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CHARACTERIZATION OF ALFALFA PROTEIN FOR RUMINANTS

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CHARACTERIZATION OF ALFALFA PROTEIN FOR RUMINANTS

by

David W. Rock

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TITLE

Characterization of Alfalfa Protein for Ruminants

BY

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TO MY GRANDFATHER

JOSEPH BRENNER

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- D. W. R.

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Introduction

Amino acids absorbed from the small intestines of ruminants are supplied by microbial protein which is synthesized in the rumen, undegraded feed protein which is resistant to microbial action and endogenous secretions. Though of reasonable quality, microbial protein may not meet the protein requirements of ruminants with high levels of production such as the growing calf or the lactating dairy cow.

The partial utilization of dietary protein in the lower portion of the ruminant digestive tract (abomasum, small intestine) is preferred to the rumen degradation of that protein. By decreasing the amount of ruminal degradation of a protein, efficiency of protein utilization is increased. Methods used to increase the amount of dietary protein which escapes rumen degradation include heat, chemical treatment, forming protein complexes and encapsulation. The amino acid profile of the bypass protein is of particular importance. High quality proteins would provide the most improvement of utilization if made to bypass.

Various techniques have been used to quantify the bypass value of various slowly degraded protein sources. In vitro ammonia production, in vitro protein solubility and in situ techniques have been considered as methods to estimate ruminal degradability. However, there exists variability between these techniques. Actual animal performance is necessary to confirm laboratory data.

Use of alfalfa as a supplemental protein source can be an important factor in many livestock operations. When available, alfalfa is an important part of rations for all ruminants. The protein of alfalfa can be made to resist ruminal degradation and bypass to the lower tract. Dehydration is an important method of

treatment in many states where alfalfa is grown. Because of this fact, alfalfa protein is of considerable interest in Nebraska.

These studies were conducted to evaluate the utilization of various alfalfa products and characterize alfalfa protein for ruminants.

REVIEW OF LITERATURE

Nitrogen Utilization

The intervention of microorganisms at the start of the digestive process in ruminants has a profound influence on the amino acid nutrition of ruminants (Satter and Roffler, 1975). Fermentation of feedstuffs results in a large amount of dietary protein and other nitrogenous products being degraded (for proteins probably 30 to 70%, depending on the nature of the protein). This process is followed by the incorporation of much of the nitrogen into microbial protein, the major nitrogenous component of the microbial biomass (Sutherland, 1976).

Proteolytic rumen microorganisms include species of Bacteriodes, Selenomonas and Butyrivibrio with the bacterial proteases being cell-membrane bound in nature (Allison, 1970). Protein catabolism by rumen protozoa is usually by engulfment and subsequent enzymatic hydrolyzation of microbial protein. Blackburn and Hobson (1960) demonstrated that proteolysis by rumen bacteria was pH dependent, with maximum activity occurring at 6.5. This was confirmed indirectly by Lewis and Emery (1962) when rumen ammonia production was maximized at pH 6.5.

The ammonia (NH_3 and/or NH_4^+) appears to be incorporated rapidly into rumen bacteria in the form of amide groups and used for synthesis: first of glutamate, aspartate and alanine, and then other amino acids (Smith, 1979). Rumen ammonia has been shown to cycle in a rumen pool (NH_3 — microbial N — NH_3) by Nolan and others (1976) and also to recycle with the blood stream (rumen ammonia — portal blood — blood urea — rumen ammonia) to conserve dietary nitrogen (Cocimano and Leng, 1967; Ford and Milligan, 1970; Nolan and Leng, 1972). Ammonia transfer across the rumen wall is pH and concentration dependent with no appreciable amounts absorbed under normal conditions (Smith,

1975). Passage of plasma-urea to the rumen pool is negatively correlated to rumen ammonia concentrations (Kennedy and Milligan, 1978). Ammonia recycling in the form of urea is thought to have developed as a survival mechanism when dietary nitrogen and energy are in short supply and the animal is in negative nitrogen balance. Recirculation of urea will not overcome the deficit, but allows the ruminant to survive longer (Phillipson, 1964).

