamic acid, however, significantly increased with time after feeding. In the present work a continuous feeder was used and differences between values before and after feeding were not determined, but it was found that glutamic acid was always higher for urea containing diets than for diets which contained no urea. The nutritional significant of the elevated blood plasma level of glutamic acid is not clear. However, Hepburn *et al.* (1960) found that the omission of glutamic acid from the diet of rats resulted in greatly reduced rates of gain whether it was omitted alone or in combination with aspartic acid. The stimulation of growth rate of rats by glutamic acid has been studied by other workers. Womack and Rose (1947) showed that glutamic acid led to increased gains when fed with an amino acid diet devoid of arginine. Rose *et al.* (1948) found that when the ten essential amino acids were supplied, glutamic acid was

effective as the sole source of nonspecific nitrogen, but its omission from a mixture of 19 amino acids was without deleterious effect upon rat growth. Schelling and Hatfield (1968) reported that abomasal infusion of either lysine or glutamic acid increased N retention by the same magnitude as that of the mixture of arginine, histidine, lysine, phenylalanine and methionine, but the response was less than that observed from infusion of all 10 essential amino acids.

In all the reports cited above as well as the results of this study the reductions of blood plasma levels of certain amino acid associated with urea feeding was also associated with a reduction in N retention. In this study blood plasma levels of glutamic acid, aspartic acid, citrulline and ornithine were increased on the urea-containing diets. These compounds are related to urea synthesis in animal tissue and the increase may be an indication of increased urea synthesis under these conditions. It may be possible, since N digestibility was increased for the urea-containing diet with time on feeding, that the increased levels of these compounds was a mechanism for the animal to increase urea utilization and adaptation. The results of Clifford and Tillman (1968) may support this hypothesis since they found that plasma glutamic and aspartic acid concentrations were greater in lambs fed diets containing urea rather than soybean protein as the sole source of N.

Amino Acid Contents of Rumen Microorganisms

All the amino acid contents of the rumen microorganisms with the exception of arginine and threonine were increased on the 50% urea-N diet. However, the differences among treatments were not significant, 0% urea-N diet always yielded higher levels of amino acids in the rumen microorganisms than the 100% urea-N diet. Slyter *et al.* (1971) fed steers either urea or biuret with or without starch and concluded that the amino acid content of ruminal bacteria hydrolysates revealed no significant change by the omission or addition of starch. However, they reported that for steers fed biuret, rumen bacteria hydrolysates contained more alanine and blood plasma contained more glutamic acid. Feeding starch could have increased the availability of these amino acids to the steers by increasing the total output of ruminal microbes or by improving the biological value of the microbes synthesized in the rumen. Neither were determined in the present study.

The amino acid composition of rumen bacteria and protozoa has been studied by Weller, 1957; Bergen *et al.*, 1967; and Meyer *et al.*, 1967. Purser and Buechler (1966) analyzed 22 strains of rumen bacteria growing in pure culture. The organisms used were selected to represent some of the predominant ones found in the rumen when either concentrates or roughages were fed. Similarities in amino acid composition of mixed populations of rumen bacteria or protozoa were remarkable when one considers the environmental

experimental, feed, and species variables involved. The amino acid content of rumen microorganisms in the present work indicated similarities between the soybean meal diet (0% urea) and diet containing 50% urea-N. There was, however, a large reduction of almost all the amino acids for the diet which contained 100% urea-N. Some of this variation, however, may be associated with sampling techniques. This was supported by the data of Bergen *et al.* (1967) since they found considerable variations among individual strains of rumen microorganisms when studying protein quality of individual rumen bacteria using an *in vitro* enzymatic digestion system. Furthermore, the proportion of essential amino acids released during the digestion of different bacterial strain also varied markedly, which suggests that modification of the bacterial population may be an important factor in the N status of an animal and the response to dietary changes.

Analyses of essential amino acids by Weller (1957) showed that rumen protozoa contained greater amounts of isoleucine, leucine, phenylalanine, and lysine than bacteria. Meyer *et al.* (1967) also found differences between amino acid compositions of protozoa and of bacteria. In the present experiment the 100% urea-N diet had the lowest level of isoleucine, leucine, phenylalanine and lysine in the rumen microorganisms. In this work the proportion of these amino acids due to the rumen bacteria or due to the rumen protozoa

were not determined. The reduction of amino acids on the 100% urea-N diet was probably associated with a lowered protozoal population with this diet.

The rumen microbial population was determined in steers fed high fiber purified diets with either urea, biuret or isolated soy protein as the dietary nitrogen source (Slyter *et al.*, 1971). The same major groups of rumenal bacteria and protozoa were present in the steers, regardless of the dietary N source. That could have occurred in the present experiment since there were no significant differences in the amino acid contents of rumen microorganisms for the soybean diet and the 50% urea-N diet. This also suggested that the level in which urea was fed could influence the results. Oltjen *et al.* (1971) reported that the molar percentage of amino acids in bacterial hydrolyzates obtained from steers fed either urea or soybean meal diets as the sole source of N did not differ with diet and was very similar to the patterns found in a steer fed a natural diet. Purser and Buechler (1966) reported that the amino acid composition of the bacterial protein presented to the host for digestion was relatively constant, irrespective of the composition of the diet.

