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PROTEIN AND NONPROTEIN NITROGEN
UTILIZATION IN THE HORSE.**

**The Ohio State University, Ph.D., 1969
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PROTEIN AND NONPROTEIN NITROGEN
UTILIZATION IN THE HORSE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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* * * * *

The Ohio State University
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INTRODUCTION

The horse has been almost completely neglected for many years in terms of nutritional research. The reasons for this situation are many. With the advent of mechanization in agriculture, the numbers of draft horses and mules declined rapidly. Even with the increased popularity of pleasure horses during the past few years, the economic impetus to do research with the horse has been lacking. As a result, most universities have not been active in this area. This has resulted in a general lack of specialized horse nutritionists in the United States. Another reason for the lack of research done with the horse is the difficulty involved in using them as experimental animals. Problems cited in the literature include such things as the horse's high susceptibility to fatal peritonitis in establishing gastrointestinal fistulas and the difficulty of installing blood vessel catheters (Linerode, 1966).

Most of the more recent research with horses has been in the areas of treatment and prevention of disease and unsoundness. Little effort has been directed toward reproductive physiology or nutrition. Along with the present increase in horse numbers there has been a significant increase in their value per head. Noting the strides made by nutritional research with other farm animals, today's horsemen have become increasingly concerned over the lack of sound research upon which most current nutritional recommendations for the horse are based.

In the past few years, several horse breed associations have made available limited funds for horse research. There has also been an

increased interest on the part of some of the land grant universities to do research in this area. An increase in the number of horse racing days in some states has resulted in additional tax revenues being available for horse research.

Much of the information upon which the current nutrient requirements of the light horse are based have been extrapolated from research done with other animals, both monogastric and ruminant. Ensminger (1966) concluded that the nutritive requirements of the horse more nearly parallel those of the pig than the cow. The data presented in the 1966 National Research Council Bulletin (Publication No. 1401) are derived, in many cases, from experimental results obtained with cattle. The authors of this bulletin observed that this was an unfortunate necessity, since it modified the strength of their conclusions. Due to this situation, they suggested the data presented were intended only as a guide. These extrapolations and comparisons have no doubt led to some errors and inconsistencies and lend validity to the old adage that "feeding horses is more an art than a science".

One must continually remember that the horse is unique among farm animals in more ways than just the anatomy of his digestive tract. The horse is fed for rapid growth, development, soundness, athletic ability, stamina, and longevity, not for the production of meat and/or fiber. He is the athlete among farm animals and should be fed accordingly. It is not at all unusual for a rapidly growing yearling or two year old to be put into training. An adequate nutritional level depends, not only on the nutrient requirements for maintenance and growth, but also an additional allowance for the amount of work being performed.

Protein is an essential nutrient for both the growing and mature animal, regardless of specie. The quality and digestibility of a protein source directly affects its nutritional worth. Before sound recommendations can be made concerning the protein nutrition of the horse more research is needed concerning this animal's digestive physiology. In addition, the importance of protein quality and the role of the cecum and its microorganisms in nitrogen utilization must be evaluated.

The research presented in this dissertation is an attempt to help clarify some of these points. The major objectives of this research were to study in mature ponies:

- 1.) The effect of protein quality and solubility on ration digestibility and nitrogen balance.
- 2.) The effects of feeding urea as the major dietary nitrogen source.
- 3.) The effect of nitrogen source on the blood plasma and cecal levels of urea and ammonia.

LITERATURE REVIEW

Comparative Anatomy and Digestive Physiology

Although the horse is considered to be a monogastric, the anatomy of his digestive tract falls somewhere in between that of a simple monogastric such as the pig, and the more complex ruminant animal. Dukes (1955) compared the relative and average absolute capacities of the different parts of the digestive tracts in the horse, the cow, and the pig (Table 1). The typical rations fed to horses and ruminants contain large amounts of cellulose, whereas those fed to pigs do not. No mammals have been found that secrete the enzyme, cellulase, which is necessary for the hydrolysis of cellulose (Cole, 1966). The ability to utilize cellulose arises from the extensive microbial populations present in the digestive tracts of horses and ruminants. The main site of microbial fermentation in the ruminant is the reticulo-rumen, while in the horse, it is in the cecum and large colon. In addition to cellulose utilization, the microorganisms also have the ability to synthesize protein and B-complex vitamins (Maynard and Loosli, 1962).

Extensive microbial populations exist in the cecum, colon, and rectum of monogastrics (Dukes, 1955; Mitchell, 1964; Olsson and Ruudvere, 1955; Lloyd et al., 1958). In the large intestine (cecum and colon) of nonruminant herbivora, bacterial fermentation is important for the digestion of fibrous feeds. No other segment of the gastrointestinal tract provides the necessary volume and relatively immobile conditions for prolonged bacterial fermentation and synthesis. Proteolytic

TABLE 1
CAPACITY OF DIFFERENT PARTS OF THE DIGESTIVE TRACT

Segment of GI Tract	Horse		Cow		Pig	
	Relative Capacity (per cent)	Average Absolute Capacity (liters)	Relative Capacity (per cent)	Average Absolute Capacity (liters)	Relative Capacity (per cent)	Average Absolute Capacity (liters)
Stomach	8.5	17.96	70.8	252.5	29.2	8.0
Small intestine	30.2	63.82	18.5	66.0	33.5	9.2
Cecum	15.9	33.54	2.8	9.9	5.6	1.6
Large colon	38.4	81.25	7.9	28.0	31.7	8.7
Small colon & rectum	7.0	14.77	----	----	----	---
	<hr/> 100.0	<hr/> 211.34	<hr/> 100.0	<hr/> 356.4	<hr/> 100.0	<hr/> 27.5

(putrefactive) bacteria are present in large numbers and attack proteins and their degradation products which may have escaped digestion and absorption in the small intestine. The bacterial fermentation of cellulose and other carbohydrates in the large intestine yield the lower volatile fatty acids also found in the rumen. Dukes (1955) noted that significant amounts of these lower fatty acids have been found in the blood draining the cecum and colon of herbivores which is indicative of absorption. Bacterial synthesis of the B-complex vitamins also occur in the cecum and colon. Linerode (1966) found that ponies were able to absorb approximately 25 per cent of the thiamine produced in the cecum.

Blackburn (1965), in a discussion of nitrogen metabolism in the rumen, noted that the rumen microorganisms possess proteolytic activity, as well as, the ability to synthesize microbial protein. After a variable stay in the reticulo-rumen and omasum, the fermented ingesta, along with a multitude of microorganisms, pass on into the true stomach or abomasum and then into the small intestine. Here much of the intact dietary protein and microbial protein are digested and absorbed (Cole, 1966; Maynard and Loosli, 1965). The digestion of protein by the gastric and intestinal proteases liberate small polypeptides and dipeptides which are cleaved into their component amino acids and readily absorbed into the portal circulation (Harper, 1967; Ganong, 1967).

In the light of the above sequence of events in the ruminant, the observation has been made that the horse has an apparent disadvantage when it comes to the utilization of microbial protein. This has been attributed to the fact that microbial protein synthesis takes place after the ingesta has left the small intestine which is recognized as

the main site of proteolysis and amino acid absorption. This presumably limits the horse's ability to digest and absorb microbial protein. The validity of this observation must be investigated to establish the contribution of the cecal microorganisms to the overall nitrogen economy of the horse.

Amino Acid Balance and Protein Quality

The nutritional adequacy of the protein contained in a feedstuff is determined, to a large extent, by its amino acid composition and digestibility (Albanese, 1959). At least twenty-three chemically different amino acids have been identified in both plant and animal proteins (Naber, 1963; Dukes, 1955). These amino acids have been classified by Rose (Maynard and Loosli, 1965) into essential and non-essential amino acids. An essential amino acid being one that cannot be synthesized by the animal's body, out of the materials normally available, at a rate rapid enough to promote normal growth. An amino acid that is essential for one specie of animal may not be so for another.

A high quality protein is one that contains the proper complement of essential amino acids for the animal in question. It also infers that these amino acids are readily available to that animal (Brown, 1967). Many methods have been devised to express the nutritive value of proteins (Albanese, 1959; Crampton and Harris, 1969). Biological value measures the percentage of the absorbed nitrogen from a protein that is retained in the body. This is a useful value, but it does not consider a protein's digestibility. In other words, it is possible to have a protein with a high biological value and a low digestibility.

Such a protein would be of little value in meeting the protein needs of an animal. A much more useful expression of the nutritional worth of a protein is net protein utilization. This is arrived at by multiplying a protein's biological value times its digestibility and gives a more accurate estimation of the value to the animal.

Peraino et al. (1963) reported an experiment designed to study free amino acids in the blood plasma of rats force fed zein, casein or their hydrolysates. They showed that the rate of protein digestion was usually more limiting than absorption for the rate of entry of amino acids into the circulation. However, the concentrations in portal plasma did show that the amino acids in whole casein, which has a higher biological value than zein, were more available for absorption than those of zein. Munro and Allison (1964) observed that the efficiency of protein utilization depends on the pattern of amino acids presented to the tissues after absorption from the gut. They also noted that if only one of the essential amino acids is absent from an otherwise well-balanced ration, it will prevent tissue protein synthesis and result in the utilization of the other amino acids as an energy source. It is not necessary for an amino acid to be completely lacking from a protein to disrupt tissue protein synthesis. This can also be caused by an amino acid imbalance which Munro and Allison defined as a change in the proportion of amino acids in the diet which results in a reduction in feed intake. This amino acid imbalance has been shown, within twenty-four hours, to result in a loss of appetite and growth failure in young animals and severe negative nitrogen balance in mature animals which indicated a loss of

body proteins. Such an imbalance can be overcome by supplementing the first-limiting amino acid.

The reason an imbalanced protein causes a reduction in food intake has been a point of controversy among researchers for some time. Sanahuja et al. (1964) concluded that the rejection of an imbalanced diet was due to some physiological effect produced by the amino acid imbalance and not due to a lowered nutritive value per se. Mitchell (1968) demonstrated that the dietary amino acid pattern can alter the blood plasma amino acid pattern. Munro and Allison (1964) observed that the amino acid pattern of blood and body fluids is frequently more out of balance than the pattern of the diet producing the effect. Jacobson (1969) reviewed work which indicated an altered plasma amino acid pattern may be able to produce a feeling of satiety, in both monogastrics and ruminants, via a neural response affecting the satiety center located in the ventro-medial hypothalamus.

Alexander (1967) observed that protein for horses and beef cattle does not have to be of high quality, since the cecal and rumen microorganisms have the ability to utilize low quality protein. He observed that the microflora can use low quality protein in the synthesis of microbial protein of a higher biological value. Other workers have shown that the amino acid composition of rumen bacteria and protozoa varies little even under widely different dietary regimes (Purser and Buechler, 1966; Weller, 1957; Bergen et al., 1968). Ensminger (1966) concluded that, in comparison to the cow, the horse should be fed less roughage, higher quality protein (never nonprotein nitrogen products, such as urea) as well as added B-vitamins and vitamin K.

Amino Acid Absorption and Protein Solubility

Due to the lack of specific information at the present time, it is difficult to assess the extent of amino acid absorption from the cecum of the horse. In monogastrics, such as the pig, dietary proteins are subjected to the action of gastric and intestinal proteolytic enzymes which result in the release of peptides and free amino acids in the small intestine. As a result of this process, the major portion of protein digestion occurs in the small intestine of animals (Davenport, 1966). Mitchell (1964) noted that the small intestine is the most active site of absorption in both ruminants and non-ruminants. He further noted that absorption from the large intestine of the monogastric is similar to that of the small intestine, though much less important, since most of the material available to the animal has been absorbed from the small intestine.

As proteins enter the duodenum of the small intestine they are rapidly digested. It has been determined that a high rate of digestion does not limit the rate of absorption. Davenport (1966) states that the amino acids arising from dietary and endogenous proteins are absorbed by the small intestine as rapidly as they are liberated. Free amino acids cross the cells of the intestinal mucosa easily and enter the portal blood. L-amino acids are absorbed more rapidly than the corresponding D-isomers (Ganong, 1967). Suda et al. (1963) concluded that the L-amino acids are actively transported from the mucosal side of the intestine. The D-amino acids are apparently absorbed solely by passive diffusion (Ganong, 1967). Absorption of amino acids is rapid in the

duodenum and jejunum, but slow in the ileum. About 15 per cent of the ingested protein in man enters the colon and is eventually passed out in the feces.

The sequence of events in protein digestion in the ruminant is quite different from those in a monogastric, such as the pig. As noted previously, Blackburn (1965) reviewed research which demonstrated that the microorganisms were responsible for the proteolytic activity in the rumen. He noted that protein solubility has long been suspected to be one of the factors which influences the rate of rumen hydrolysis. Intraruminal degradation of insoluble zein has been shown to be 40 per cent (McDonald, 1954), while that of casein is 90 per cent (McDonald and Hall, 1957). Even though there is strong proteolytic activity in the rumen fluid, the ruminal concentration of free amino acids is low (Annison, 1956). There is a rapid deamination of amino acids by microbial deaminases which leads to the production of ammonia (Warner, 1956). Blackburn (1965) observed that the major disadvantage of deamination within the rumen, is that the amino nitrogen is converted to ammonia which cannot be resynthesized to protein nitrogen by the animal. The horse may have an advantage over the ruminant in this respect, since intestinal proteolysis and absorption precedes cecal fermentation and presumably, absorption.

High correlations have been reported between protein solubility and the rate of ammonia production by the rumen microorganisms (Chalmers et al., 1965; Little et al., 1963). Similar correlations have also been reported between the level of ruminal ammonia and absorption of ammonia nitrogen from the rumen (Lewis et al., 1957). If nitrogen is

absorbed from the rumen as ammonia more rapidly than the microorganisms can use it in protein synthesis, a portion is wasted because of its excretion in the urine. This loss is reduced to some extent in ruminants by a urea recycling mechanism which will be discussed later. Proteins which are rapidly converted to ammonia by the rumen microorganisms would have high apparent digestibilities when fed (Little and Mitchell, 1967). Nitrogen retention would reflect the amino acid balance of the synthesized microbial proteins, but might be reduced by the loss of ammonia nitrogen.

If the same proteins were not subjected to microbial degradation in the rumen and were well digested posterior to the rumen, apparent protein digestibility would not be subject to the error caused by ammonia production. Also, in this situation, nitrogen retention would reflect the amino acid balance of the administered protein. Little and Mitchell (1967) demonstrated that the utilization of some proteins can be improved in ruminants by abomasal administration. Little et al. (1968) reported the nature and quantity of nitrogen compounds reaching the abomasum were significantly affected by the solubility of the dietary protein source.