The amount and composition of nitrogen compounds that pass from the stomach depend on: 1) extent of dietary protein digestion; 2) rate of microbial synthesis; 3) composition of microbes; 4) time spent by microbes in the rumen; and 5) extent of addition or removal of nitrogen in the omasum and abomasum (Hogan, 1975). Amino acids absorbed from the small intestine of ruminant animals are supplied by microbial protein synthesized in the rumen, undegraded or protected food proteins and amino acids which bypass the rumen and endogenous secretions (Chalupa, 1975). Little can be done to influence directly the amino acids supplied by endogenous secretions. Amino acids from microbial proteins and materials which bypass the rumen can be manipulated, with a desirable situation being maximum production of microbial protein with inexpensive non-protein nitrogen sources and the supplementation of this microbial protein with amino acids or protein bypassing the rumen (Chalupa, 1973).

Microbial Protein Synthesis

Digestion of fiber and microbial protein production from non-protein compounds provides a system to make human food out of materials which cannot be used directly by man (Chalupa, 1977).

Quality of rumen bacterial protein isolated from sheep on widely different diets has been shown to be very similar (Hoogenroad, 1970; Weller, 1957; Purser

and Buechler, 1966). The biological value of this protein has been calculated to be 80, with a true digestibility being 64 to 74% (Bergen et al., 1968a; McNaught et al., 1954; Oxford, 1955). Identification of limiting amino acids in bacterial protein may vary depending on the method of determination. Nimrick et al. (1970a,b) determined the limiting amino acids of rumen synthesized protein by infusing individual amino acids into the abomasum of growing lambs fed semipurified diets containing urea as the sole nitrogen source. The suggested order of limiting amino acids was: 1) methionine, 2) lysine and 3) threonine. Bergen et al. (1968a), using a plasma amino acid score, showed that cystine was the most limiting amino acid, followed by arginine, histidine, leucine and lysine. In an earlier study these same authors (Bergen et al., 1966) used an in vitro enzymatic degradation system. They determined that the digestibility of cellulolytic and non-cellulolytic organisms were 69 and 77% respectively. The limiting amino acid of cellulolytic strains was methionine. Leucine was most limiting in non-cellulolytic bacteria. Histidine has been shown to be first limiting in protozoa (Bergen et al., 1968a).

Various factors, both nutritive and non-nutritive, have been shown to depress or enhance rumen microbial synthesis. Among these, energy and ammonia have been considered to be most important.

Adenosine triphosphate (ATP) is the chief source of energy for rumen microbes (Bergen and Yokoyama, 1977). This energy is derived from the fermentation of carbohydrates (starch, cellulose, pectins and hemicellulose) to volatile fatty acids (VFA). Bauchop and Elsden (1960) showed that the growth of organisms (streptococcus faecalis) was proportional to the energy concentration of an in vitro medium. The Y_{atp} (grams dry weight of organism produced per mole of ATP) was equal to 10.5 (8.3 to 12.6). When considering that bacteria contain

approximately 10.5% nitrogen, this represents 1.1 gram bacterial N per mole ATP. The value of 10.5 has been substantiated by other workers with a variety of organisms (Forrest, 1969; Forrest and Walker, 1971; Payne, 1970).

Though Y_{atp} values had been shown to average 10.5, wide variations have been observed. Smith (1975) stated that it appeared that predictions of cell yield in terms of energy production and substrate consumed were uncertain due to the complexity of the system. Stauthammer and Bettenhausen (1973), Owens and Isaacson (1977) and Hespell and Bryant (1979) cited four basic reasons why Y_{atp} values may deviate and could not be a biological constant: 1) the variable chemical composition of the cells; 2) transfer of metabolic intermediates between species; 3) availability of cell components; and 4) energy expended by the organisms for cell maintenance and replacement of lysed cells.