Loosli *et al.* (1949) fed a purified diet containing urea as the N source to sheep and goats. Rumen contents of the animals contained 9 to 20 times more amino acids than the diet fed and all 10 essential amino acids were synthesized

in large amounts. Similar results were obtained by Virtanen and Lampila (1962). Duncan *et al.* (1953) also presented quantitative evidence that rumen microorganisms can utilize urea-N to synthesize amino acids. They found that except for histidine the amino acid pattern of the protein in the rumen contents of animals fed a urea-containing diet was fundamentally similar to that of animals fed a natural diet. In our results no significant differences in the amino acid content of rumen microorganisms from the diets fed were observed.

Liver Amino Acids Pattern

Liver free amino acids concentrations in this study were higher than those obtained with rats (Alam *et al.*, 1966; Alam *et al.*, 1966; Hoshino, 1962, Solomon *et al.*, 1951; Schurr *et al.*, 1950; and Wannemacher and Allison, 1968).

All the essential amino acids except histidine and phenylalanine decreased on the urea containing diets. The diet which contained 100% urea-N depressed essential amino acids more than the diet which contained 50% urea-N. In studies with rats Wannemacher and Allison (1968) found that the essential amino acids level rapidly decreased to 50% in protein depleted rats as compared to the nondepleted control level at which time the rats had lost 10% of their body N. After this initial decrease, the values remained relatively constant even after the rats had lost 50% of their body N. This period of rapid loss correlated with an

increased rate of protein synthesis; when the anabolic rate decreased and the free amino acid level remained constant. Thus, the increased rate of amino acid incorporation into protein was not stimulated by an elevated FAA (free amino acid) level but was affected by some other regulatory mechanism. However, this elevated rate of protein biosynthesis was reflected by a marked diminution of the cellular essential FAA. An apparent adaptive mechanism altered the protein metabolism so that protein biosynthesis would correspond to the decreased FAA pool present in the liver of rats which had lost 10% of their body N. This change in protein metabolism would result in a conservation of body N. The previously described mechanism could apply to the results of this experiment. On urea diets there would be insufficient amount of the essential amino acid available for protein synthesis, which would result in a reduced level of free amino acids in the liver.

Nonessential amino acids in the liver increased on the urea containing diets. The increased level of the amino acids which are related to urea synthesis (glutamic acid, aspartic acid, citrulline, and ornithine) may provide an adaptive mechanism for N recycling in the animal. It could also indicate that the liver cells adapted for the amination of the organic acids using the excess ammonia present for the synthesis of the nonessential amino acids. However, Wannemacher and Allison (1968) in studies on protein depleted

rats found an initial rise of the nonessential FAA in liver, the nonessential FAA values slowly decreased until in the severely protein depleted rats the values were 50% of the nondepleted control. This initial elevation in non-essential FAA supports the concept that there was a flow of amino acids toward liver and vice versa, and that the concentration of nonessential FAA was in excess of that needed for rapid synthesis of protein demanded as much of the essential amino acids as of nonessential amino acids. Since the essential amino acids were in short supply for incorporation with the nonessential amino acids for the synthesis of protein, there would be no excess of the latter appearing in the free form. Thus, all the adaptation response of the animal tissue to the urea diets was to aminate the organic acids using the excess ammonia produced on the urea diets for the synthesis of the nonessential amino acids. By assuming that there was an amino acid imbalance in the urea-fed animals, the protein synthesis process would not proceed at the normal rate and this may be one of the reasons for the lowered N retentions of those animals.

Rumen Epithelium Amino Acid Patterns

The concentrations of all essential amino acids in the rumen epithelium with the exception of threonine and phenylalanine decreased for the urea containing diets when compared to the soybean meal (0% urea). Aspartic acid,

citrulline and ornithine were increased for the urea containing diets. The increase of these amino N compounds would suggest that the amination of the organic acids in the rumen epithelium of the urea fed animals was greater than in animals fed no urea (soybean meal). This had also been suggested by Hoshino *et al.* (1966) and McLaren *et al.* (1961).

As previously discussed regarding the liver, protein synthesis required a balanced concentration of all amino acids (essential and nonessential) in any given tissue to proceed at a normal rate. Since there was a lack of the essential amino acids in the rumen epithelium, protein synthesis would not proceed at an adequate rate. The N balance data indicated that urea-fed animals had lower N retention than the soybean fed animals. Then the increased levels of the nonessential amino acids in the rumen epithelium along with the lower N retention obtained for the urea fed animals indicated that the adaptation response of these animals to the urea diets was in the utilization of the ammonia produced from urea in the amination of the organic acids to nonessential amino acids. These nonessential amino acids were directed for urea synthesis which would be partially excreted in the urine.

In general, the essential amino acids were depressed in the liver, rumen epithelium, and plasma of the urea-fed animal. On the other hand, the nonessential amino acids were

increased in the same animals. It is suggested that when urea was fed an amino acid imbalance existed which contributed to a reduced protein synthesis and increased urea synthesis. This could explain the lowered N balance obtained for the urea-fed animals when compared to the soybean-fed animals. This situation may be corrected by the addition of certain amino acids to the urea diets.