Annison (1965) emphasized that in determining ruminal absorption, it is necessary to distinguish between absorption rates and production rates. For substrates which are the end-products of fermentation and are absorbed rapidly from the rumen without uptake or modification by the rumen epithelium, rates of production and absorption will be similar. An example of such a substance is ammonia. On the other hand, the production rate will exceed the absorption rate, if the substrate is in-

completely absorbed or converted into other compounds in the rumen or metabolized by the rumen epithelium during absorption.

Absorption from the rumen can be measured by concentration changes occurring within it. This type of information is difficult to interpret if the substance being studied is produced by rumen fermentation or if it enters the rumen via the saliva or by diffusion from the blood across the rumen wall. If these things are not occurring, it is still necessary to correct for volume changes resulting from the addition of saliva, passage of ingesta out of the rumen and the two-way transfer of water across the rumen wall in response to changes in osmotic pressure. Rumen absorption rates are known to be affected by concentration, pH, effectiveness of mixing of rumen contents, and blood flow to and from the rumen.

Absorption from the rumen can also be measured by the analysis of blood draining this organ (Annison, 1965; Lewis, 1961). The substrate concentration in the venous drainage relative to the arterial blood supply provides a measure of the absorption rate, if the rate of blood flow is known. Samples of arterial and venous blood must be taken simultaneously by procedures which cause no change in the blood flow or metabolic rate.

Based on arterial-portal differences after adding casein digest to the rumen, Annison (1956) concluded that absorption of amino acids from the rumen did not occur. However, Waldo (1968) noted that Tsuda (1956) found a 20 per cent decline in glycine concentration in the minature rumen in four hours. Waldo also cited the work of Demaux et al. (1961) in which they found an increase in ruminal vein amino

nitrogen when solutions containing amino acids were placed in a washed out sheep rumen. Lewis and Emery (1962) showed that lysine was absorbed from the rumen. Ion exchange separation of amino acids has demonstrated the ruminal absorption of several acidic and neutral amino acids (Cooke et al., 1965). Waldo (1956) noted that the amino acids seem to compete with each other for absorption. He concluded that the absorption of amino acids from the rumen must be small. Thus, it seems that the major portion of amino acids made available to the tissues of the ruminant result from the gastric (abomasal) and intestinal degradation of intact dietary and microbial proteins after they leave the rumen. Hogan and Phillipson (1960) indicated that nitrogen passes from the rumen to the lower part of the digestive tract somewhat continuously. A fairly constant absorption of amino acids seems consistent with the present knowledge of digestion (Theurer et al., 1966).

Annison and Lewis (1959) divided the pathways of protein digestion in ruminants into (1) the activities in the reticulo-rumen and (2) those taking place posterior to it. The former activities are mainly concerned with the breakdown of the nitrogenous part of the feed by microorganisms and the anabolism of microbial protein. The activities posterior to the reticulo-rumen are concerned with the digestion of microbial protein and the dietary protein which has not been converted by the microorganisms. McDonald (1954) and McDonald and Hall (1957) suggest that the extent of digestion of different feed proteins in the reticulo-rumen may differ considerably and significant quantities of dietary protein may pass into the abomasum under certain feeding conditions.

From the above observations, it seems likely in the horse that the majority of amino acid absorption arising from the proteolysis of dietary protein should occur in the small intestine. One would not expect a significant amount of intact dietary proteins, polypeptides, dipeptides or amino acids to reach the cecum. However, this is in direct opposition to the findings of Reitnour et al. (1968) in their work with ponies. Their data indicate that net protein digestion anterior to the cecum appeared to be inefficient. They suggested that a major portion of the protein digestion occurred in and posterior to the cecum of the equine. The amount of dietary protein that escapes intestinal proteolysis is no doubt affected by its solubility. In addition, the rate of passage from the mouth to the cecum in the horse has been shown to be quite rapid. Howell and Cupps (1950), using tracers in horses with permanent cecal fistulas, found that feed appeared at the fistula about one and one-half hours after feeding. By the end of the sixth hour, most of the feed eaten at one time had passed through the cecum. Also investigated by these workers was the possibility that antiperistalsis could result in the movement of ingesta from the cecum back into the small intestine, where absorption of amino acids could readily take place. They were unable to detect the occurrence of antiperistalsis through the use of carbon granules as tracers.

Ammonia Metabolism

Ammonia is produced in the rumen by bacterial action on dietary proteins, peptides, amino acids, amides, urea, nitrates, and other non-protein nitrogen compounds (Warner, 1956 and 1964; Abou Akkada and

Blackburn, 1963; Hoshino et al., 1966; Pearson and Smith, 1943; Lewis, 1951 a and b). Ruminal ammonia concentration represents a balance between utilization by rumen bacteria, metabolism in the rumen wall, absorption into the portal vein and passage into the omasum. Johns (1955) observed that rumen ammonia levels vary from 0-130 mg. per 100 ml. of rumen fluid. The concentration of ammonia in the rumen has been used as an index of the usefulness of dietary protein to the animal (Blackburn, 1965).

Ammonia is the primary soluble nitrogen source utilized by the microbial population for protein synthesis (Blackburn, 1965; Lewis, 1961; Tillman and Sidhu, 1964). It has been shown to be an essential nutrient for the growth of many rumen microorganisms (Bryant, 1963; Hungate, 1966). The addition of nitrogen sources yielding ammonia have been shown to stimulate the in vitro digestion of cellulose (Belasco, 1954; Chalupa et al., 1963; Little et al., 1963) and starch (Accord et al., 1966 and 1968). Therefore, it appears that ammonia is important in the nutrition of both cellulolytic and amylolytic bacteria. The work of el-Shazly et al. (1961) indicated that the inhibition of cellulose digestion by the addition of starch was mainly due to competition between the cellulolytic and amylolytic bacteria for nitrogen. This inhibition was overcome by the addition of urea which is broken down by bacterial urease to yield two molecules of ammonia and one molecule of carbon dioxide.

The nitrogen requirements for bacteria are quite simple. Bryant (1961) examined 44 strains of rumen bacteria and found 80 per cent could be grown with ammonia as the only source of nitrogen. He also noted

that 26 per cent could not be grown unless ammonia was present and 56 per cent could use either ammonia or amino nitrogen. Quantitatively, ammonia may be a growth limiting nutrient in the case of a nitrogen deficiency (Conrad and Hibbs, 1968). At dietary levels less than 1.2 per cent nitrogen, rumen function was impaired, feed intake was reduced and growth rate of the host animal was stopped or greatly reduced (Harris et al., 1941 a and b; Moir and Harris, 1962; Williams and Moir, 1951).

Amino acids synthesized by rumen microorganisms require the presence of ammonia, carbon skeletons, certain minerals, and energy (Tillman and Sidhu, 1969). Utilization of carbon from carbohydrates (McNaught, 1951; Hoover et al., 1963), carbon dioxide (Huhtanen et al., 1954; Otagaki et al., 1955), isovaleric acid (Allison et al., 1955), acetate and other volatile fatty acids (Hoover et al., 1963) indicates that carbon from many sources can be used for amino acid synthesis. Other workers have indicated that the rumen microorganisms require specific carbon skeletons for the synthesis of certain amino acids, such as leucine, isoleucine, valine, phenylalanine, and tryptophan (Allison et al., 1966; Hungate, 1966; Allison and Bryant, 1963; Allison, 1965; Allison and Robinson, 1967). Energy for amino acid synthesis is provided by dietary carbohydrates and other organic compounds. Hogan and Weston (1967) calculated that about 15 grams of microbial protein was synthesized per 100 grams of organic matter utilized in the rumen.

McDonald (1948) was the first to show that ammonia absorption occurred from the rumen. He estimated the quantity of ammonia nitrogen absorbed from the rumen of sheep to be about 4-5 grams per day. Lewis (1957) demonstrated the ammonia concentration in the portal blood

increased as a curvilinear function of rumen ammonia concentration. Waldo (1968) cited the work of Gaertner et al. (1963) in which they found increased absorption with increasing rumen ammonia levels and concluded that the absorption occurred by diffusion. This group also noted that the permeability of the rumen wall to ammonia was inversely related to urine flow. Hogan (1961) found that water flux across the rumen wall had no effect on ammonia absorption. His data showed ammonia absorption to be dependent upon the concentration gradient at pH 6.5, but noted that practically no absorption occurred at pH 4.5. Bloomfield et al. (1963) also showed a reduction in ammonia absorption with a decreased rumen pH. Hogan (1961) concluded that free ammonia is absorbed as a function of concentration gradient.

Therefore, ammonia absorption across the rumen wall is influenced by both concentration gradient and pH. Ammonia is a weak base with a pKa of about 8.80-9.15 (Visek, 1968). Coombe et al. (1960) showed that an increase in pH causes the ammonium ion to be converted to free ammonia which is rapidly absorbed. The ammonia is absorbed into the portal blood, but under normal conditions, only small quantities are found in the peripheral blood. Ammonia is removed from the blood by one or another of several reactions (Harper, 1967). One of these, is the amination of keto acids, principally oxaloacetate and alpha-ketoglutarate, forming aspartate and glutamate, respectively. Another mechanism for ammonia removal, is the amidation of glutamate to form glutamine. Finally, the best known reaction is the conversion of ammonia to urea in the liver. Lewis et al. (1957) reported that the liver was able to convert all of the absorbed ammonia to urea until

the level in the portal blood reached 0.8 mM which was observed with ruminal fluid ammonia concentrations of 55-60 mM.

Ammonia is converted to urea in the liver via the urea cycle which has only been slightly modified since it was proposed by Krebs and Henseleit in 1932 (Dukes, 1966; Harper, 1967). Except for the brain, the liver is believed to be the only organ in the body capable of synthesizing urea. Simply expressed, this cycle involves the formation of citrulline by the addition of carbon dioxide and ammonia to the amino acid, ornithine. Citrulline is then converted to the amino acid, arginine, which involves two intermediate steps. Arginine is then hydrolyzed by the enzyme, arginase, to yield ornithine and urea. Ornithine is then recycled and used in subsequent urea formations. Urea, carbon dioxide, and water are the principal end-products of protein metabolism in mammals and urea excretion is directly related to nitrogen intake (Harper, 1967). Urea nitrogen comprises the following percentages of total urinary nitrogen: 86.2 in the dog, 81.9-84.5 in sheep, 70.0-79.2 in goats, and 87.5 in man (Dukes, 1955).

The ammonia content of the blood leaving the kidney by way of the renal vein is always higher than that of the renal artery, indicating that the kidney produces ammonia and adds it to the blood (Harper, 1967). However, a much more important aspect of renal ammonia metabolism is the excretion into the urine of the ammonia produced by the tubular cells of the kidney. This reaction is a part of the renal tubular mechanism for the regulation of the acid-base balance, as well as, the conservation of cation. The ammonia produced in the kidney is not derived from urea. The intracellular amino acids serve

as the source of ammonia with glutamine being the most important amino acid used for this purpose. This amino acid accounts for a larger fraction of the total alpha-amino nitrogen of the plasma than any other one amino acid. Ammonia normally constitutes 2.5-4.5 per cent of the total urinary nitrogen in man. However, it makes up only 0.101-0.272 per cent and 0.071-0.096 per cent of the urinary nitrogen in cattle and horses, respectively (Dukes, 1955).

Urea Recycling

Endogenous urea enters the rumen via the saliva and by diffusion across the rumen epithelium (McDonald, 1948; Houpt, 1959; Packett and Groves, 1965; Waldo, 1968; Caffrey *et al.*, 1967; Tillman and Sidu, 1969; Kay and Hobson, 1963). Diffusion through the rumen wall seems to be the main route of entry into the gastrointestinal tract, but blood urea has also been shown to diffuse into the abomasum and intestines (Cocimano and Leng, 1967). Houpt and Houpt (1968) observed that the urea diffusing into the rumen from the blood was almost completely hydrolyzed to carbon dioxide and ammonia before it entered the rumen fluid. These workers suggested that a possible explanation for this phenomena was the penetration of ruminal urease into the ruminal epithelium. If this is so, the transfer of urea across the rumen wall would also depend on the concentration gradient of ammonia. Waldo (1968) cited work which indicated that the total endogenous urea influx into the rumen is primarily a function of blood urea concentration. If nonessential amino acid synthesis is ignored, this implies a rumen wall transfer of some four to six times the amount of urea secreted in the parotid saliva.

McDonald (1948) demonstrated the presence of salivary urea in the saliva of sheep. Lewis (1957) showed that the concentration of urea in ruminant saliva increased when higher rumen ammonia levels occurred. Tillman and Sidhu (1969) noted Somers (1961 a, b and c) reported that urea made up 60-70 per cent of the total nitrogen in sheep saliva, while Baily and Balch (1961) observed that in cattle it made up 77 per cent of the total nitrogen in mixed saliva. The urea content of mixed saliva was about 65 per cent of the plasma urea concentration, which ranged from 4-19 mg. per 100 ml. of urea nitrogen. During times of low dietary nitrogen intake, the secretion of urea in the saliva increases (Somers, 1961d) and urinary urea excretion decreases (Schmidt-Nielsen et al., 1957; Schmidt-Nielsen and Osaki, 1958). This recycled urea is hydrolyzed to ammonia by bacterial urease in the rumen and may be used to maintain an active microbial population (Moir and Harris, 1962). Dietary carbohydrate has been shown to improve the microbial utilization of recycled urea (Houpt, 1959; Packett and Groves, 1965). As mentioned previously, urea also diffuses into the abomasum and intestines where urease activity has also been shown to occur (Sidu et al., 1968). McDonald (1948) noted that ammonia can be absorbed from the intestines. Phillipson (1964) observed that qualitatively, both ruminants and non-ruminants recycle urea.

This nitrogen recycling mechanism is made even more effective by a conservation mechanism in the kidney. It allows urea to be retained in the blood when the animal is subjected to a low dietary nitrogen intake (Kay and Hobson, 1963). This seems to be accomplished via an active reabsorption of urea by the kidney tubules. The stimulus for the

activation of this mechanism is associated with the low nitrogen intake itself and not with the blood urea concentration. Urea conservation cannot operate at high urine flows (Kay and Hobson, 1963; Waldo, 1968). Most of the nutritional studies show an increase in nitrogen retention with restricted water intake at low protein levels (Livingston et al., 1962; Topps and Elliott, 1964). The fact that water restriction with adequate nitrogen intake does not affect urine nitrogen excretion is consistent with the work of Schmidt-Nielson et al. (1958).