Dilution rate, or turnover rate, of both in vitro and in vivo systems has been shown to affect microbial synthesis by affecting the energy available to the organism for production. Dilution rate may influence the redistribution of microbial species between overlapping niches, the pattern of metabolites produced or the efficiency of microbial protein synthesis (Sutherland, 1976). An additional effect of increased turnover rate would be an increased growth rate, which is generally associated with increased microbe size. Thus, the maintenance energy requirement per unit of microbial biomass becomes increasingly smaller. Similar results were observed by Hobson and Summers (1967) and Isaacson et al. (1975). By altering the dilution rate in vivo with the addition of water, Harrison et al. (1975) observed a correlation of r=-.89 between propionate production and dilution rate. Line of fit for the individual diets studied (rye grass vs concentrate) was more significant than the means of all diets, showing that diets responded differently to varying dilution rates.

Ammonia is quantitatively the most important nitrogen nutrient for rumen bacteria (Smith, 1975). Pilgrim et al. (1970) used N^{15} as a marker to determine the amount of rumen ammonia incorporated into microbial cells. On a low protein diet (75% wheat hay, 25% lucerne), 77% of the bacterial N and 53% of the protozoal N passed through the rumen ammonia pool. These figures were reduced to 63 and 38 percent respectively for animals consuming a higher protein diet (100% lucerne), prompting the authors to conclude that synthesis of microbial protein was more dependent on ammonia as a starting point with a low nitrogen diet than with a high nitrogen diet.

Ammonia can become limiting for microbial protein synthesis in diets where sufficient energy is available. Hume et al. (1970a) found a linear increase in rumen protein production when wethers consuming a protein-free diet were infused with urea (2, 4, 9, 16 g N/day). Rumen ammonia level increased as the level of nitrogen infusion increased and was at a level of 133 mg NH_3/l of rumen fluid when maximum protein production was observed. Higher levels of ruminal ammonia (235 mg NH_3/l) were observed to be optimal for maximum dry matter disappearance of barley in trials with sheep (Mehrez et al., 1977). The authors stated that this level may be pH dependent relative to the type of ration which the animal is consuming. Satter and Slyter (1974) used continuous fermentors to study the effect of ammonia concentration on microbial synthesis and found a much lower level (50 mg NH_3-N/l) to be optimal for microbial yield. Slyter and coworkers (1979) used ruminally fistulated steers to substantiate their earlier work. By infusing graded levels of urea into the rumen (0 to 140 g), varied ruminal ammonia levels were produced. Nitrogen retention increased until the level of ammonia in the rumen reached 4.5 mg/100 ml. Slight non-significant increases in nitrogen retention were observed at higher levels.

Microbial yields have also been shown to be affected by pH (Hobson, 1965), fermentability of a feed or processing method (Meyer et al., 1967; Cole et al., 1976), roughage level (Cole et al., 1976), VFA (Wegner et al., 1960; Hume, 1970a; Umunna, 1972), site of fermentation (Mann and Ørskov, 1973), the addition of sulfur or sulfur containing amino acids (Patton et al., 1968; Hume and Bird, 1970), and protein source (Hume, 1970b).

Requirements for amino acids by the rumen microbial population have been demonstrated by Maeng et al. (1976a,b). These workers showed that growth rate of rumen bacteria was increased two times and mean doubling time was halved when amino acids replaced 25% of the urea-N in a growing medium (mixture of 18 amino acids). Results with the substitution of 18 amino acids was greater than 10 essential amino acids, 8 non-essential amino acids and the sulfur-containing amino acids respectively.

Protozoal protein can make up a considerable portion of the microbial pool. The biological value of this protein is similar to bacterial protein (80) (McNaught et al., 1954; Oxford, 1955). The digestibility of protozoal protein has been shown to be greater than bacterial protein (91 vs 74%) (Oxford, 1955). Equal biological value along with increased digestibility would result in an increase in the net protein utilization of protozoal nitrogen (Bergen et al., 1968b).

Factors which affect the protozoal population in the rumen were outlined by Hume (1973). Protozoa are somewhat pH specific (Allison et al., 1975), having an optimum range from 6.0 to 7.0, with inhibition being observed at 5.5 and protozoal death below 4.5. This pH sensitivity could somewhat explain the diurnal changes which the protozoal population goes through. Other possible causes would be nutrient availability, rumen osmolality and dilution rate of feed, saliva production and water intake.