CHAPTER V

GENERAL DISCUSSION

The results in this study indicate that N digestibility was improved by time on the experimental diets. The improvement was relatively higher for urea-supplemented diets than for the soybean diet. This was in agreement with the results of Hatfield *et al.*, 1955 and 1959; Repp *et al.*, 1955; Gaither *et al.*, 1955; McLaren *et al.*, 1959; Smith *et al.*, 1960; Anderson *et al.*, 1959; and Schaadt *et al.*, 1966.

N retention was improved somewhat with time but was not consistent and soybean diet was superior to urea in that respect. This was supported by the results of Johnson and McClure, 1964 and Oltjen and Putnam, 1966.

Dry matter digestibility was depressed by urea supplementation particularly when 100% of the total N in the diet was from urea. Dry matter digestibility has been reported to decrease on a urea diet (Hatfield *et al.*, 1955; 1959; Ewan *et al.*, 1958; and Oltjen *et al.*, 1968).

Ammonia-N concentration in the rumen was significantly ($P<0.01$) higher for the urea-supplemented diets than the soybean meal diet.

Blood ammonia and urea-N concentrations were also higher for the urea-supplemented diets than the soybean diet. The differences in the concentration of blood urea-N among diets were significant ($P<0.01$) but for blood ammonia the differences were not significant.

It was shown in this study that there was a high level of ammonia in the rumen and blood of the urea-fed animals. For more efficient utilization of the N source under these circumstances urea must be utilized for protein synthesis either by rumen microorganisms or by the animal tissue. Generally, there are three major enzyme systems responsible for the incorporation of ammonia into organic-N compounds. The three systems are glutamate dehydrogenase, glutamine synthetase, and carbamyl phosphate synthetase in animal tissue or carbamyl phosphokinase in bacteria. The enzymes utilize either one molecule of ammonia for the synthesis of glutamate or carbamyl phosphate or two molecules of ammonia for the synthesis of glutamine. These compounds can donate there amino-N thereafter for the synthesis of all other amino acids as well as purine and pyrimidine for protein synthesis. The activities of these enzymes were expected to increase on the urea supplemented diets for the utilization of the excess ammonia presented in the rumen. There was, however, no significant difference among diets in the activity of glutamate dehydrogenase from liver or rumen epithelium. The time during which urea was

fed also had no effect on the activity of this enzyme in the liver. Rumen microorganisms responded differently in that the 50% urea-N diet had higher glutamate dehydrogenase activity than the 100% urea-N and 0% urea diets. The higher activity of glutamate dehydrogenase of rumen microorganisms for the urea containing diet (50% urea-N diet) indicated that amination reactions were important pathways in the assimilation of ammonia by rumen microorganisms and glutamate dehydrogenase plays an important role in this process. The 100% urea-N diet had a lower activity of glutamate dehydrogenase than the soybean diet, which is supported by studies by Chalupa *et al.* (1970). They reported that 4.3% urea in the diet (92% of the diet N) decreased the activity of glutamate dehydrogenase of rumen microorganisms when compared to a soybean diet, however, Chalupa *et al.* (1970) found an increased activity of liver glutamate dehydrogenase for urea-fed animals. Also, Whanger and Church (1970) had observed that glutamate dehydrogenase activity for urea-fed steers was higher than steers fed cottonseed meal in both liver and rumen epithelium. Results obtained were not statistically significant. The results obtained for the activities of glutamate dehydrogenase of the liver and rumen epithelium may suggest that the adaptation response of these tissues to the urea-containing diet occurred within one to 10 days or after the 40 day period which was not measured.

Liver glutamine synthetase activities were slightly higher for urea-fed animals than animals fed the soybean diet (0% urea). In spite of the increased activities of liver glutamine synthetase there were no significant differences among the three diets. Chalupa *et al.* (1970) found that the activity of liver glutamine synthetase was slightly higher for the soybean-fed animals when compared with animals fed 4.3% urea diet. Similar results were found in this study when 100% urea-N diet was compared with the soybean diet, but the 50% urea-N diet had a higher glutamine synthetase activity than the soybean diet. Duda and Handler (1958) administered ^{15}N ammonia, glutamine, L and D-leucine to rats and found that glutamine synthesis was the major fate of ammonia in the liver. When the peak of activity was attained, the specific activity of glutamine was approximately 7 times that of either urea or glutamic acid. The same type of discussion could be applied for rumen epithelium activities of glutamine synthetase since the results showed the same trend as previously described in the liver. Cook *et al.* (1965) observed that there was a fourfold increase in concentrations of glutamine in the rumen vein blood 20 min after addition of amino acid mixtures to the rumen. They suggested that glutamine was rapidly synthesized in the rumen wall and absorbed into the blood stream via the rumen wall.

It has been found that rumen microbial glutamine

synthetase activity was significantly ($P<0.05$) higher for animals fed 50% urea-N diet than those fed 0% or 100% urea-N diets. This and other data obtained by Chalupa *et al.* (1970) in which they found increased activity of this enzyme with urea-fed sheep when compared to soybean-fed sheep indicated that this enzyme system is a very important pathway for ammonia metabolism in urea-fed animals.