Urea as a Dietary Nitrogen Source

Urea is being used in continuously increasing amounts as a nonprotein nitrogen source in ruminant rations. Conrad and Hibbs (1968) cited several reasons for the increased use of urea for feeding. First is the economic advantage of partially replacing a protein supplement with urea. Secondly, is the increased demand for natural protein sources (e.g. soybean meal) for human consumption. There has also been an increased acceptance of urea by feeders as a result of research which has improved the results derived from its use.

Urea is the diamide of carbonic acid which liberates two molecules of ammonia and one molecule of carbon dioxide when it is hydrolyzed by bacterial urease. The ammonia can then be utilized by the rumen microorganisms in protein synthesis provided a suitable energy source is available (Blackburn, 1965). Purified and semi-purified diets are generally used to test a nonprotein source against a purified protein source. This has proven to be a useful technique because the nonprotein nitrogen source can supply essentially all of the dietary nitrogen. In

this manner, it can be compared directly to the protein source without altering the other nutrients in the diet.

Oltjen et al. (1969) reported purified diet studies in which various nonprotein nitrogen sources have supplied 98 per cent of the total nitrogen in the diet. Virtanen (1966) used a purified diet fed to dairy cows in which urea and diammonium salts supplied 99.5 per cent of the total nitrogen in the diet. Belasco (1954) conducted in vitro studies and found that 35 per cent replacement of protein by urea gave maximum urea utilization. Other workers have shown that high producing dairy cows responded to high levels of urea (Colvolos, et al., 1963 and 1967; Conrad and Hibbs, 1967). Thus, recent research has indicated that the old rule of thumb for using urea in ruminant rations may be in need of revision. This rule states that urea should not exceed 3 per cent of the concentrate portion or 1 per cent of the dry matter of a ration to be fed to ruminants (Conrad and Hibbs, 1968).

Growth and Nitrogen Balance Studies With Urea

The growth rates of ruminants fed purified diets ad libitum which supplied all of the dietary nitrogen as nonprotein nitrogen have been lower than those of ruminants fed protein containing diets (Oltjen, 1969). Oltjen et al. (1962 a and b) reported gains and feed efficiency of lambs fed urea containing diets to be 65 per cent of those fed isolated soy protein. Ellis et al. (1956) conducted metabolism trials with lambs fed purified diets in which he reported biological values of 54 for urea, 57 for gelatin, 73 for casein, and 84 for isolated soy protein. Oltjen and Putnam (1966) found the nitrogen retention of steers

fed urea to be 60 per cent of that of steers fed isolated soy protein in purified diets. Clifford and Tillman (1968) observed that the nitrogen retention of sheep fed a urea containing diet was about 70 per cent as good as sheep fed an isolated soy protein purified diet. Matrone et al. (1964) reported that gains and feed efficiency were about 50 per cent as good when urea was compared to casein in purified diets fed to sheep. A complete substitution of soybean nitrogen with urea nitrogen resulted in a reduced rate of gain in sheep (Oltjen et al., 1962; Clifford and Tillman, 1967) and reduced milk production in cows (Virtanen, 1966). These results indicate that when a nonprotein nitrogen source completely replaces the protein in purified diets, growth rate and feed efficiency are reduced by about 35 per cent. There appears to be a trend for a further reduction in performance in extended growth trials. Oltjen (1969) suggests that this may be related to a nutritional inadequacy of the purified diets, as well as, an actual effect of the nonprotein nitrogen source itself.

In general, feed intake has been similar with both nonprotein and protein nitrogen sources in the purified diets. This is contrary to the depression often seen with urea in natural diets. Oltjen (1969) felt that the lowered palatability of the purified diets may have tended to hide this reduction in intake. Various substances have been added to urea containing purified diets in an effort to improve animal performance. As a result of experiments in which plasma amino acid patterns have been altered in response to urea in the diet, various dietary amino acid additions have been tested. Lower plasma concentrations of leucine, lysine, alanine, tyrosine, glutamic acid, proline, and

methionine have been observed by various workers when cattle were fed urea rather than soybean protein as the dietary nitrogen source (Oltjen and Putnam, 1966; Virtanen, 1966; Frietag et al., 1966; Little et al., 1966).

For the most part, amino acid additions, both singly and in combination, have not resulted in improved animal performance. Some of the possible reasons for the lack of a positive response include: (1) the use of DL-amino acids instead of the natural L-forms, (2) they may have been supplied in too small a quantity, (3) they may have been catabolized by the rumen microorganisms instead of being incorporated into microbial protein, (4) the amino acids used may not have been limiting for growth (Oltjen, 1969).

Marked depressions in the ruminal concentrations of isobutyric and isovaleric acids have been observed when ruminants were fed protein-free diets containing urea (Orskov and Oltjen, 1967; Cline et al., 1966; Clifford and Tillman, 1968). These branched-chain volatile fatty acids are present in the ruminal fluid of ruminants fed natural diets mainly through the degradation of dietary protein and the deamination of the branched-chain amino acids (el-Shazly, 1952 a and b; Annison, 1954). Cline et al. (1966) reported a significant increase in ration digestibility and nitrogen retention of lambs fed a urea containing purified diet with the addition of isovaleric, isobutyric and n-valeric acids. They also observed a decrease in ruminal ammonia concentration which was interpreted to indicate increased microbial protein synthesis. However, Clifford et al. (1968) concluded that ruminal synthesis of branched-chain fatty acids was not the major growth limiting factor in lambs fed

a purified diet in which urea was the only nitrogen source. Orskov and Oltjen (1967) observed that replacing isolated soy protein with urea resulted in lowered proportions of acetic acid and increased proportions of butyric acid.

McLaren et al. (1962) reported results which indicated a microbial requirement for peptide nitrogen to improve nitrogen utilization of semi-purified diets containing urea. Bunn et al. (1968) got a significant growth response with lambs from the addition of 5 per cent alfalfa or a combination of amino acids to a purified diet. McLaren et al. (1965) found that nitrogen retention was improved when one-third of the urea nitrogen, which supplied 75 per cent of the total dietary nitrogen, was replaced by an isonitrogenous amount of creatine. These workers also noted that the addition of vitamin B₁₂, folic acid, or both, did not affect nitrogen utilization. Oltjen et al. (1962) reported that urea as the only nitrogen source in a purified diet does not appear to reduce the synthesis of B-vitamins by the rumen microorganisms. In fact, Oltjen (1969) cited work which indicates that dietary urea seems to stimulate B-vitamin synthesis in the rumen, but that the availability of readily fermentable carbohydrates and certain amino acids may limit vitamin synthesis.

Conrad and Hibbs (1968) reviewed the mineral requirements of bacteria utilizing urea containing rations. They noted that phosphorus is probably the most important mineral lacking on this type of dietary regime. Most of the mineral elements needed by rumen microorganisms were found in alfalfa, alfalfa ash, molasses ash, soybean protein, and the ash of soybean protein (Bentley et al., 1952 a and b; Burroughs

et al., 1951 a and b; Woods and Tillman, 1956). The mineral mixture used by Virtanen (1966) to meet the needs of the rumen microorganisms and the cows fed purified diets, contained sodium, potassium, calcium, magnesium, copper, selenium, boron, cobalt, iodine, and molybdenum. Burroughs et al. (1951b) stated that phosphorus and sulfur are apparently needed in larger amounts than other minerals for optimum utilization of urea and ammonia nitrogen.

Sulfur is used by the rumen bacteria in the synthesis of the sulfur-containing amino acids, cystine and methionine (Kay and Hobson, 1963). The general microbial pattern of sulfur amino acid metabolism differs from the mammalian pattern in that methionine is derived from cystine (Moir et al., 1967-68). The main form in which sulfur is normally used by the rumen microflora in the synthesis of these amino acids is sulfide, which can be provided either by a reduction of sulfate or from the sulfur-amino acids in the feed. Brown et al. (1960) and Jacobson and Barnett (1967) suggested that sulfur may be deficient in heifers and high-producing dairy cows fed rations high in both corn plants and urea. Usually, a ratio of 1 part sulfur to 15 parts of nitrogen from a non-protein source is considered adequate to meet this requirement (Dawe's Laboratories, 1966). However, Moir et al. (1967-68) reported data from experiments in which urea nitrogen made up 40 per cent or more of the dietary nitrogen and sulfur either as sulfate or methionine was added. They strongly suggested that for maximum efficiency of urea nitrogen utilization, the ratio of sulfur to nitrogen should be slightly narrower than 1:10.

Feeding a urea containing purified diet twice a day has been

reported to result in ruminal ammonia concentrations which tend to peak at 1.0-1.5 hours post-prandial (Chalupa et al., 1964; Oltjen and Putnam, 1966; Oltjen et al., 1968). Ruminal ammonia values averaged 35-55 mg. of ammonia nitrogen per 100 ml. of rumen fluid. This was below the toxic level, but resulted in high urinary nitrogen losses. This may account for part of the reduction in performance of ruminants fed urea versus protein containing diets on a once or twice a day basis. Oltjen (1969) observed that if ruminants are fed the urea containing diets ad libitum, ruminal ammonia concentrations are lowered and nitrogen retention is usually improved.

Bloomfield et al. (1960) reported the rate of urea hydrolysis was about four times greater than the uptake of ammonia by rumen micro-organisms. They suggested that it might be possible to improve urea utilization by decreasing ruminal urease activity. Increased growth rate and feed efficiency have been obtained by inducing urease immunity in calves, lambs, chicks, and rats (Harbers et al., 1965; Glimp and Tillman, 1965; Dang and Visek, 1960). Sidhu et al. (1968) reported results which indicate a decreased urea hydrolysis in urease immunized sheep. They cited work showing that antibodies are secreted into the saliva and suggested the possibility that the urease-antiurease reaction may have occurred in the rumen.

Several workers have investigated the influence of rumen ammonia levels on the nitrogen retention of ruminants fed highly soluble proteins. Chalmers and Synge (1954) and Lewis et al. (1957) found a negative correlation between nitrogen source solubility and nitrogen retention by ruminants fed poor quality roughages. Heat treatment of

casein (Chalmers et al., 1954), groundnut meal (Whitelaw et al., 1961; Chalmers et al., 1964), and soybean meal (Tagari et al., 1962) improved nitrogen retention in ruminants. This was attributed to a decrease in solubility which resulted in decreased proteolysis which lowered ammonia production. Sherrod and Goldman (1962), using cottonseed and soybean meals, found that autoclaving decreased nitrogen solubility, protein digestibility, rumen ammonia, and urinary nitrogen and improved nitrogen retention.

Oltjen et al. (1968) observed that, in general, ruminal pH values are similar for urea and isolated soy protein containing rations fed ad libitum to ruminants. However, when they were fed once or twice per day, the ruminal pH may be considerably higher 1-2 hours after feeding on the urea diet. This is due to the rapid hydrolysis of urea to ammonia by the rumen microorganisms. Hogan (1961) noted that a low ruminal pH of 5.4-6.0 may be beneficial to animals fed urea containing purified diets. He based this on the fact that ammonia transfer through the rumen wall is much less rapid at a low pH. This situation would provide more free ammonia for microbial protein synthesis. Salivary flow is known to affect the buffering capacity of the rumen. Oltjen et al. (1965 and 1967) indicated that high ruminal ammonia concentrations can markedly reduce salivary flow.

Gall et al. (1951) reported similar microbial populations in the rumen of sheep fed urea and casein containing diets. Chalupa et al. (1964) found a difference in rumen redox potentials in sheep fed urea versus corn gluten meal containing rations which indicated greater microbial activity when urea was the dietary nitrogen source. However,

nitrogen retention favored the corn gluten meal fed animals. Virtanen (1966) also indicated that bacterial protein synthesis was greatly increased in the rumens of cows fed a urea-ammonium salts purified diet. On the other hand, he reported a drastic reduction in protozoa numbers along with an increase in bacterial numbers in the rumen. He suggested that this was due to the urea in the ration. Other workers have shown that the ruminal ingesta of cattle fed purified diets are often free of protozoa and have increased bacterial concentrations (Oltjen et al., 1966; Slyter et al., 1968). This was true for both protein and nonprotein containing purified diets. This disappearance of protozoa, may be partly responsible for the reduced performance of ruminants fed purified diets (Oltjen, 1969). Abou Akkada and el-Shazly (1965) reported increased nitrogen retention in faunated lambs when compared with defaunated lambs.

Adaptation Response to Urea Utilization

Nitrogen utilization of urea-containing diets is also influenced by the length of time the animals have been consuming the ration (Caffrey et al., 1967; Chalupa, 1968; Little et al., 1966; McLaren, 1964). McLaren (1959 and 1960) reported the utilization of absorbed nitrogen was increased by increasing the adaptation period during which sheep received nonprotein nitrogen. Regression analysis indicated that nitrogen retention was improved 3 per cent for each ten day period of urea feeding and 2 per cent for each 100 kcal. of readily available carbohydrate in the diet. Smith et al. (1960) found that nitrogen utilization improved 0.2 per cent per day during a fifty day feeding period. Other workers have also observed an increase in nitrogen retention with

sheep and cattle fed either isolated soy protein, ammonium salts or urea as a result of decreased urinary nitrogen loss (Clifford and Tillman, 1968; Oltjen and Putnam, 1966; Virtanen, 1966; Johnson et al., 1964; Schaadt et al., 1966).

Various reasons have been given to explain this adaptation response (Chalupa, 1958). Lewis (1960) suggested it was due to an adaptation of the rumen microorganisms themselves. (Caffrey et al. (1967a) and Chalupa et al. (1968) reported that sheep fed a urea diet had lower ruminal urease activity than sheep receiving a similar soybean containing diet. McLaren et al. (1962) indicated that the adaptation was due to increased utilization of ammonia by the rumen mucosa. On the other hand, Holzochuh and Wettera (1965) and Caffrey et al. (1967) postulated the cause to be increased conversion of ammonia to urea in the liver. McLaren (1964) revised his first proposal and believed that the adaptation was due to an increased urea absorption in the glomerular filtrate by the renal tubules of the kidney.

Ammonia Toxicity

When the rate of ammonia absorption from the rumen via the portal system exceeds the liver's capability to convert it to urea, increasing amounts of ammonia will be found in the peripheral blood (Lewis et al., 1957). When the concentration of ammonia in the peripheral blood reaches 1-4 mg. per 100 ml., symptoms of ammonia toxicity usually appear (Chalupa, 1968). Bartley (1969) reported it took 30 gm. of urea per 50 kg. body weight to produce toxicity and death in animals not fully adapted to a urea containing diet. However, it required 50 gm. of urea

per 50 kg. of body weight before toxicity and death resulted in animals that had received urea for several months. Toxicity occurred when the ruminal ammonia concentration approached 100 mg. per 100 ml. of rumen fluid thirty minutes after feeding. The concentration of ammonia would often rise above 100 mg. per 100 ml. of rumen fluid in subsequent samples without toxicity resulting, providing the thirty minute concentration was much below 100 mg. per cent.