The role of protozoa has been discussed at great length in the literature. Oxford (1955) argued that their importance was to convert plant protein into animal protein. Klopfenstein et al. (1966) reported increased dry matter digestibility with faunated lambs. Abou Akkoda (1975) and Abou Akkoda and el-Shazly (1964) cited experiments where the performance of faunated sheep was superior to their defaunated counterparts. These animals showed a superior ability to digest food constituents, with more reducing sugars, ammonia N and VFA apparent in the rumen fluid. These animals had a greater propionate:acetate ratio and increased nitrogen retention. These researchers concluded that rumen ciliate protozoa were essential for the metabolism and growth of young lambs. Klopfenstein et al. (1964) suggested that protozoa may be involved in favorable responses to antibiotics.

However, the protozoal population may be detrimental to the animal's utilization of nutrients, particularly N. Protozoa engulf bacteria (Thomas, 1973) with a waste of N, as some of the bacteria are not utilized. Also, protozoa and bacteria compete for the same substrates within the rumen (Eadie and Hobson, 1962). Work cited by Weller and Pilgrim (1974) showed that protozoa were sequestered within the rumen with passage of 6 to 29% of predicted protozoa flow to the omasum. Bergen and Yokoyama (1977) stated that it was a possiblity that there exists within the rumen a cycling protozoa pool with little net protein passage to the lower tract. In this case, ruminal protozoa may actually limit ruminal protein production and depress the supply of amino acids to the host.

Quantification and Manipulation of Slowly Degraded Protein

In addition to microbial protein, the ruminant's requirement for protein is met by protein that has escaped rumen degradation. Because of this important

role in the nutrition of the whole animal, the ability to quantify and manipulate this fraction is the object of considerable research.

Rumen ammonia levels are a reliable indication of rumen protein degradation in most cases. el-Shazly (1958) stated that protein solubility is the most important factor controlling rumen ammonia production. Solubility values for various feedstuffs have been determined (Wohlt et al., 1973), with these feedstuffs grouped according to type (roughage, grain). Insoluble proteins were utilized more efficiently than soluble sources in diets for dairy cattle (Atchison et al., 1976). However, a linear response was not observed when proteins were combined in these trials. Rumen ammonia and solubility were highly correlated (Wohlt et al., 1976).

There exists a need for a proper method to determine rumen protein degradability. Miller (1973) suggested metabolism trials with cannulated animals as a means of obtaining information as to the specific characteristics of a protein source. One major problem of this type of approach is the inability to distinguish between the plant and microbial protein fractions in the digesta.

Actual degradation of a protein source has been considered a method of estimating the amount of protein which escapes rumen degradation (Ørskov and Mehrez, 1977; Mehrez and Ørskov, 1978). At short incubation times (4 hours), the polyester bag technique gave estimates of nitrogen disappearance similar to estimates of degradation in the rumen obtained from measurements of non-ammonia nitrogen flow to the small intestine (Horton and Miller, 1977). Variation in results from bag measurements was caused by sample size to bag relationship (Mehrez and Ørskov, 1977) with three animals and two measurements necessary to eliminate variability of animals and days. Ørskov and McDonald (1979) proposed

that two trials, a degradation trial (fiber bag) and a rate of passage trial were necessary to accurately predict true degradability.

Problems exist with both solubility and degradability measurements. Solubility can be influenced by factors associated with the solvent such as pH, ionic strength, temperature, degree of agitation and extraction time (Wolt et al., 1973) and by different solvents (Whitelaw and Preston, 1963; Crooker et al., 1978). Values obtained with the nylon bag vary due to pore size of the bag, sample size, flow of particles into the bag and lack of gas release which may cause bag floatation (Uden et al., 1974). The nylon bag technique measures the loss of soluble material from the bag. Apparent digestibility is a measure of net absorption of soluble material from the gastrointestinal tract (Baily and Hironaka, 1970). Care must be taken in interpretation of these results. Degradation in the rumen does not specifically indicate utilization of the degraded or undegraded portions.