Katunuma *et al.* (1966) found that ammonia inhibits the conversion of isocitrate to α-ketoglutarate which is a very important compound for the reaction of glutamate dehydrogenase. These workers have also shown that ammonia *in vitro* activates pyridine nucleotide oxidase which oxidizes NADH and NMNH to NAD and NMN, respectively. Matsuda and Katunuma (1969) have shown that ammonia also activates a pyridine nucleotide pyrophosphatase in rat liver mitochondria or microsomes which cleave NADH and NADPH to NMNH. Thus, if ammonia activates the pyrophosphatase pathway of NADPH breakdown, a decrease in the total $NADP^+$/NADPH would be expected and this was observed in the liver of urea-fed lambs by Prior *et al.* (1970).

The previous argument indicated that in urea-fed animals the activity of glutamate dehydrogenase could be decreased due to the lack of substrates required for the enzyme reaction, at the same time glutamine synthetase activity was increased as an adaptation response to the urea diet. That could have occurred only if there was a

mechanism for the synthesis of glutamine without passing the glutamate step during its synthesis which could be suggested under these circumstances. This is supported by the results of Rerard and Wiame (1964) and Kalman *et al.* (1966) who have reported a glutamine-dependent carbamyl phosphokinase in *Escherichia coli*. This could be an important pathway for glutamine synthesis in rumen microorganisms without passing the glutamate synthesis step as was suggested above.

Liver carbamyl phosphate synthetase was significantly ($P<0.1$) higher than that of rumen epithelium or rumen microorganisms, but there was no significant differences among diets. The only clear differences observed for the activity of carbamyl phosphokinase in this study were for the rumen microorganisms. Urea-fed animals had a higher activity of this enzyme than the soybean fed animals. Chalupa *et al.* (1970) reported that urea-fed sheep had more carbamyl phosphokinase activity in the rumen microorganisms than soybean fed animals. However, they postulated that this enzyme system was of minor importance in the ruminal assimilation of ammonia by the mixed rumen microbial population. At any rate the increased activity of carbamyl phosphokinase of rumen microorganisms of urea-fed animals could indicate an increase in urea synthesis which was partially excreted in the urine and led to the lowered N retention obtained for these animals.

The essential amino acids were depressed in the liver, rumen epithelium, and plasma of the urea-fed animals. On the other hand, the nonessential amino acids were increased in the same animals. It is suggested that when urea was fed, an amino acid imbalance existed which contributed to a reduced protein synthesis and increased urea synthesis. This could explain the lowered N balance obtained for the urea-fed animals when compared to the soybean-fed animals. This was not expected since there was an increase in glutamine and glutamate synthesis in these animals and it was expected that glutamine and glutamate would donate amino-N for the synthesis of all other amino acids and subsequently for the synthesis of protein. It appeared that glutamine and glutamate donated amino N for the synthesis of the nonessential amino acids more than for the essential amino acids which led to an amino acids imbalance and increased urea synthesis, which was partially excreted in the urine and led to the lowered N balance obtained. This situation may be corrected by the addition of certain essential amino acids to the urea diets to enable the animals to utilize the excess of the nonessential amino acids present.

CHAPTER VI

SUMMARY

Three rumen fistulated beef bulls, averaging 240 kg, were used in a latin square design. The experimental periods consisted of a 15 day basal period in which all animals were fed ground alfalfa hay containing 2.42% N. During the last five days of the basal period a N balance trial was conducted. On the first, third and fifth day of the collection period blood samples were taken from the jugular vein and rumen samples were taken through the rumen fistula. On the fourth day of the collection period liver samples were taken by the biopsy technique and on the fifth day of the collection period rumen fluid and rumen epithelium samples were taken.

All animals were fed on a continuous feeder which provided 7 kg of feed in 24 hr and water was provided free choice.

Following the basal period the animals were fed the experimental diets which contained 0%, 50% or 100% of the N as urea over a 40-day period. All diets were formulated to be isonitrogenous. The 40-day experimental period was divided into four consecutive 10-day periods. During the last 5 days of each 10-day period a N balance trial was

conducted and, in addition, blood, rumen, liver and rumen epithelium samples were obtained in the same manner previously described for the basal period. After the first 40-day experimental period the animals were fed the basal ground alfalfa diet for 15 days as previously described and then switched to the experimental diets for the second period of the experiment. This procedure was followed a third time to complete the latin square.

Blood ammonia-N, blood urea-N and rumen ammonia-N and plasma, liver, rumen epithelium and rumen microorganisms amino acids were determined. The activities of glutamate dehydrogenase, glutamine synthetase and carbamyl phosphate synthetase were determined in liver, rumen epithelium and rumen microorganisms.

Nitrogen digestibility was improved by time on the experimental diets. The improvement was relatively higher for urea-supplemented diets than the soybean diet. For N retention there was some improvement with time, but it was not consistent and the soybean diet was superior to the urea diet. The 100% urea-N diet resulted in the lowest N retention. Dry matter digestibility was depressed by urea supplementation, particularly when 100% of the total N in the diet came from urea.

Ammonia-N concentration in the rumen was significantly ($P<0.01$) higher for the urea-supplemented diets than the soybean meal diet. Blood urea-N concentration was significantly

(P<0.01) higher for the urea-supplemented diets than the soybean meal diet. There was no significant differences among diets in the concentrations of blood ammonia-N.

There was no significant differences among diets in the activity of liver or rumen epithelium glutamate dehydrogenase. The 0% urea diet (soybean) had higher activities than the urea-containing diets. Glutamate dehydrogenase activity followed the same trend.