The characteristic symptoms of ammonia toxicity are uneasiness, muscular tremors, excess salivation, muscular incoordination, bloat, and tetany (Bartley, 1969; Chalupa, 1968). This is usually followed by death within 1.5-2.5 hours after the initial appearance of the symptoms. Animals normally die when the blood ammonia levels reach 5 mg. per 100 ml.. Rumen pH will rise to about 8 and normal rumen function will cease. Muherer et al. (1967) found ammonia toxicity to be characterized by high peripheral blood ammonia levels, acidosis and hypoxic hypoxia (reduced pO_2 of arterial blood). These workers concluded that the acidosis was of respiratory origin and that death was due to ventricular fibrillation. Bartley (1969) recommended animals showing toxic symptoms should be given 1-2 gallons of a 5 per cent solution of acetic acid either by stomach tube or as a drench. For a diagrammatic summary of nitrogen metabolism in the ruminant refer to Figure 1, page 33.

Utilization of Nonprotein Nitrogen by Monogastrics

The monogastric species studied are unable to efficiently utilize urea or other nonprotein nitrogen sources to replace a portion of their dietary protein requirement. The adequacy of a dietary protein for a

FIGURE 1

A DIAGRAMMATIC SUMMARY OF NITROGEN
METABOLISM IN THE RUMINANT

From: Digestive Physiology and Nutrition of the Ruminant
Edited by D. Lewis. Page 127.

monogastric animal, such as the pig, is determined by its content and balance of essential amino acids and amino nitrogen for the synthesis of nonessential amino acids and its digestibility (National Research Council, 1968). Diammonium citrate and diammonium phosphate are satisfactory nonprotein nitrogen sources for nonessential amino acid synthesis in the pig. It is quite possible that urea could also act in this capacity. However, urea would first have to be hydrolyzed by urease to liberate ammonia.

Isotopic studies have shown that urea is hydrolyzed in the digestive tract of pigs with a small, but definite amount, of the ingested urea nitrogen found incorporated into body protein (Kornegay et al., 1965). No symptoms of urea toxicity were observed when pigs were fed 2.5 per cent urea in addition to or in place of, part of the protein in the ration. These workers noted increased blood urea and decreased plasma ammonia concentrations of pigs fed the urea-containing diets. Hays et al. (1957) stated the results of three experiments conducted by them indicated that urea had no place in practical swine rations.

Olcese et al. (1948) concluded that urea could not be used to replace part of the dietary protein in rabbits. The rabbit and the guinea pig are the laboratory animals that possess digestive tracts most comparable to the horse, being nonruminant herbivores with well-developed ceca. Becker (1924) found that feeding rabbits 1-2 grams of urea per kilogram of body weight produced severe urea toxicity symptoms. Houpt (1961) reported that when rabbits were injected with 61 micromoles of urea, 16 micromoles could not be accounted for in the blood, body water,

or urine. Since some was found to have moved into the cecum, it was concluded that rabbits could utilize some endogenous urea.

Very little definitive work concerning the utilization of nonprotein nitrogen by the horse has been conducted. Conflicting recommendations can be found concerning the use of urea containing rations for the horse. Ensminger (1963) observed that nonprotein nitrogen sources such as urea have a protein replacement value when fed to mature horses and may be used to advantage in small quantities. Ensminger (1966) later reported that mature horses can consume urea containing rations without any apparent detrimental effects. He cited a situation, which had occurred in the field, where four mature horses consumed an average of 0.55 pounds of urea per head per day in a urea containing cattle supplement for five months with no ill effects. Bonnell and Wells (1968) incorporated 1, 2, 3, and 5 per cent by weight of urea in a 25 per cent corncob and 75 per cent alfalfa ration which was fed to mature ponies. They observed that the blood urea nitrogen levels did not fluctuate within the periods tested, palatability was not depressed, and no toxicity symptoms were observed under the conditions of their experiment.

Intestinal Absorption of Urea in Monogastrics

Wilson (1962) stated that the rate of intestinal absorption of urea is a linear function of the concentration gradient. Urea is able to diffuse in both directions across the intestinal epithelium. A linear relationship between absorption rate and concentration gradient, while consistent with diffusion, does not exclude absorption by enzymatic mechanisms or by involving some type of absorption reaction. Lindemann

and Wilson (1960) estimated the pore size in the intestinal wall to be 4.0 angstroms, while Guyton (1966) gives the effective diameter of the urea molecule as 3.6 angstroms. This lends further support to the belief that urea absorption occurs via simple diffusion through the intestinal pores. These pores are water filled and under certain conditions, bulk fluid movement will "drag" small solute molecules through the pores in the moving stream. Fisher (1955) showed increased fluid movement across rat intestine in vitro was associated with a large increase in the movement of urea, creatinine, and mannitol across the gut wall.

In light of the preceding discussion, it seems reasonable to conclude that in a urea containing ration fed to a horse, most of the urea would diffuse out of the small intestine before reaching the cecum. This urea should then be picked up by the portal circulation and cause an increase in urea levels in the peripheral blood and the urine. If this sequence of events occurs, then it is unlikely the horse would be able to efficiently use urea in the formation of bacterial protein. However, there is a distinct possibility that a portion of the urea picked up by the circulatory system might be recycled via the saliva and also across the intestinal wall. If any urea is recycled in the saliva, it would no doubt diffuse out of the small intestine before reaching the cecum. Therefore, only the endogenous urea being returned to the large intestine (viz. the cecum and colon) would be available for utilization in the synthesis of microbial protein.

If endogenous urea in the horse is utilized by the intestinal microorganisms for protein synthesis, the question still remains whether

this protein can be utilized by the host animal. From research conducted with both ruminants and nonruminants, it seems unlikely that any significant amount of amino acids derived from the proteolysis of the microorganisms would be absorbed from the large intestine. No proteolytic enzymes have been found to be secreted in this portion of the gastrointestinal tract. If in fact, this is the case, the bacterial protein would be excreted in the feces and would result in a net loss of nitrogen to the host animal. One contribution that the recycled urea could make, would be the stimulation of cellulose digestion in the large intestine.

MATERIALS AND METHODS

Experimental Procedure

A 3 x 3 split-split plot Latin square design was used to measure the effects of dietary nitrogen source on selected variables in three mature Shetland pony stallions fitted with permanent cecal fistulas (Table 2, page 38). The ponies were randomly allotted to each of three pelleted, semi-purified diets (Tyznik, 1968) which contained casein, corn gluten meal, and urea as the nitrogen sources (Tables 3 and 4, pages 39 and 40). The ponies were fed approximately a maintenance allowance of digestible energy as calculated from metabolic body size. The equation used states that the digestible energy in kilocalories for maintenance is equal to 75.5 times the bodyweight in pounds to the three fourths power or $D.E._{maint.} = 75.5 Wt.^{0.75}$ (National Research Council, 1966). The bodyweight of the three ponies at the initiation of this experiment ranged from 319-344 pounds. Water and trace mineralized salt were available on a free-choice basis at all times. All three ponies were dewormed by means of the stomach tube administration of a combination of appropriate anthelmintics prior to the start of the experiment.

Initially, the diets were formulated to contain approximately 50 per cent cellulose and 7.5 per cent crude protein. Severe acceptability problems were encountered when these rations were fed to the ponies. Therefore, the diets were reformulated to contain approximately 25 per cent cellulose and 12 per cent crude protein which is comparable to the composition of a good quality grass hay (Crampton and

TABLE 2
EXPERIMENTAL DESIGN

	Pony 1	Pony 2	Pony 3
Period I	Ration A	Ration B	Ration C
Period II	Ration C	Ration A	Ration B
Period III	Ration B	Ration C	Ration A

Nitrogen Source

Note: Ration A = Casein

Ration B = Corn gluten meal

Ration C = Urea

TABLE 3
PER CENT COMPOSITION OF DIETS^a

Ingredients	Ration A	Ration B	Ration C
Solka Floc (BW-40) ^b	24.4	24.8	25.4
Corn Starch	38.7	30.1	44.1
Cerelose	14.3	10.6	18.4
Casein	14.3	-----	-----
Corn Gluten Meal	-----	26.6	-----
Urea (45% N)	-----	-----	4.0
Corn Oil	2.9	2.7	2.8
Dicalcium Phosphate	1.4	1.4	1.4
Vitamin-Mineral Premix	4.0	3.8	3.9
Totals	100.0	100.0	100.0
Crude Protein	14.7	17.4	12.2
Ash	1.9	0.1	0.8
Cellulose	21.1	22.2	22.8
Gross Energy (Kcal/lb)	1907	1929	1806

a/The above rations were pelleted into 3/16 inch diameter pellets.

b/Purified wood cellulose obtained from Brown Company, Berlin, New Hampshire.

TABLE 4
VITAMIN - MINERAL PREMIX

Ingredient	Amount Supplied Per Pound Of Complete Ration
Brewer's Yeast	0.25%
Magnesium as magnesium oxide	400 ppm
Vitamin A	2000 I.U./lb.
Vitamin D	400 I.U./lb.
Vitamin E	3 I.U./lb.
Trace Mineralized Salt ^a	1.0%

^a/Guaranteed analysis: (1) NaCl, 95-99%, (2) Mn, not less than 0.200%, (3) Fe, not less than 0.160%, (4) Cu, not less than 0.033%, (5) sulfide sulfur, not less than 0.011%, (6) Co, not less than 0.010%, (7) Zn, not less than 0.010%, and (8) I, not less than 0.007%. Manufactured by Diamond Crystal Salt Co., St. Clair, Michigan.

Harris, 1969). In spite of this reformulation, ration acceptability remained a problem. The topical application of molasses was tried to no avail. Finally, in order to achieve the desired level of intake, it was necessary to replace three-quarters of a pound of the experimental diet with an equivalent amount of pelleted dehydrated alfalfa meal in the daily ration allowance fed twice a day. The dehydrated alfalfa meal contained 14.5 per cent crude protein as determined by the Kjeldahl method. Therefore, 0.75 pound (1b.) supplied 0.11 pounds of crude protein and 1411 kilocalories (kcal.) of gross energy.

The original intention was for all three rations to be both isonitrogenous and isocaloric. However, this did not turn out to be the case (Table 3, page 39). This no doubt resulted from using average nutrient values from feed composition tables in formulating the diets. An additional factor could possibly have been inaccurate mixing at the feed mill.

Each of the three experimental periods was preceded by a minimum of two weeks for an adjustment period and included a five day total collection period. Although four days appears to be long enough for a total collection period for a digestion trial with horses, five days has been recommended to reduce the influence of animal variation (Vander Noot et al., 1967). In order to make the necessary fecal and urine collections, the ponies were confined to metabolism stalls for each of three, five day collection periods. This necessitated the construction of both metabolism stalls and collection harnesses (Vander Noot et al., 1965). The metabolism stalls were constructed by the modification of cages originally designed to house sheep. The collection harnesses

were based on the plans, with several modifications, presented by Vander Noot et al. (1965). Photographs of the stalls and harnesses constructed by the author for this experiment are included as Plates I through V, pages 97 through 103. The cecal samples were taken in the morning of the fifth day of the total collection period at 0, $\frac{1}{2}$, 1, 3, and 6 hours after feeding. Jugular blood samples were taken the morning following the last day of the collection period at 0, 1, 2, 3, 4, 5, and 6 hours after feeding. During each of the two week adjustment periods, the ponies were housed in wire enclosed box stalls and runs with concrete floors without bedding.

The effects of nitrogen source in a semi-purified diet on the following parameters were measured: (1) nitrogen balance, (2) coefficients of apparent digestibility for crude protein, cellulose and energy, (3) urinary urea and total urinary nitrogen, (4) cecal ammonia, urea, protein and residual nonprotein nitrogen (defined as total nonprotein nitrogen less ammonia and urea nitrogen), and (5) blood plasma ammonia, urea and residual nonprotein nitrogen (NPN) levels.

Problems were encountered with the cannulae used to maintain the cecal fistulas. At the time of fistulation, Jarrett sheep cannulae¹ were installed. This type of cannula proved to be unsuitable for this purpose with ponies. The neck of the cannula was not long enough to provide for adequate adjustment. As a result, the cannulae resulted in localized necrosis and granuloma formation and were also continually

¹Normally used to maintain rumen fistulas in sheep. Manufactured by the South Australian Rubber Mill, Adelaide, South Australia.

coming out. Linerode (1966) also experienced similar problems with this type of cannula.

The continued use of the Jarrett cannulae resulted in a thickening of the abdominal wall surrounding the cecal fistulas. The author designed a new cannula which could be adjusted so that no pressure was put on the already irritated area. The use of this type of cannula reduced the swelling and thickening of the wall and the incidence of cannula loss. The success of these cannulae was increased by further modification of the design. The three cannulae discussed above are shown in Plate VI, page 102.

The author believes that the design of the cecal cannula can still be greatly improved. A promising possibility would be the adaptation of the Silastic-type cannula¹ employed by Diershke and Hyatt (1969) to observe ovarian activity in ewes. A cannula constructed in such a manner allows the peritoneal tissue to actually grow into the dacron material which was bonded to the cannula's base. In addition to making the cannula immovable, this seal should lower the incidence of granuloma formation and keep the leakage of cecal fluid to a minimum.

Data is also included from a fourth animal and is treated as a separate experiment. This was a mature pony stallion fitted with a permanent indwelling catheter in the lateral cecal vein. Comparisons of blood samples taken from the jugular vein and lateral cecal vein were made for ammonia, urea and total nonprotein nitrogen. The blood

¹Silastic (medical-grade silicone elastomer). The cannulae were fabricated by the Dow Corning Center for Aid to Medical Research, Midland, Michigan.

samples were taken from this animal when he was consuming the casein diet at 0, 1, 2, 3, 4, 5, and 6 hours after feeding. These samples were taken after a two week adjustment period. The cecal vein catheter stopped functioning shortly after the samples were taken and it was, therefore, impossible to get further samples to compare the three rations. For a more complete description of the surgical technique and type of catheters used, refer to the Appendix, page 90 and Plate VII, page 103.