The rumen microorganisms on the 50% urea-N diet had a higher glutamate dehydrogenase activity than the 100% urea-N and 0% urea diets.

Liver and rumen epithelium glutamine synthetase activity was slightly higher for urea-fed animals than animals fed the soybean meal diet. Rumen microbial glutamine synthetase activity was significantly higher (P<0.05) for animals fed 50% urea-N diet than those fed either 0% or 100% urea-N diets.

Liver carbamyl phosphate synthetase was significantly (P<0.01) higher than that of rumen epithelium or rumen microorganisms, but there was no significant differences among diets. Rumen epithelium carbamyl phosphate synthetase activity followed the same pattern as that of the liver, however, the activity was very low.

All the essential amino acids were depressed in the liver, rumen epitheliun, and plasma of the urea-fed animals. Glutamic acid, citrulline, ornithine, glycine

were increased on the urea diets. There was no differences in the concentrations of all the amino acids of rumen microorganisms among the three diets.

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APPENDIX

Table 1A. Blood urea-N at 1st, 3rd, and 5th day of each collection period.

% N_2 from Urea	Days on Diets	1st Collection Period	3rd Collection Period	5th Collection Period	Average
		(mg/100 ml plasma		)	
0	0	22.233	22.233	22.233	22.233
	10	15.167	16.667	16.667	16.167
	20	15.333	19.667	15.067	16.633
	30	20.433	17.167	17.500	18.367
	40	22.833	19.733	17.567	20.067
50	0	22.833	22.833	22.833	22.833
	10	21.167	21.500	23.500	22.067
	20	22.833	26.067	25.833	24.900
	30	23.733	25.000	26.333	25.067
	40	26.833	31.567	31.333	29.867
100	0	21.667	22.000	23.000	23.000
	10	34.333	36.400	27.733	32.833
	20	34.067	38.167	30.400	34.233
	30	40.667	36.667	37.167	38.167
	40	39.667	42.500	37.500	39.867
	[1]S.E. of means	±2.9214	±2.4086	±2.7121	±1.9719

[1] Each value is the mean of three animals.

Table 2A. Rumen ammonia nitrogen at 1st, 3rd, and 5th day of each collection period.

% N_2 from Urea	Days on Diets	1st Collection Period	3rd Collection Period	5th Collection Period	Average
		(mg/100 ml rumen fluid		)	
0	0	2.403	2.403	2.403	2.403
	10	2.887	5.530	1.203	3.200
	20	4.570	3.850	2.887	3.810
	30	8.413	5.290	1.923	4.820
	40	6.490	4.090	1.683	4.087
50	0	3.847	3.847	3.847	3.847
	10	8.653	6.250	11.063	7.990
	20	14.187	22.360	5.770	14.100
	30	9.617	11.300	7.453	9.447
	40	7.213	8.893	11.297	9.123
100	0	2.887	2.887	2.887	2.887
	10	22.360	22.600	24.043	23.000
	20	12.743	22.643	23.323	23.303
	30	27.407	18.513	27.893	24.603
	40	19.477	34.380	17.793	23.900
	[1]S.E. of means	±2.5326	±4.9454	±4.5141	±2.9917

[1]Each value is the mean of three animals.

Table 3A. μ Unit of enzyme/min/mg soluble protein of the liver.

% N_2 from Urea	Days on Diet	Glutamate Dehydrogenase	Glutamine Synthetase	Carbamyl Phosphate Synthetase
0	0	53.0	50.0	262.0
	10	51.0	81.0	310.0
	20	49.0	58.0	368.0
	30	32.0	43.0	17.0
	40	31.0	40.0	726.0
50	0	20.0	33.0	198.0
	10	27.0	65.0	230.0
	20	41.0	50.0	338.0
	30	52.0	98.0	601.0
	40	38.0	43.0	647.0
100	0	35.0	30.0	170.0
	10	39.0	113.0	260.0
	20	34.0	68.0	400.0
	30	35.0	57.0	469.0
	40	32.0	34.0	569.0
	[1]S.E. of means	±10.0	±22.3	±101.4

[1] Each value is the mean of three animals.

Table 4A. μ Unit of enzymes/min/mg soluble protein of rumen epithelium.

% N_2 from Urea	Days on Diet	Glutamate Dehydrogenase	Glutamine Synthetase	Carbamyl Phosphate Synthetase
0	0	6.0	50.0	12.0
	10	7.0	68.0	3.0
	20	20.0	183.0	18.0
	30	18.0	127.0	19.0
	40	10.0	72.0	16.0
50	0	5.0	45.0	12.0
	10	9.0	64.0	12.0
	20	12.0	266.0	9.0
	30	22.0	106.0	18.0
	40	7.0	85.0	17.0
100	0	3.0	33.0	9.0
	10	10.0	60.0	5.0
	20	14.0	170.0	11.0
	30	21.0	97.0	33.0
	40	13.0	71.0	17.0
	[1]S.E. of means	±0.00	±30.0	0.00

[1]Each value is the mean of three animals.

Table 5A. μ Unit of enzyme/min/mg soluble protein of rumen micro-organisms.