Nitrogen Balance and Digestion Trial Determinations

The feces were collected in fifteen gallon galvanized cans lined with disposable plastic bags. Collections were weighed and a grab sample taken once every twenty-four hours and frozen each day. The daily fecal samples for each five day collection period were composited for each pony. After thorough mixing, each composite fecal collection was sampled in duplicate. Each of these samples were weighed and dried in a forced-air oven at 58°C for a minimum of thirty-six hours. Dry matter determinations were made from these samples which were then finely ground in a Thomas-Wiley mill and stored in glass jars for subsequent determinations. Grab samples of each diet were taken daily during each total collection period, composited and also finely ground. Determination of ash and crude protein were performed on both the feed and fecal samples using techniques approved by the Association of Official Agricultural Chemists (AOAC, 1960). The cellulose determinations on the feed and feces were made by the use of a modification of the method of Crampton and Maynard (1938). Coefficients of apparent

digestibility were calculated for crude protein, cellulose and energy. The energy values of the feed and feces were determined in an oxygen bomb calorimeter.

The urine was collected daily in two gallon plastic jugs acidified with 10 ml. of concentrated hydrochloric acid to prevent bacterial fermentation. Each twenty-four hour urine collection was measured in a graduated cylinder and 10 per cent of this volume was saved each day. The urine samples were composited for each pony for each five day collection period and 250 ml. of this was frozen at -20°C for subsequent determination of urea and total nitrogen.

Urinary urea was determined by the use of an auto-analyzer¹ which employs a modification of the carbamido-diacetyl reaction which is a colorimetric method. No prior treatment of urine specimens is necessary as ammonia does not enter into the chemical reaction. Total nitrogen in the urine samples was determined using the standard Kjeldahl procedure as approved by the AOAC (1960).

Analysis of Cecal Contents

Cecal samples were collected from permanent cecal fistulas during each of the three total collection periods on the morning of the fifth day at 0, $\frac{1}{2}$, 1, 3, and 6 hours after feeding. An unsuccessful attempt was made to train the ponies to consume their feed in a one hour period. This necessitated leaving the feed in front of them throughout the time

¹Techicon Autoanalyzer. Techicon Corporation. Tarrytown, New York 10591

the cecal samples were being taken. As a result, the feed consumption patterns varied between both ponies and rations on the sampling days.

Immediately after being taken, the cecal samples were strained through four layers of cheesecloth and placed in plastic bottles containing mercuric chloride to stop bacterial fermentation. Approximately 2.5 ml. of mercuric chloride was used per 50 ml. of strained cecal contents. Ammonia determinations were performed as soon as possible after collection using the colorimetric method of Chaney and Marbach (1962) which employs the catalyzed indophenol reaction. The remainder of the samples were then frozen at -20°C for later determination of urea, protein and total nitrogen. The urea nitrogen was determined on an auto-analyzer.

The determination of protein nitrogen in the cecal contents required the separation of the protein and nonprotein fractions. This was accomplished by precipitating the protein nitrogen with 10 per cent tungstic acid as described by Winter (1962). His method was followed with the exception that the precipitate was washed only once rather than twice. Once this separation was accomplished, urea nitrogen was determined in the supernatant using an autoanalyzer. Total nonprotein nitrogen in the supernatant was also determined with an autoanalyzer, employing a modification of the Kjeldahl procedure. The protein nitrogen in the precipitate was determined using the standard Kjeldahl procedure as approved by the AOAC (1960).

Analysis of Blood Samples

Blood samples were taken by means of indwelling 18 gauge x 10 cm. teflon catheters¹ in the jugular vein. The catheters were installed shortly after the ponies were removed from the metabolism stalls on the evening of the fifth day of the total collection period. The blood samples were taken the next morning at 0, 1, 2, 3, 4, 5, and 6 hours after feeding. Again, the feed remained in front of the ponies throughout the sampling period.

The plasma ammonia was determined as soon as possible after collection to minimize the loss of ammonia. The sampling procedure was based on the recommendations of Visek (1969). Approximately 10-15 cc. of blood was collected in a clean, dry glass syringe which had been cooled in ice. The syringe was iced to lower the vapor pressure and reduce the production of carbon dioxide. Excess carbon dioxide production would raise the pH of the blood and result in a more rapid release of ammonia. Care was taken to prevent foaming of the blood as it was being drawn. Once the sample had been taken, it was immediately placed in a clean, dry, iced centrifuge tube which contained the anti-coagulant, ethylenediaminetetraacetic acid (EDTA). The whole blood samples were centrifuged to separate the cells from the plasma. The plasma was decanted and ammonia was determined immediately, using the method of Chaney and Marbach (1962).

¹Longdwel catheter. Beckton, Dickinson and Company. Rutherford, New Jersey.

The remaining portion of the plasma samples were frozen at -20°C for the subsequent determination of urea and residual NPN by means of an autoanalyzer. Residual NPN was determined on the protein-free supernatant of tungstic acid (10 per cent) precipitated plasma (Oser, 1965).

Methods of Statistical Analysis

Nitrogen Balance and Digestion Trials: These trials employed a 3×3 Latin square design. These data were analyzed by use of an analysis of variance as described by Snedecor and Cochran (1967) with ponies, periods and treatments as the three sources of variation. The use of this analysis allows the determination of any significant differences across all treatments. In order to make comparisons among treatment means, Duncan's New Multiple Range Test (Steel and Torrie, 1960) was used.

Analysis of Cecal and Blood Data: The Least-Squares and Maximum Likelihood General Purpose Program of Harvey (1968) which is written in the Fortran IV computer was used to analyze these data. This experiment was based around a 3×3 split-split Latin square design with periods, ponies and treatments as the three main sources of variation and sample time as the sub-plot source of variation. The interaction of sample time with each of the three main effects was also investigated.

An additional analysis was performed with sample time nested within each treatment. This analysis provided the opportunity to observe the effect of sample time on the variables within each treatment. Orthogonal polynomials were fitted to determine the appropriate equation

that would describe the trend of sample time within each treatment. The resulting regression coefficients can be used to plot the line that best fit the data.

Analysis of Jugular Vein vs. Cecal Vein Blood Samples: These data represent an analysis of the blood samples collected from the jugular vein and cecal vein of the pony fitted with the permanent catheter in the lateral cecal vein. The Least-Squares and Maximum Likelihood General Purpose Program of Harvey (1968) was used to analyze this simple two factor design with sample site and sample time as the two sources of variation.

RESULTS AND DISCUSSION

Levels of Significance

Levels of significance through $P \leq .25$ are reported in these results. Steel and Torrie (1960) state that the levels of 5 per cent and 1 per cent are most often used in reporting the results of agricultural research. These authors noted that the choice of these specific levels of significance is arbitrary. In the case of experiments with a limited number of animals, it is possible to conclude that the treatments had no effect if these levels are required (Type II error), unless a large real difference exists. This suggests that the choice of another level of significance, such as 10 per cent, may be valid for experiments with limited numbers such as this one.

Results which are not significant at a predetermined level cannot be completely ignored as though the treatment had no effect at all. Little and Hills (1966) note that if such results give some indication of a real treatment effect, the experiment might be worth repeating with a greater effort being made to reduce the error.

Nitrogen Balance and Digestion Trials

The results of the nitrogen balance and digestion trials are presented in Tables 5 and 6, pages 52 and 53. Analysis of variance and Duncan's New Multiple Range Test (Steel and Torrie, 1960) failed to show any significant ($P \leq .05$) differences due to treatment in feed intake, nitrogen balance, total nitrogen excretion, urea excretion, or in the

apparent digestibilities of cellulose, protein, or energy. Feed intake continued to be a problem throughout this experiment with all diets, in spite of the inclusion of 0.75 lb. of dehydrated alfalfa meal per head per day. There was a trend for feed consumption to be the poorest with the corn gluten meal ration. This may have been a reflection of the poor quality of corn gluten meal protein which is low in the amino acids arginine, lysine and tryptophan (Naber, 1963). This reduction in feed intake is consistent with work cited by Munro and Allison (1964) in which monogastrics were fed a ration containing an imbalanced protein.

Despite the lack of a statistical difference, the rations can be ranked, on the basis of observation, from the most to the least acceptable as follows: (1) casein ration, (2) urea ration, and (3) corn gluten meal ration. Casein is a more soluble protein which has a higher biological value for monogastrics than does corn gluten meal. However, Albanese (1959) notes that the biological value of casein for growing rats is much below that of egg albumin (commonly used as a reference protein), being 69 and 97, respectively. The limiting factor in casein is a relative deficiency in the sulfur-containing amino acids, methionine and cystine.

Possible real differences in nitrogen utilization in this study may well have been masked by the fact that the three rations turned out to be neither isonitrogenous nor isocaloric (Table 3, page 39). This is further reflected by the rations' respective calorie:protein ratios which differed from each other at the 5 per cent level (Tables 5 and 6, pages 52 and 53). The significance of the calorie:protein ratio in the nutrition of the horse has apparently not been investigated. An

TABLE 5
AVERAGE FEED CONSUMPTION, NITROGEN BALANCE AND EXCRETION AND
COEFFICIENTS OF APPARENT DIGESTIBILITY^b

Treatment	Feed ^a Intake (lb./day)	Nitrogen Balance (gm./day)	Total Nitrogen Excreted (gm./day)	Urea Excretion (gm./day)	Coefficients of Apparent Digestibility			Calorie: Protein Ratios ^c
					Cellulose	Protein	Energy	
Casein diet	3.19	-1.61	26.98	21.17	68.25	72.45	74.84	97 ^b
Corn gluten meal diet	2.90	-5.29	30.16	23.27	67.69	70.86	66.73	74 ^b
Urea diet	3.25	-2.83	26.41	21.71	81.49	78.72	82.76	123 ^b
S. E. $\bar{x}$	± 0.28	± 4.76	± 19.07	± 17.07	± 7.72	± 5.59	± 4.41	± 3.65

^a/Figures include 0.75 lb. dehydrated alfalfa meal.

^b/All of above means were compared within each variable using Duncan's New Multiple Range Test. None of the means differed significantly at the 5% level with the exception of the calorie: protein ratio means with each differed significantly from the other.

^c/Calorie ratio = $\frac{\text{Kcal. digestible energy/lb.}}{\text{Crude protein \%}}$

TABLE 6
ANALYSIS OF VARIANCE FOR FEED CONSUMPTION, NITROGEN BALANCE
AND EXCRETION AND DIGESTION TRIAL DATA

Source of Variation	d.f.	Mean Squares							Calorie: Protein Ratios
		Feed Consumption	Nitrogen Balance	Total N Excretion	Urea Excretion	Cellulose Dig.	Protein Dig.	Energy Dig.	
Total	8								
Periods	2	0.42	11.29	38.48 ^f	16.59	73.27	130.79	166.37	244.00 ^f
Ponies	2	0.57	14.50	77.71 ^f	44.33 ^f	367.16	513.74 ^f	341.03 ^f	279.00 ^e
Treatments	2	0.11	11.62	12.23	3.56	179.05	51.77	192.73 ^f	1828.00 ^c
Error	2	0.23	68.02	10.91	8.74	178.72	93.74	58.40	40.00

a/P < 0.005

d/P < 0.05

b/P < 0.01

e/P < 0.10

c/P < 0.025

f/P < 0.25

additional factor that complicates the interpretation of these data was the inclusion of the alfalfa meal in the daily ration allowance of each animal.

The lack of a significant difference in the apparent utilization of the urea diet as compared to the casein and corn gluten meal diets has some interesting implications. This suggests that, under the conditions of this experiment, urea is being retained and utilized to some extent. Apparently, it is reaching the cecum and/or colon in some manner, thereby providing the microflora with a nitrogen source. This, in turn, enables the bacteria to maintain their cellulolytic activity. It is unlikely that the small amount of crude protein from the dehydrated alfalfa meal (viz. about 0.11 lb. per head per day) would supply sufficient nitrogen to do this by itself. The National Research Council (1966) states that a 400 lb. mature horse should receive 0.44 lb. of total protein per day.

Urea retention in the body is indicated by the observation that less than one-half of that ingested per day, appeared in the urine. The average feed consumption of the ponies receiving the urea diet was 2.75 lb. per day (3.25 lb. total feed less 0.75 lb. alfalfa meal). The 2.75 lb. contained approximately 45 gm. of urea while only about 22 gm. of urea was excreted per day in the urine.

In spite of the fact that casein is a more soluble protein and has a higher biological value than corn gluten meal, no significant differences were found in nitrogen balance or in coefficients of apparent digestibility (Tables 5 and 6, pages 52 and 53). This suggests that, within the parameters and under the conditions of this experiment, these two protein sources do not differ in value for mature ponies.

Cecal Fluid Measurements

The average cecal concentrations of ammonia nitrogen, urea nitrogen, residual NPN (defined as total nonprotein nitrogen less ammonia and urea nitrogen) and the protein nitrogen are presented in Table 7, page 56 and Figures 2 through 5, pages 57 through 60. The least-squares analysis of variance for these variables are reported in Tables 8 and 9, pages 61 and 62. The least-squares means were compared ($P < .05$) for all treatments within each sampling time and over all sampling times, using Duncan's New Multiple Range Test (Steel and Torrie, 1960).

Ammonia: Ammonia nitrogen levels ranged from a low of 2.0 mg. per 100 ml. of cecal fluid to a high of 13.8 mg. per 100 ml.. Least-squares analysis of variance (Table 8, page 61) showed sample time to be statistically different ($P < .05$), which indicates a variation in concentration with time after feeding. The periods by sample time interaction differed at the 25 per cent level of significance. The ponies by sample time interaction was also significantly different ($P < .005$), indicating a variation in the response of ponies regardless of treatment. The treatments by sample time interaction was different at the 10 per cent level. The treatment means showed the cecal ammonia nitrogen concentration to be greater ($P < .05$) with the casein treatment than with either the corn gluten meal or urea treatment.

A step-wise polynomial regression analysis was conducted from the first through fourth degree (Harvey, 1968) on the mean postprandial cecal ammonia nitrogen concentrations. This was done to determine the

TABLE 7
AVERAGE CECAL CONCENTRATIONS BY TREATMENTS
(LEAST-SQUARES MEANS)

Treatment	Sample ^a Time	Ammonia Nitrogen (mg./100ml.)	Urea Nitrogen (mg./100ml.)	Residual NPN (mg./100ml.)	Protein Nitrogen (mg./100ml.)
Casein diet	0	9.2	1.1	28.9	50.3
	0.5	10.4 ^c	1.9	39.4	33.0
	1	10.8 ^d	1.7	38.5	35.6
	3	8.9	3.8	54.1	21.4
	6	5.0	3.1	44.6	25.3
Treatment Means & S.E. _{$\bar{x}$}		8.9±0.44 ^e	2.3±0.23	41.1±2.67 ^f	33.1±3.95
Corn gluten meal diet	0	5.3 ^b	0.6	32.1	45.0
	0.5	5.5	1.1	31.7	38.1
	1	7.2	0.8	36.9	43.2
	3	6.1	2.3	47.3	30.8
	6	4.8	2.9	38.3	38.1
Treatment Means & S.E. _{$\bar{x}$}		5.8±0.44	1.6±0.23	37.3±2.67	39.0±3.95
Urea diet	0	8.8	1.4	32.4	42.0
	0.5	7.1	1.3	23.9	51.8
	1	8.3	1.7	27.0	54.8
	3	6.6	3.0	35.5	54.8
	6	5.0	2.5	29.0	46.7
Treatment Means & S.E. _{$\bar{x}$}		7.2±0.44	2.0±0.23	29.6±2.67	50.0±3.95 ^g
S.E. (Treat.XSample Time)		±0.99	±0.51	±5.97	±8.85

^a/Hours after feeding.

^b/Differs significantly ($P < .05$) from other treatments at time 0.

^c/Differs significantly ($P < .05$) from other treatments at time 0.5.

^d/Differs significantly ($P < .05$) from corn gluten meal diet, but not from the urea diet at time 1.

^{e/f}/Differ significantly ($P < .05$) from both the corn gluten meal and urea diets.

^g/Differs significantly ($P < .05$) from the casein and corn gluten meal diets.

FIG. 2. - AVERAGE CECAL CONCENTRATIONS OF AMMONIA NITROGEN BY TREATMENTS

FIG. 3. - AVERAGE CECAL CONCENTRATIONS OF UREA NITROGEN BY TREATMENTS

FIG. 5.—AVERAGE CECAL CONCENTRATIONS OF PROTEIN NITROGEN BY TREATMENTS

TABLE 8
LEAST-SQUARES ANALYSIS OF VARIANCE ACROSS
ALL TREATMENTS FOR CECAL CONTENTS

Source of Variation	d.f.	Mean Squares			
		Ammonia Nitrogen	Urea Nitrogen	Residual NPN	Protein Nitrogen
Total	45				
Periods	2	4.956	2.731	131.244	1528.982 ^f
Ponies	2	50.215 ^f	3.777	207.356 ^f	2286.668 ^f
Treatments	2	35.417 ^f	2.096	520.072 ^e	1103.467 ^f
Error A	2	10.348	2.018	43.906	301.188
Sample Time(ST)	4	18.254 ^a	7.444 ^a	317.064 ^f	183.792
Periods X ST	8	2.717 ^f	1.494 ^e	156.841	400.224 ^f
Ponies X ST	8	9.419 ^a	1.469 ^e	54.751	186.546
Treatments X ST	8	3.433 ^e	0.361	60.699	188.850
Error B	8	1.111	0.470	122.708	218.620

a/P < 0.005

b/P < 0.01

c/P < 0.025

d/P < 0.05

e/P < 0.10

f/P < 0.25

TABLE 9

LEAST-SQUARES ANALYSIS OF VARIANCE FOR NESTED CECAL DATA

Source of Variation	d.f.	Mean Squares			
		Ammonia Nitrogen	Urea Nitrogen	Residual NPN	Protein Nitrogen
Total	45				
Periods	2	4.956	2.731	131.244	1528.982 ^f
Ponies	2	50.215 ^f	3.777	207.356 ^f	2286.668 ^f
Treatments	2	35.417 ^f	2.096	520.072 ^e	1103.467 ^f
ST/Casein	4	15.768 ^a	3.496 ^b	256.028 ^f	376.006 ^f
Linear	1	50.918 ^a	8.127 ^a	398.400 ^f	788.833 ^e
Quadratic	1	8.234 ^d	4.648 ^a	563.567 ^e	516.925 ^f
Cubic	1	3.909 ^e	0.570	1.265	40.223
Quartic	1	0.0120	0.638	60.878	158.042
ST/CGM	4	2.455 ^f	3.109 ^c	120.444	91.369
Linear	1	1.637	11.433 ^a	137.934	87.758
Quadratic	1	3.958 ^e	0.403	302.292 ^f	173.469
Cubic	1	2.299 ^f	0.216	29.601	21.175
Quartic	1	1.926 ^f	0.384	11.950	83.075
ST/Urea	4	6.897 ^c	1.562 ^e	61.989	94.116
Linear	1	23.087 ^a	3.637 ^c	9.834	0.001
Quadratic	1	0.005	2.096 ^e	34.578	273.659
Cubic	1	0.111	0.473	165.870	96.106
Quartic	1	4.387 ^e	0.040	37.675	6.698
Periods X ST	8	2.717 ^f	1.494 ^e	156.841	400.224 ^f
Ponies X ST	8	9.419 ^a	1.469 ^e	54.751	186.546
Error	8	1.111	0.470	122.708	218.620

a/P < 0.005b/P < 0.01c/P < 0.025d/P < 0.05e/P < 0.10f/P < 0.25

TABLE 10

LISTING OF POLYNOMIAL REGRESSIONS AND STANDARD ERRORS FOR CECAL VARIABLES

Treatment	Variable	Standard Error	Significant Polynomial	Non-orthogonal regression coefficients				
				B ₀	B ₁	B ₂	B ₃	B ₄
Casein diet	Ammonia Nitrogen	0.00920	Cubic P .10	10.01449	-0.62886	-0.14147	0.01727	
	Urea Nitrogen	0.01307	Quadratic P .025	2.74024	0.32138	-0.04110		
	Residual NPN	0.21119	Quadratic P .10	44.73788	2.86797	-0.45260		
	Protein Nitrogen	0.86765	Linear P .10	40.72222	-1.64814			
	Ammonia Nitrogen	0.02009	Quadratic P .10	8.00992	0.06713	-0.03793		
Corn gluten meal diet	Urea Nitrogen	0.04002	Linear P .005	1.94444	0.19842			
	Residual NPN		N.S. ^b					
	Protein Nitrogen		N.S.					
	Ammonia ^a	0.01684	Linear P .005	7.27556	-0.28196			
Urea diet	Nitrogen	0.01410	Quartic P .10	11.18092	-0.40177	-1.07941	-0.08643	0.02802
	Urea Nitrogen	0.01307	Quadratic P .10	2.47880	0.21538	-0.02760		
	Residual NPN		N.S.					
	Protein Nitrogen		N.S.					

^a/Quadratic polynomial drops below zero. When plotted; disregarded.

^b/N.S. = Not significant ($P < .10$)

regression coefficients which can be used to predict the curve which best fits the data. The generalized form of the prediction equation used in this analysis was:

$$Y = B_0 + B_1 (X - \bar{X})^1 + B_2 (X - \bar{X})^2 + B_3 (X - \bar{X})^3 + B_4 (X - \bar{X})^4$$

The term X in this equation is equal to sampling time. The postprandial sample times were coded as follows: (1) 0 hr. = 1, (2) 0.5 hr. = 2, (3) 1 hr. = 3, (4) 3 hr. = 7, and (5) 6 hr. = 13. Therefore, in this case, $\bar{X} = 5.2$

The results of the least-squares analysis of variance with treatments nest within samples times are presented in Table 9, page 62. The listing of the significant ($P < .10$) non-orthogonal polynomial regression coefficients are reported in Table 10, page 63. Cecal ammonia nitrogen was significantly different with different sample times for the casein treatment ($P < .005$), for the corn gluten meal treatment ($P < .25$), and for the urea treatment ($P < .025$). The third degree polynomial (cubic) was significant ($P < .10$) for the casein ration. The second degree (quadratic) was significant ($P < .10$) for the corn gluten meal ration. Although the fourth degree polynomial (quartic) was significant ($P < .10$) for the urea ration, it was disregarded because it was not physiologically possible. When the regression coefficients for this polynomial were plotted, the curve went below 0 mg. per 100 ml.. Because of this, it was decided that the first degree (linear) best described the data.

The changes in cecal ammonia nitrogen are graphically presented in Figure 2, page 57, using the least-squares means. Duncan's New Multiple

Range Test (Steel and Torrie, 1960) showed that ammonia nitrogen levels of the corn gluten meal treatment to be significantly lower ($P \leq .05$) than those of either the casein or urea treatments at the time zero. The casein treatment showed higher ($P \leq .05$) levels than the other treatments at one-half hour. At one hour after feeding, the ammonia level with the casein ration remained higher ($P \leq .05$) than with the corn gluten meal ration. The difference between the casein and urea treatments at one hour were not significantly different ($P \leq .05$).

The cecal concentration of ammonia nitrogen reached its highest level (10.8 mg. per 100 ml.) at one hour postprandial with the casein treatment. This implies that not all the protein, despite its high solubility, was hydrolyzed and absorbed in the small intestine. The ammonia concentration peaks at one hour after feeding with all treatments. This illustrates the rapid rate of passage from mouth to cecum in the pony.

The ammonia level in the ceca of the ponies receiving the urea ration appeared to decline with time after feeding. This suggests that the urea is not reaching the cecum, probably as a result of being absorbed from the small intestine. The linear decline in ammonia nitrogen per 100 ml. may also be partially due to the entrance of the cellulose portion of the diet into the cecum.

In spite of the higher crude protein level (17.4 per cent) of the corn gluten meal diet as compared to the casein (14.7 per cent) and urea (12.2 per cent) diets, the cecal ammonia levels with the corn gluten meal diet were consistently lower over the period sampled. This is consistent with work with ruminants (McDonald, 1954; McDonald and

Hall, 1957) which indicates that a less soluble protein (e.g. corn gluten meal) will be hydrolyzed more slowly by the rumen microorganisms than a readily soluble protein (e.g. casein). Lichtenwalner and Fontenot (1968) observed in cattle that rumen fluid ammonia levels reflect the availability of nitrogen in a dietary protein (or nitrogen) source. These workers reported that rumen ammonia levels were the highest with urea containing rations, lowest with corn gluten meal containing rations, and intermediate with soybean meal containing rations.

Urea: The average cecal concentrations of urea nitrogen ranged from 0.8 to 3.8 mg. per 100 ml. of cecal fluid (Table 7, page 56). No significant differences were found between treatments when compared within specific sampling times. Cecal urea peaked at three hours after feeding for both the casein and urea diets, whereas, it peaked at six hours postprandial for the corn gluten meal diet (Figure 3, page 58). Urea nitrogen levels differed ($P < .005$) between sample time-treatment means. Both the period by sampling time and ponies by sampling time interactions differed at the 10 per cent level of significance.

The step-wise polynomial regression analysis indicated that the urea nitrogen concentration differed over sampling time for the casein diet ($P < .01$) and at the 25 per cent level for both the corn gluten meal and urea diets. The quadratic curve was significant for the cecal urea nitrogen levels of the casein ($P < .005$) and the urea ($P < .10$) treatments. However, the corn gluten meal treatment resulted in a linear ($P < .005$) increase over the six hour sampling period. For a graphic representation of this data, refer to Figure 3, page 58.

Residual Nonprotein Nitrogen: This measurement was made on the supernatant of the 10 per cent tungstic acid precipitated cecal fluid. Total nitrogen was determined in this protein-free fraction and then the ammonia and urea nitrogen were deducted (referred to as residual nonprotein nitrogen or residual NPN). This fraction should contain free amino acids, nucleotide nitrogen, small peptides, and any other nitrogenous compounds not precipitated as protein. The least-squares analysis of variance (Table 8, page 61) indicated a difference when this variable was tested across treatments ($P \leq .10$) and across ponies ($P \leq .25$). This analysis failed to show any significant differences when treatments were compared within specific sampling times. However, the treatment mean for the casein diet differed ($P \leq .05$) from those of the corn gluten meal and urea diets (Table 7, page 56).

The step-wise polynomial regression analysis showed the quadratic function to best describe the data for the casein ($P \leq .10$) treatment (Table 9, page 62). There was an indication ($P \leq .25$) that the corn gluten meal treatment data also fit the quadratic curve. No degree of polynomial regression was found to be significant for the urea treatment. Periods by sample time and ponies by sample time interactions were not different at the levels of significance tested. These data are presented graphically in Figure 4, page 59. There was a trend for the casein diet to result in the highest levels of residual NPN, followed by the corn gluten meal and urea diets, respectively. The higher levels could be due to: (1) an increased rate of proteolysis, (2) a decreased rate of microbial protein synthesis, (3) a reduced rate of absorption from the cecum, or (4) a combination of these factors.

Protein Nitrogen: No differences were found between treatments within specific sampling times at the levels of significance tested (Table 9, page 62). The treatment mean for protein nitrogen, was higher ($P < .05$) for the urea diet as compared to the other two treatments (Table 7, page 56). This implies a stimulation of microbial protein synthesis, possibly via urea recycling in the blood supplying the cecum. Virtanen (1966) indicated that bacterial protein synthesis was increased in the rumen of cows fed urea containing diets. Phillipson (1964) reported that urea recycling occurs in both ruminants and nonruminants. As previously noted in the results of the nitrogen balance study, a portion of the ingested urea appeared to be retained in the body. In addition, the total nonprotein nitrogen levels tended to be lowest in the ceca of the ponies receiving the urea containing ration. These observations would further support the hypothesis of increased microbial protein synthesis in the cecum.

Least-squares analysis of variance showed periods, ponies and treatments to each be different at the 25 per cent level (Table 8, page 61). The polynomial regression analysis indicated that the linear curve best described the data for the casein treatment ($P < .10$). The data for the corn gluten meal and urea treatments appeared to be randomly distributed through the fourth degree (Table 9, page 62). The periods by sample time interaction was significant at the 25 per cent level. For a graphic representation of these data refer to Figure 5, page 60.

Blood Plasma Measurements

The average blood plasma concentrations of ammonia nitrogen, urea nitrogen, and residual NPN are presented in Table 11, page 70. The least-squares means are presented graphically in Figures 6, 7, and 8, pages 71 through 73. These means were compared for all treatments within each sampling time and over all sampling times using Duncan's New Multiple Range Test (Steel and Torrie, 1960) at the 5 per cent level of significance. The least-squares analysis of variance is reported in Table 12, page 74. A step-wise polynomial regression analysis was conducted from the first through the fifth degree (Table 13, page 75). A listing of the significant ($P < .10$) polynomial regressions is given in Table 14, page 76.