% N_2 from Urea	Days on Diet	Glutamate Dehydrogenase	Glutamine Synthetase	Carbamyl Phosphate Synthetase
0	0	38.0	116.0	72.0
	10	58.0	420.0	91.0
	20	70.0	663.0	57.0
	30	111.0	204.0	324.0
	40	111.0	189.0	67.0
50	0	26.0	110.0	54.0
	10	27.0	280.0	75.0
	20	57.0	616.0	60.0
	30	126.0	300.0	306.0
	40	61.0	156.0	98.0
100	0	24.0	106.0	79.0
	10	24.0	333.0	102.0
	20	28.0	805.0	95.0
	30	70.0	165.0	255.0
	40	96.0	181.0	181.0
	S.E. of means	±31.6	±224.7	±64.0

[1]Each value is the mean of three animals.

Table 6A. Amino acids and amino nitrogenous compounds of rumen microorganisms (mg/100 ml of rumen fluid).

% N from Urea	Days on Diet	Lysine	Histidine	Arginine	Aspartic Acid	Threonine	Glutamic Acid	Glysine	Alanine
0	0	0.173	0.012	0.034	0.125	0.054	0.217	0.051	0.152
	10	0.444	0.038	0.130	0.156	0.177	0.788	0.167	0.553
	20	0.114	0.017	0.079	0.072	0.073	0.229	0.058	0.214
	30	0.298	0.035	0.139	0.098	0.233	0.467	0.187	0.272
	40	0.082	0.010	0.023	0.062	0.037	0.159	0.039	0.118
50	0	0.206	0.017	0.040	0.078	0.078	0.256	0.063	0.166
	10	0.088	0.271	0.042	0.302	0.123	0.737	0.196	0.592
	20	0.350	0.013	0.088	0.154	0.108	0.528	0.152	0.536
	30	0.385	0.006	0.138	0.202	0.157	0.496	0.142	0.489
	40	0.099	0.012	0.037	0.076	0.047	0.219	0.069	0.417
100	0	0.167	0.024	0.044	0.092	0.066	0.176	0.065	0.112
	10	0.137	0.014	0.040	0.118	0.069	0.176	0.057	0.199
	20	0.282	0.033	0.117	0.170	0.146	0.550	0.112	0.399
	30	0.070	0.029	0.013	0.076	0.036	0.153	0.042	0.097
	40	0.032	0.008	0.012	0.028	0.015	0.058	0.017	0.062
	S.E. of means	±0.134	±0.067	±0.048	±0.063	±0.066	±0.110	±0.053	±0.156

Each value is the mean of three animals.

Cystine	Valine	Methionine	Iso-Leucine	Leucine	Thyrosine	Phenyl-alanine	Citrulline	Ornithine
0.000	0.063	0.022	0.050	0.063	0.032	0.028	0.017	0.128
0.014	0.0281	0.104	0.172	0.252	0.124	0.125	0.064	0.062
0.008	0.045	0.048	0.027	0.049	0.028	0.030	0.027	0.048
0.039	0.101	0.064	0.081	0.143	0.100	0.058	0.043	0.034
0.005	0.042	0.016	0.021	0.037	0.006	0.006	0.015	0.020
0.001	0.070	0.027	0.056	0.070	0.027	0.028	0.032	0.145
0.036	0.206	0.111	0.139	0.210	0.108	0.113	0.198	0.181
0.013	0.174	0.073	0.121	0.149	0.043	0.066	0.050	0.214
0.010	0.195	0.078	0.137	0.196	0.084	0.088	0.023	0.067
0.055	0.077	0.049	0.044	0.080	0.039	0.044	0.132	0.027
0.002	0.044	0.021	0.042	0.063	0.032	0.030	0.037	0.240
0.017	0.080	0.038	0.057	0.081	0.028	0.015	0.017	0.139
0.062	0.081	0.071	0.085	0.134	0.052	0.061	0.059	0.030
0.059	0.021	0.047	0.014	0.016	0.002	0.002	0.007	0.010
0.003	0.015	0.005	0.005	0.006	0.002	0.002	0.002	0.016
±0.024	±0.078	±0.036	±0.052	±0.076	±0.045	±0.042	±0.054	±0.076

Table 7A. Amino acids and amino nitrogenous compounds of plasma (mg/100 ml plasma).

% N from Urea	Days on Diet	Lysine	Histidine	Arginine	Aspartic Acid	Threonine	Glutamic Acid	Proline	Glycine	Alanine
0	+0	1.124	0.959	1.616	0.312	1.021	1.842	0.420	1.596	1.880
	10	1.644	1.442	1.748	0.283	0.868	3.054	0.023	2.308	1.880
	20	1.681	1.043	1.938	0.201	0.956	2.531	0.534	1.986	2.425
	30	1.438	0.923	1.611	0.167	0.915	2.457	0.400	1.962	1.953
	40	1.804	1.145	1.553	0.228	0.913	2.110	0.750	1.710	1.939
50	0	1.247	0.814	1.806	0.282	1.036	1.866	0.379	1.753	2.163
	10	1.095	0.873	1.217	0.198	0.799	2.366	0.078	1.932	1.460
	20	1.472	0.943	1.790	0.177	0.906	2.785	0.449	1.963	2.149
	30	1.656	0.875	1.866	0.254	0.961	2.770	0.485	2.147	2.135
	40	1.296	1.013	1.533	0.233	0.762	2.018	0.518	1.712	1.968
100	0	1.382	1.076	1.342	0.638	0.958	1.956	0.512	2.040	2.160
	10	1.257	1.208	1.858	0.172	0.742	2.914	0.135	1.982	1.752
	20	1.406	0.969	1.935	0.320	0.956	2.700	0.795	2.647	1.924
	30	1.299	0.997	2.085	0.223	1.008	2.744	0.446	1.967	1.904
	40	1.082	0.827	1.707	0.301	0.770	2.609	0.714	1.967	1.590
	S.E. of means	±0.173	±0.185	±0.170	±0.096	±0.081	±0.306	±0.238	±0.241	±0.286