Ammonia: Plasma ammonia nitrogen levels in the jugular vein varied very little regardless of dietary treatment. These values ranged from a low of 0.23 mg. of ammonia nitrogen per 100 ml. to a high of 0.34 mg. per 100 ml.. Least-squares analysis of variance failed to show any significant differences due to treatment within specific sampling times (Table 12, page 74). However, the overall treatment mean of plasma ammonia nitrogen for the urea treatment was significantly lower ($P < .05$) than for the casein or corn gluten meal treatments. The analysis of variance indicated that periods were different ($P < .10$) and that ponies differed at the 25 per cent level.

Sample time and the periods by sample time and ponies by sample time interactions were all different ($P < .10$). The step-wise polynomial regression indicated that the quadratic curve best described ($P < .05$)

TABLE 11
AVERAGE BLOOD PLASMA CONCENTRATIONS BY TREATMENTS
(LEAST-SQUARES MEANS)

Treatment	Sample ^a Time	Ammonia Nitrogen (mg./100 ml.)	Urea Nitrogen (mg./100 ml.)	Residual NPN (mg./100 ml.)
Casein diet	0	0.27	13.5	74.4
	1	0.26	13.9	32.9
	2	0.31	14.3	52.6
	3	0.32	13.9	32.4
	4	0.34	14.0	41.1
	5	0.24	13.6	38.1
	6	0.25	13.8	52.6
Treatment Means & S.E. $\bar{x}$		0.28 $\pm$ 0.01	13.8 $\pm$ 0.77	45.7 $\pm$ 7.73
Corn, gluten meal diet	0	0.30	15.7	27.3
	1	0.24	16.2	28.7
	2	0.26	15.5	46.8
	3	0.26	16.0	34.1
	4	0.33	14.5	31.4
	5	0.26	13.8	35.7
	6	0.28	13.7	43.4
Treatment Means & S.E. $\bar{x}$		0.28 $\pm$ 0.01	15.1 $\pm$ 0.77	35.3 $\pm$ 7.73
Urea diet	0	0.23	15.4	68.5
	1	0.25	16.0	25.6
	2	0.26	16.3	36.4
	3	0.26	16.7	33.7
	4	0.28	17.0	32.3
	5	0.26	13.8	35.7
	6	0.29	16.5	24.4
Treatment Means & S.E. $\bar{x}$		0.26 $\pm$ 0.01 ^b	16.7 $\pm$ 0.77 ^c	38.4 $\pm$ 7.73
S.E. (Treat.XSample Time)		$\pm$ 0.03	$\pm$ 2.03	$\pm$ 20.45

^a/Hours after feeding.

^b/_{,c}/Differ significantly ($P < .05$) from the casein and corn
gluten meal treatment means.

FIG. 6. AVERAGE BLOOD PLASMA CONCENTRATIONS OF AMMONIA NITROGEN BY TREATMENTS

FIG. 7. AVERAGE BLOOD PLASMA CONCENTRATIONS OF UREA NITROGEN BY TREATMENTS

FIG. 8. AVERAGE BLOOD PLASMA CONCENTRATIONS OF RESIDUAL NONPROTEIN

NITROGEN

TABLE 12
LEAST-SQUARES ANALYSIS OF VARIANCE
FOR BLOOD PLASMA

Source of Variation	d.f.	Mean Squares		
		Ammonia Nitrogen	Urea Nitrogen	Residual NPN
Total	63			
Periods	2	0.072 ^e	87.622	895.223
Ponies	2	0.018 ^f	325.413 ^f	667.986
Treatments	2	0.002	35.155	730.043
Error A	2	0.005	76.968	2222.611
Sample Time (ST)	6	0.004	0.903	798.133
Periods X ST	12	0.004	0.304	576.649
Ponies X ST	12	0.004	0.672	618.885
Treatments X ST	12	0.002	1.734	416.132
Error B	12	0.002	1.526	1092.960

a/P < 0.005

b/P < 0.01

c/P < 0.025

d/P < 0.05

e/P < 0.10

f/P < 0.25

TABLE 13
LEAST-SQUARES ANALYSIS OF VARIANCE FOR NESTED PLASMA DATA

Source of Variation	d.f.	Mean Squares		
		Ammonia Nitrogen	Urea Nitrogen	Residual NPN
Total	63			
Periods	2	0.072 ^e	87.622	895.223
Ponies	2	0.018 ^f	325.413 ^f	667.986
Treatments	2	0.002	35.155	730.043
ST/Casein	6	0.005 ^e	0.191	756.531
Linear	1	0.001	0.004	612.360
Quadratic	1	0.016 ^b	0.463	2043.451 ^f
Cubic	1	0.001	0.376	173.601
Quartic	1	0.008 ^d	0.028	637.496
Quintic	1	0.002	0.000	379.874
Residual	1	0.002	0.275	692.400
ST/CGM	6	0.003 ^f	3.169 ^f	161.725
Linear	1	0.001	14.334 ^b	236.679
Quadratic	1	0.001	1.834	11.063
Cubic	1	0.006	0.934	299.309
Quartic	1	0.003 ^f	0.328	37.943
Quintic	1	0.002	0.200	285.016
Residual	1	0.004 ^f	1.382	100.343
ST/Urea	6	0.001	1.011	712.141
Linear	1	0.007 ^e	3.943 ^f	898.334
Quadratic	1	0.000	1.750	545.900
Cubic	1	0.000	0.245	1953.125 ^f
Quartic	1	0.000	0.128	22.830
Quintic	1	0.000	0.000	852.509
Residual	1	0.000	0.000	0.149
Periods X ST	12	0.004 ^e	0.304	576.649
Ponies X ST	12	0.004 ^e	0.672	618.885
Error	12	0.002	1.526	1092.960

^a/P < 0.005; ^b/P < 0.01; ^c/P < 0.025; ^d/P < 0.05; ^e/P < 0.10; ^f/P < 0.25

TABLE 14
LISTING OF POLYNOMIAL REGRESSIONS AND STANDARD ERRORS
FOR BLOOD PLASMA VAIRABLES^a

Treatment	Variable	Standard Error	Significant Polynomial	Non-orthogonal regression coefficients				
				B ₀	B ₁	B ₂	B ₃	B ₄
Casein diet	Ammonia Nitrogen	0.00109	Quartic P .05	0.33153	0.00333	-0.03090	-0.00083	0.00239
	Urea Nitrogen		N.S. ^b					
	Residual NPN		N.S.					
Corn gluten meal diet	Ammonia Nitrogen	0.00158	Cubic P .10	0.26873	0.02377	0.00151	-0.00306	
	Urea Nitrogen	0.13479	Linear P .01	15.11111	-0.41310			
	Residual NPN		N.S.					
Urea diet	Ammonia Nitrogen	0.00438	Linear P .10	0.27476	0.00893			
	Urea Nitrogen		N.S.					
	Residual NPN		N.S.					

^a/Sample times coded as 1 through 7 = $\bar{X} = 3$.

^b/N.S. = Not significant ($P \geq .10$).

the pattern of plasma ammonia nitrogen due to the casein treatment. The cubic function was significant ($P \leq .10$) for the corn gluten meal treatment, while the linear curve best fit the data from the urea treatment. It is interesting to note that the urea ration did not result in a significant rise in the peripheral blood ammonia levels over those of the other two diets.

Urea: The plasma urea nitrogen levels ranged from 13.5 to 17.1 mg. per 100 ml. (Table 11, page 70). This falls within the range of 12.1 to 23.8 mg. per 100 ml. of plasma as determined by Fennesbeck and Symons (1969) in their work with mature horses. No significant differences were found between treatments when they were compared within each of the sample times. However, the treatment mean for the urea ration was significantly ($P \leq .05$) higher than the means of the other two treatments. Fennesbeck and Symons (1969) cited work which indicated that the major causes of high plasma urea concentrations are: (1) protein intake in excess of the body's requirement, (2) low protein quality, (3) ingestion and absorption of urea, and (4) renal failure. These workers suggested that the plasma urea concentration may indicate the extent to which the amino acids from the ingested protein are wasted by the body. It is quite possible that the higher crude protein levels of the casein and corn gluten meal diets (14.7 and 17.4 per cent, respectively) as compared to the urea diet (12.2 per cent) and the poor protein quality of the corn gluten meal may have masked the presence of some additional real differences in blood urea nitrogen in this experiment.

Least-squares analysis of variance (Table 12, page 74) indicated ($P \leq .25$) that there was more variation due to ponies than from

treatments for this variable. The polynomial regression analysis (Table 13, page 75) showed the linear curve to be significant ($P \leq .01$) for the corn gluten meal treatment. The trend ($P \leq .25$) was for the linear curve to also be significant for the urea treatment. The casein treatment appeared to follow a random distribution as none of the functions tested proved to be significant. These data are presented in graphic form in Figure 7, page 72.

Residual NPN: The nonprotein nitrogen fraction of the blood includes all of the nitrogen containing constituents of the blood which are not precipitated as protein. Included in this fraction of blood plasma are urea, ammonia, uric acid, creatinine, creatine, amino acids, glutathione, and other compounds in small amounts, some of which are of unknown structure (Oser, 1965). The residual NPN fraction of the blood plasma was determined to investigate the effect of dietary nitrogen source on this fraction.

No significant differences were observed for this variable when treatments were compared within each sampling time or across treatment means (Tables 11 and 12, pages 70 and 74). The step-wise polynomial regression analysis suggested ($P \leq .25$) that the quadratic curve best fit the data for the casein treatment, while the cubic curve was significant ($P \leq .25$) for the urea treatment. These data are graphed in Figure 8, page 73. It is concluded that the determination of this plasma fraction was of little value in evaluating the effects of the dietary treatments employed in this experiment.

Comparison of Blood Plasma From the Jugular Vein and Lateral Cecal Vein

As noted in the Materials and Methods section, these data were obtained from a fourth pony stallion receiving the casein diet and were treated as a separate experiment. Comparisons are made between two sampling sites, the jugular vein and lateral cecal vein (Tables 15 and 16, pages 80 and 84 and Figures 9, 10, and 11, pages 81 through 83).

Ammonia: Least-squares analysis of variance showed sampling site to be significant ($P < .005$) for ammonia nitrogen (Table 16, page 84). Plasma ammonia values were about three times as great in the lateral cecal vein as compared to the jugular vein (Figure 9, page 81). The concentration of plasma ammonia in the cecal vein ranged from 0.98 to 1.60 mg. per 100 ml., while that of the jugular vein ranged from 0.30 to 0.45 mg. per 100 ml.. The higher levels of ammonia in the cecal vein are very indicative of ammonia absorption from the cecum. The lower levels measured in the jugular plasma illustrate the liver's ability to convert this ammonia to urea, which is less toxic to the animal.

Urea: Least-squares analysis of variance failed to show any significant difference ($P < .25$) in plasma urea nitrogen due to sampling site. The mean concentrations over all sampling times were almost identical for both sites, 7.33 mg. per 100 ml. for the jugular vein and 7.26 mg. per 100 ml. for the cecal vein (Table 15, page 80). It would be very interesting to know the urea nitrogen level in the arterial

TABLE 15
COMPARISON OF BLOOD PLASMA FROM THE JUGULAR VEIN
AND THE LATERAL CECAL VEIN (CASEIN DIET)

Sample ^a Time	Ammonia Nitrogen (mg./100 ml.)		Urea Nitrogen (mg./100 ml.)		Residual NPN (mg./100 ml.)	
	Jugular Vein	Cecal Vein	Jugular Vein	Cecal Vein	Jugular Vein	Cecal Vein
0	0.39	0.98	6.5	6.0	12.7	30.7
1	0.32	0.88	7.5	6.5	16.3	139.3
2	0.45	1.14	6.5	6.8	15.6	28.6
3	0.38	1.15	7.5	7.0	9.7	21.4
4	0.36	1.02	7.3	9.0	47.6	6.8
5	0.30	1.09	8.5	7.0	7.3	0.9
6	0.40	1.60	7.5	8.5	6.0	33.4
Mean	0.37	1.12	7.3	7.3	16.5	37.3

^a/Hours after feeding.

FIG. 9. COMPARISON OF AMMONIA NITROGEN LEVELS IN BLOOD PLASMA FROM THE JUGULAR VEIN AND LATERAL CECAL VEIN

FIG. 10. COMPARISON OF UREA NITROGEN IN BLOOD PLASMA FROM THE JUGULAR VEIN AND THE LATERAL CECAL VEIN

FIG. 11. COMPARISON OF RESIDUAL NONPROTEIN NITROGEN IN BLOOD PLASMA FROM THE JUGULAR VEIN AND THE LATERAL CECAL VEIN

TABLE 16
LEAST-SQUARES ANALYSIS OF VARIANCE FOR JUGULAR
VEIN VS. LATERAL CECAL VEIN

Source of Variation	d.f.	Mean Squares		
		Ammonia Nitrogen	Urea Nitrogen	Residual NPN
Total	15			
Sample Site	1	1.976 ^a	0.018	1520.486
Sample Time	6	0.033	1.011	1113.826
Linear	1	0.076	4.862 ^d	1570.921
Quadratic	1	0.020	0.110	0.229
Cubic	1	0.035	0.083	1478.520
Quartic	1	0.055 ^f	0.063	1220.029
Quintic	1	0.008	0.930	2412.259 ^f
Residual	1	0.002	0.016	1.001
Error	6	0.023	0.641	1264.523

a/P < 0.005

b/P < 0.01

c/P < 0.025

d/P < 0.05

e/P < 0.10

f/P < 0.25

blood supplying the cecum. This would provide useful information in evaluating the extent of urea recycling in the horse.

Residual NPN: Least-squares analysis of variance also failed to show any significant difference ($P < .25$) for this plasma fraction due to sampling site (Table 16, page 84). The concentration varied from 0.9 to 139.3 mg. per 100 ml. of plasma in the cecal vein and from 6.0 to 47.6 in the jugular vein. The distribution of these values appeared to be random, although the polynomial regression analysis (Table 16, page 84) suggested ($P < .25$) that the data described a quintic function. These data are graphed in Figure 11, page 83.

As previously discussed, this measurement appears to be of little value in evaluating the protein nutrition of mature ponies. A more appropriate measurement would no doubt be that of alpha-amino nitrogen for estimation of free amino acids. In terms of a comparison of the peripheral circulation with the cecal drainage, this measurement would provide useful information concerning amino acid absorption from the cecum.

SUMMARY

A 3 x 3 split-split plot Latin square design was employed to measure the effects of dietary nitrogen source on mature Shetland pony stallions with permanent cecal fistulas (average weight, 332 pounds). The ponies were randomly allotted to each of three pelleted, semi-purified diets which contained casein, corn gluten meal, and urea as the nitrogen sources. Jugular blood and cecal contents were sampled in conjunction with nitrogen balance and digestion trials.