Cystine	Valine	Methion-ine	Leucine	Iso-leucine	Tyrosine	Phenyl-alanine	Tourine	Citrul-line	Orni-thine	Trypt-ophane
0.087	2.658	0.323	1.595	1.323	1.058	0.869	0.224	0.903	0.958	0.554
0.309	3.380	0.343	1.746	1.827	0.912	0.739	0.213	0.540	0.858	0.628
0.339	3.964	0.339	1.895	2.004	1.128	0.777	0.445	1.037	0.989	0.827
0.266	3.544	0.268	1.800	1.688	1.016	0.741	0.395	0.852	0.887	0.628
0.225	3.715	0.231	1.920	1.914	1.022	0.771	0.386	0.838	1.026	1.141
0.130	3.062	0.309	1.517	1.581	1.271	0.947	0.176	0.868	0.923	0.360
0.177	2.168	0.238	1.121	1.161	0.548	0.418	0.210	0.688	0.645	0.511
0.332	2.940	0.337	1.387	1.491	0.798	0.695	0.309	0.939	0.878	0.637
0.202	3.254	0.345	1.491	1.636	1.007	0.741	0.319	0.992	0.971	0.853
0.279	3.535	0.776	1.286	1.317	0.765	0.564	0.265	0.821	0.803	0.321
0.052	2.940	0.288	2.177	1.664	1.341	1.121	0.249	1.019	1.929	0.324
0.175	2.123	0.296	0.979	1.089	0.642	0.494	0.152	0.997	0.851	0.610
0.150	2.896	0.268	1.170	1.296	0.711	0.513	0.225	1.397	1.041	0.798
0.125	2.640	0.338	1.237	1.315	0.681	0.560	0.215	1.182	1.020	0.740
0.104	1.901	0.250	0.831	1.000	0.625	0.338	0.194	0.713	0.832	0.321
±0.079	±0.238	±0.132	±0.203	±0.141	±0.130	±0.101	±0.063	±0.182	±0.244	±0.206

Table 8A. Amino acids and amino nitrogenous compounds of liver (mg/100 g of fresh liver).

% N from Urea	Days on Diet	Lysine	Histidine	Aspartic Acid	Threonine	Cystine	Valine	Methion-ine	Iso-leucine	Leucine	Tyrosine
0	0	3.675	5.125	10.841	5.056	4.985	3.318	0.756	1.597	3.375	2.585
	10	5.156	8.438	18.671	4.212	3.863	4.659	0.618	2.069	4.431	3.558
	20	5.589	5.145	10.046	3.408	1.293	4.385	0.737	2.077	4.398	2.639
	30	4.753	6.686	13.127	4.332	1.645	4.341	0.893	2.193	4.883	2.393
	40	6.244	6.894	13.607	3.265	5.900	5.802	0.694	2.817	5.1 2	3.507
50	0	3.946	6.072	7.881	3.533	2.167	4.278	0.522	1.840	3.985	2.493
	10	5.856	9.502	22.816	4.102	3.896	4.201	0.823	2.079	5.197	2.788
	20	4.591	5.526	13.359	3.272	3.622	5.000	0.711	1.604	3.859	2.329
	30	4.970	6.263	16.838	3.738	1.667	4.281	0.835	2.020	3.962	2.079
	40	5.402	6.521	9.170	3.817	2.153	4.373	0.792	1.939	4.808	4.678
100	0	4.817	4.435	11.112	3.375	7.601	3.794	0.659	1.294	4.020	1.020
	10	3.886	5.800	14.501	3.752	4.262	3.748	0.527	1.600	4.263	4.263
	20	5.580	6.819	16.741	3.636	1.939	3.197	0.672	1.544	3.664	3.664
	30	4.327	5.013	10.951	3.692	1.809	3.124	0.452	1.323	2.847	2.235
	40	3.995	5.519	8.498	1.901	4.672	4.061	0.688	1.572	3.597	2.636
	S.E. of means	1.325	1.422	4.388	1.115	2.233	0.962	0.250	0.464	0.941	1.187

Phenyl-alanine	Glutamic Acid	Glysine	Alanine	Taurine	Citrul-line	Orni-thine	Trypto-phane	O-phospho serine	O-phospho ethanol amine	O-phosph-thionine
0.732	85.998	23.380	4.742	10.267	3.353	7.42	1.140	0.000	0.000	0.000
2.173	105.627	34.352	18.376	3.558	0.304	9.988	8.501	2.938	5.753	0.537
2.263	79.931	19.171	19.335	2.639	2.421	8.806	2.764	1.495	11.355	0.701
2.444	98.105	32.701	21.513	36.136	1.766	10.365	3.335	2.171	6.587	0.424
2.892	92.614	30.910	19.783	45.436	2.335	11.974	2.817	1.814	3.752	0.517
2.461	77.264	24.599	17.841	9.087	0.824	8.606	0.753	0.000	0.000	0.000
2.023	104.613	32.346	16.977	20.536	4.317	11.987	2.282	1.932	11.605	0.434
3.312	76.079	25.133	15.083	22.308	4.079	8.726	2.104	1.719	11.414	0.000
1.592	98.719	27.948	24.153	25.177	6.007	10.755	3.368	2.918	13.627	0.388
3.912	72.830	30.988	22.983	27.517	10.032	0.000	1.769	1.012	8.419	0.260
0.943	68.334	22.805	10.641	8.007	7.736	8.328	0.763	0.000	0.000	0.000
1.519	105.193	28.321	14.926	10.970	1.077	12.486	1.632	1.201	9.570	0.170
2.204	85.891	22.906	13.529	21.635	2.356	10.862	1.775	1.186	8.528	0.305
1.727	109.631	27.792	21.525	16.319	3.584	10.717	1.934	3.247	11.609	0.466
3.051	137.444	34.226	20.720	12.958	1.849	11.190	3.191	1.190	8.584	0.000
±1.196	±15.223	±5.803	±3.552	±8.844	±2.018	±2.837	±1.523	±0.922	±3.964	±0.217

Table 9A. Amino acids and amino nitrogenous compounds of rumen epithelium (mg/100 g fresh rumen epithelium).

% N from Urea	Days on Diet	Lysine	Histidine	Aspartic Acid	Threonine	Glutamic Acid	Glycine	Alanine	Cystine	Valine
0	0	1.640	0.379	4.441	1.526	15.894	4.723	2.674	0.454	0.861
	10	2.229	0.378	3.609	0.919	18.693	6.341	3.273	0.701	0.841
	20	2.025	0.397	4.152	1.464	15.999	6.184	4.943	0.676	1.264
	30	1.636	0.365	5.531	1.395	18.790	6.856	4.007	1.302	1.152
	40	1.367	0.770	4.509	1.142	16.502	6.338	3.843	0.795	0.711
50	0	1.397	0.321	3.459	1.327	19.596	3.910	2.432	0.000	0.624
	10	1.412	0.374	2.702	1.351	14.940	6.818	2.778	0.824	0.682
	20	1.373	0.303	5.870	1.308	16.489	5.665	3.892	2.720	0.863
	30	0.978	0.222	4.427	1.040	12.718	4.695	3.237	0.526	0.651
	40	1.177	0.303	3.494	1.287	17.596	5.729	4.373	0.385	0.725
100	0	1.516	0.275	3.500	1.291	9.910	3.806	2.199	0.454	0.746
	10	1.287	0.368	4.014	2.002	12.842	4.877	2.154	0.002	0.768
	20	1.827	0.452	4.044	1.441	13.027	4.595	3.607	0.677	1.147
	30	1.138	0.301	3.829	1.261	12.622	4.575	2.957	1.180	0.495
	40	1.479	0.359	7.688	2.698	18.650	6.751	4.941	0.341	0.451
	S.E. of means	0.324	0.165	1.263	0.457	4.326	1.249	0.873	0.659	0.268

Methion-ine	Iso-leucine	Leucine	Tyrosine	Phenyl-alanine	Taurine	Citrul-line	Orni-thine	O-phospho-serine	O-phospho-ethanolamine
0.239	0.460	1.137	0.263	0.157	1.073	0.028	1.552	0.000	0.000
0.197	0.359	0.898	0.080	0.080	1.813	0.502	1.576	0.088	0.263
0.207	0.589	1.202	0.003	0.003	2.827	0.007	2.075	0.233	0.632
0.216	0.549	1.010	0.003	0.003	2.800	0.217	1.625	0.159	0.473
0.327	0.529	0.754	0.002	0.002	2.604	0.002	1.621	0.083	0.668
0.111	0.289	0.690	0.005	0.005	0.983	0.000	1.532	0.000	0.000
0.081	0.135	0.549	0.003	0.003	3.672	0.359	1.514	0.156	0.679
0.111	0.309	0.598	0.000	0.000	2.579	0.430	3.098	0.097	0.113
0.005	0.186	0.412	0.200	0.070	1.558	0.002	1.200	0.234	0.152
0.080	0.223	0.582	0.000	0.000	2.230	0.000	0.000	0.000	0.000
0.145	0.305	0.647	0.533	0.389	0.872	0.112	1.385	0.000	0.000
0.003	0.098	0.580	0.002	0.002	2.045	0.002	1.644	0.000	1.075
0.137	0.382	1.435	0.003	0.003	1.797	0.056	1.660	0.080	0.183
0.115	0.211	0.515	0.003	0.003	1.388	0.394	1.575	0.252	0.436
0.082	0.142	0.496	0.002	0.002	1.795	0.049	3.299	0.000	0.000
±0.128	±0.184	±0.324	±0.112	±0.072	±0.589	±0.179	±0.202	±0.109	±0.352