The effects of nitrogen source on the following variables were measured: (1) nitrogen balance, (2) coefficients of apparent digestibility for crude protein, cellulose, and energy, (3) urinary urea and total urinary nitrogen, (4) cecal ammonia, urea, protein, and residual nonprotein nitrogen (defined as total nonprotein nitrogen less ammonia and urea nitrogen), and (5) blood plasma ammonia, urea, and residual nonprotein nitrogen (NPN).

Nitrogen Balance and Digestion Trials

The results of the digestion and nitrogen balance trials failed to show any significant differences due to treatment for feed intake, nitrogen balance, total nitrogen excretion, urea excretion or in the apparent digestibilities of cellulose, protein, or energy. It appeared, under the conditions of this experiment, that the mature pony was able to utilize urea as a nonprotein nitrogen source in maintaining an active intestinal microflora. In addition, both casein and corn gluten meal seemed to be utilized equally as well.

Cecal Fluid Measurements

Measurements of cecal ammonia nitrogen ranged from 2.0 to 13.8 mg. per 100 ml. of strained cecal fluid. Ammonia levels were found to differ significantly ($P < .05$) with sample time after feeding. The ponies by sample time interaction was also significantly different ($P < .005$), indicating a variation in the response of ponies regardless of treatment. The treatment means showed cecal ammonia nitrogen to be greatest ($P < .05$) with the casein treatment. The ammonia concentration peaked at one hour after feeding for all treatments. The data from this experiment suggest that little, if any, of the dietary urea reached the cecum, probably as a result of being absorbed from the small intestine. The corn gluten meal protein appeared to be hydrolyzed more slowly in the cecum as indicated by consistently lower cecal ammonia levels.

The average cecal concentrations of urea nitrogen ranged from 0.8 to 3.8 mg. per 100 ml.. No significant differences in cecal urea concentrations were found between treatments when compared within individual sampling times. Cecal urea level peaked at three hours after feeding for both the casein and urea treatments, whereas it peaked at six hours postprandial with corn gluten meal.

There was a trend for the residual NPN in the cecum to be the highest with the casein diet and the lowest with the urea diet. These higher residual NPN levels could be due to: (1) an increased rate of proteolysis, (2) a decreased rate of microbial protein synthesis, (3) a reduced rate of absorption, or (4) a combination of these factors.

The urea diet resulted in a higher ($P \leq .05$) treatment mean for cecal protein nitrogen when compared to the other two treatments. This implies a stimulation of microbial protein synthesis, possibly via urea recycling in the blood supplying the cecum.

Blood Plasma Measurements

Plasma ammonia nitrogen levels in the jugular vein varied very little regardless of dietary treatment. The ammonia levels ranged from 0.23 to 0.34 mg. per 100 ml. of plasma. The urea treatment did not result in a significant rise in peripheral blood ammonia levels as compared to the other two treatments. In fact, the overall treatment mean of plasma ammonia for the urea diet was significantly lower ($P \leq .05$) than for the casein or corn gluten meal treatments.

The urea nitrogen levels ranged from 13.5 to 17.1 mg. per 100 ml. of plasma. The urea diet resulted in higher ($P \leq .05$) mean plasma urea levels than either the corn gluten meal or casein diets. However, no significant differences were found between treatments when plasma urea levels were compared on the basis of individual sample times. Least-squares analysis of variance indicated ($P \leq .25$) that there was more variation due to ponies than treatments.

No significant differences were found in the plasma residual NPN fraction when treatments were compared within each sampling time or across treatment means. It is concluded that the determination of this plasma fraction was of little value in evaluating the effects of dietary nitrogen source on mature ponies in this experiment.

Comparison of Blood Plasma From the Jugular Vein and Lateral Cecal Vein

Data are also included from a fourth mature Shetland pony stallion fitted with a permanent indwelling catheter in the lateral cecal vein. Comparisons are made between blood samples taken from the jugular vein and cecal vein for ammonia, urea, and residual NPN while this pony was consuming the casein-containing diet. The procedures employed in the surgical installation of cecal vessel catheters are also discussed in the Appendix.

Plasma ammonia nitrogen values were found to be about three times as great in the lateral cecal vein as compared to the jugular vein. The concentration of plasma ammonia in the cecal vein ranged from 0.98 to 1.60 mg. per 100 ml., while that of the jugular vein ranged from 0.30 to 0.45 mg. per 100 ml.. The higher ammonia levels in the cecal vein are very indicative of ammonia absorption from the cecum.

Least-squares analysis of variance failed to show any significant differences in plasma urea nitrogen or residual NPN due to sampling site. The mean urea nitrogen concentrations over all sampling times were almost identical for both the jugular and cecal veins, being 7.33 vs. 7.26 mg. per 100 ml. of plasma, respectively.

APPENDIX

Cannulation of Cecal Vessels

The original objectives of this research project included an estimation of free amino acid absorption from the cecum and of urea recycling in the blood supply to the cecum. The proposed approach involved obtaining blood samples from the lateral cecal artery and lateral cecal vein. In order to accomplish this, it was necessary to establish permanent indwelling catheters¹ in the cecal vessels. The surgery was performed by Dr. Donald R. Redman² with the author serving as his assistant. Dr. Redman had previously performed the surgery to install the cecal fistulas in the ponies used in this study using the procedure described by Linerode (1966).

Linerode (1966) had attempted to install similar catheters in the cecal vessels without success. The main problem he encountered was an inability to anchor the catheters once they were in place in the vessels. This resulted in the catheters coming out of the vessels before the peritoneum could be sutured closed. Due to this problem, Linerode abandoned the use of the Longdwell catheters and used polyethylene tubing (P.E. 160)³ instead. He anchored this tubing by placing a suture around each vessel and catheter about 1.25 inches from the point of entry. In

¹Longdwell Catheters (teflon). Made by Becton, Dickinson & Co., Rutherford, New Jersey.

²Assistant Professor in the Department of Veterinary Science, Ohio Agricultural Research & Development Center, Wooster, Ohio.

³P.E. 160 refers to intramedic polyethylene tubing with an outside diameter of 160 mm. Produced by Clay Adams, Inc., New York, New York.

addition to this, a simple overhand knot was loosely tied in the tubing. This knot was then sutured in place to the cecal serosa at the point where the tubing entered the vessel. The Longdwell catheters (Plate VII, page 103) have definite advantages over the use of the polyethylene tubing. The main advantage being that the catheter has an obturator which fits inside the catheter and extends the entire length of it. This prevents blood from entering the catheter when the obturator is in place and greatly reduces the occurrence of blood clots which will occlude the catheter.

It was felt that, if a means of anchoring the Longdwell catheters could be devised, they would be superior to the polyethylene tubing (P.E. 160). Plate VII, page 103 shows the 18 inch x 18 gauge Longdwell catheter of the type used in this study. Note the two teflon collars present on this catheter. Each of these collars has a length of synthetic suture material drawn under the collar, the free ends being used for anchoring sutures. The collar located just below the hub of the needle is permanently attached with an epoxy resin¹ made especially for teflon. The other collar, located on the body of the catheter, is designed to slide along the tubing and can be fastened at any point desired. This is accomplished by forming a double half-hitch at each end of the collar with the suture material. When the catheter is sutured in place, the half-hitches are drawn snug, being careful not to restrict the obturator by collapsing the catheter wall.

¹Epocap and Epocure. Manufactured by Hardman, Inc., Belleville, New Jersey.

Prior to surgery, feed was withheld from the pony for a period of 12 to 24 hours to reduce the amount of ingesta in the gastrointestinal tract. On the morning of surgery, the pony was clipped on the right side from a point just above the cecal fistula to the midline and from a point anterior to the last rib to the hip. At this time, the animal was administered a tranquilizer¹ intravenously. While the tranquilizer was taking effect, the surgical area was thoroughly scrubbed with soap and water and rinsed with clear water. The pony was then moved to the area in the barn where the surgery was to be performed. At this point an intravenous anesthetic² was given and the pony was laid on his left side on a rubber mat. Anesthesia was maintained by gas³ administered by an oxygen-gas machine. The surgical area was again scrubbed with soap and water and rinsed. The skin was then defatted with ether and rinsed with isopropyl alcohol. The area was draped using sterile towels.

The surgery was designed to cannulate the lateral cecal artery and vein about one-third of the way from the apex of the cecum. An incision was made in the right flank which was parallel to and about three inches posterior and ventral to the last rib. This put the incision anterior to any seepage from the cecal fistula. The muscles were separated by blunt dissection to minimize bleeding. The skin incision was approximately six inches long, while the one made in the peritoneum

¹Acepromazine maleate (injectable). Manufactured by Ayerest Laboratories, Inc., Veterinary Medical Division, New York, New York.

²Surital (sodium thiamylal). Manufactured by Parke, Davis & Co., Detroit, Michigan.

³Fluothane (brand of Halothane). Manufactured by Ayerest Lab., Inc., New York, New York.

was only about four inches in length. The peritoneum was held in place by means of forceps. The apex of the cecum was located and brought to the outside. This was accomplished without any real problem despite the adhesion of the cecum in the area of the permanent fistula.

The lateral vessels were exposed by carefully incising the lateral band longitudinally and by blunt dissection. Both the lateral artery and vein are located in this band with the artery lying above the vein. Once the artery and vein were separated from the fibrous band, a small incision was made in each vessel and the catheters were introduced. Both catheters were installed toward the animal's back with about 6 to 8 inches of the 18 inch catheter being introduced. This was done so as to locate the end of the catheters at a point where the diameter of the vessels was larger than at the point of entry. The catheters were then flushed with heparinized saline (1 U.S.P. unit of heparin per 10cc. of physiological saline) and checked to make sure that blood could be drawn easily. Once this was accomplished, the moveable collar was adjusted up to the point of entry and was sutured to the serosa of the cecum.

The cecum was then sponged off and rinsed with sterile saline before being carefully returned to the body cavity. The peritoneum was sutured to the serosa of the cecum along with the first muscle layer to establish adhesions. This was done to restrict the movement of this portion of the cecum. The remaining muscle layers were not passed directly to the outside, but were tunneled under the skin anteriorly and slightly ventrally. The catheters were then anchored by suturing the permanently attached collar to the skin. The skin incision was

closed with simple interrupted sutures that also picked up the first muscle layer to prevent the formation of a "pocket" in this area.

Once this was completed, the pony was administered a broad-spectrum antibiotic and was on his feet in about fifteen minutes after the incision had been closed. The incision was dressed and bandaged with an adhesive stretch bandage¹ to give support and to prevent the bandage from slipping. Care was taken to cover the exposed ends of the catheters with the bandage to prevent them from becoming caught. The pony was walked until he appeared to regain his equilibrium and then he was placed in a well-bedded stall.

The following postoperative care was performed twice daily: (1) administration of a broad-spectrum antibiotic which was continued for 7 to 10 days after surgery, (2) flushing of the catheters with heparinized saline which was continued for as long as the catheters remained in the pony, and (3) recording of rectal temperatures. No severe postoperative complication occurred in any of the ponies as a direct result of this surgery. However, on about the third day after the operation, all ponies developed a temperature of 103 to 103.5°F, which was brought down within 24 to 36 hours by the antibiotics administered. The skin sutures were removed in approximately 10 days after surgery at which time the skin incision appeared to be completely healed.

This surgical procedure was performed, with some modification, on six ponies in a two month period. The first three ponies operated on

¹Elasticon Bandage. Manufactured by Johnson & Johnson Co., Brunswick, New Jersey.

had catheters installed in both the lateral cecal vein and artery. These catheters failed to function within two days after being installed and were subsequently removed. The suspected cause for this stoppage was the occurrence of a fibrous tissue reaction in the vessel which resulted in occlusion of the catheters. The decision was made to install a catheter in the lateral cecal vein only, in hopes of minimizing trauma to the area. Three more ponies were operated on using this approach with variable results. The catheter in the cecal vein operated for only one day in one pony, while it functioned for approximately ten days in a second pony. The catheter in the third animal remained patent for a little over two months before it ceased to function. Following this, the animal went off feed and subsequently died from what was diagnosed as a ruptured stomach which was apparently unrelated to catheterization of the vessels.

Attempts were made with the last two ponies discussed to re-open the catheters by the administration of a combination of streptococcal enzymes combined with plasminogen of human placental origin.¹ This mixture is known to attack fibrin blood clots and to digest and liquify viscous accumulated pyogenic material (Harper, 1967). This preparation was administered in conjunction with a broad-spectrum antibiotic as prescribed by the manufacturer. The use of this enzyme mixture resulted in the liquification of a considerable amount of purulent-like material, possibly indicating the presence of a localized infection. However,

¹Varizyme (contains streptokinase, human plasminogen and streptodornase). Produced by American Cyanamid Co., Princeton, New Jersey.

its use only resulted in being able to draw one sample from an inoperative catheter. If this treatment had been initiated earlier, it may have been more successful.

Although the foregoing results are not very encouraging, it is believed that this type of catheterization is feasible. By further modification of the approach, this type of work could provide an unlimited amount of information concerning gastrointestinal physiology and function.

PLATE I

METABOLISM STALLS (REAR VIEW)

PLATE II

METABOLISM STALLS WITH LOADING RAMP IN PLACE

PLATE III

PONY WITH PERMANENT CECAL FISTULA
AND METABOLISM HARNESS

PLATE IV

METABOLISM STALLS WITH PONIES IN PLACE.
NOTE APRONS AND CONTAINERS FOR
COLLECTION OF FECES.

PLATE V

METABOLISM STALLS WITH PONIES IN PLACE.
NOTE PLASTIC BOTTLES FOR
COLLECTION OF URINE.

PLATE VI

CECAL CANNULAS. FROM LEFT TO RIGHT: (1) JARRETT SHEEP CANNULA AND
(2) ADJUSTABLE CECAL CANNULAS DESIGNED FOR THIS EXPERIMENT.

PLATE VII

INDWELLING TEFLON CATHETERS. FROM TOP TO BOTTOM: (1) 18 GAUGE X 18 INCH LONGDWEL CATHETERS USED IN THE CECAL VESSELS, (2) OBTURATOR FOR 18 GAUGE X 18 INCH CATHETER, (3) 18 GAUGE X 4 INCH LONGDWEL CATHETER USED IN THE JUGULAR VEIN, (4) INNER NEEDLE WHICH FITS INSIDE OF 18 GAUGE X 4 INCH CATHETER, AND (5) STYLET WHICH FITS INSIDE OF INNER NEEDLE.

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