There has been a tendency to use solubility and degradation as synonymous terms. While ruminal degradation of a protein is necessarily dependent on the ability of that protein to "solubilize" in the rumen medium, solubility alone does not insure degradation (Bull et al., 1977). Correlation coefficients of soluble nitrogen in Burrough's mineral buffer, .15M sodium chloride solution, and autoclaved rumen fluid to dacron bag degradation determinations were .66, .47 and .54, respectively (Crawford et al., 1978).

Other factors which will control the amount of protein escaping ruminal degradation include level of feed intake (Owens and Isaacson, 1977), level of protein in the diet (Walker et al., 1975) and inherent differences of amino acid degradation (Chalupa, 1974). Scheifinger and coworkers (1976) determined that

not all amino acids are degraded by all strains of rumen bacteria and the subsequent proteolytic qualities of the rumen microflora were the result of extensive bacterial interaction.

Feeding proteins that are relatively resistant to microbial degradation in the rumen (bypass protein) can improve the animal's nitrogen economy by minimizing losses that are incurred in the metabolism of dietary protein to microbial protein and by enhancing opportunities for utilization of non-protein nitrogen chemicals in the rumen (Chalupa, 1977). Some proteins, such as zein, are naturally slowly degraded (Ely et al., 1967; Hume, 1974). Improving the bypass properties of many proteins has received considerable interest in early, as well as recent, literature.

Heat treatment is probably the most widely studied method of decreasing the rumen degradability of a protein. With soybean meal, heat treatment decreases protein solubility, decreases rumen ammonia production and increases nitrogen retention (Tagari et al., 1962; Sherrod and Tillman, 1962; 1964). Hudson and coworkers (1970) heated soybean meal at 149 C for 4 hours. This treatment increased ($P<.05$) the concentration of both dry matter and non-protein nitrogen (NPN) in the abomasum. Amino acid nitrogen may have been measured as NPN in this study. This would indicate an increased amount of protein reaching the lower tract.

Nitrogen digestibilities of heated and unheated soybean meal were similar. Soybean meal and sunflower meal were heated by Schingoethe and Ahrar (1979). Solubility of both sources was decreased (27 and 35%, respectively) with no change in the amino acid pattern of each protein. Digestibility of soybean meal is not affected by heat treatment (Yu, 1978a) and is utilized more efficiently than untreated material as measured with milk production in dairy cows (Ahrar and

Schingoethe, 1979). Increased growth was observed with heat treated soybean meal supplementation in 12% crude protein rations, but not 17% crude protein (Glimp et al., 1967).

Heat treatment of groundnut meal was successful in stimulating nitrogen retention with no effect on digestibility (Chalmers et al., 1964) and heat was shown to decrease the protein degradability of fish meal (Mehrez et al., 1980). However, Whitelaw and Preston (1963) have shown that with early weaned calves, heat treatment increased nitrogen retention of fish meal but not groundnut meal. The differing amino acid composition of the two supplements was cited as a possible explanation for the differences in nitrogen retention.

Forage protein responds to heat treatment. Utley and McCormick (1980) evaluated dehydrated bermuda grass in whole shelled corn based rations. In a 120-day feeding trial with yearling steers, animals fed dehydrated bermuda grass gained faster ($P<.05$) than similar steers supplemented with raw cottonseeds or a urea-cottonseed supplement.

Solubility of alfalfa protein can be decreased by dehydration. Krause and Klopfenstein (1978) oven-dried alfalfa at 80 C for 4 hours. In vitro ammonia release was less ($P<.05$) for alfalfa dried at 80 C or above than for soybean meal with freeze-dried or oven-dried alfalfa products being intermediate. In a steer growth trial, a complementary effect between dehydrated alfalfa and urea was observed. If large amounts of protein escape rumen degradation and are bypassed to the lower tract, rumen ammonia levels could drop below that necessary for maximum fiber digestion. Urea addition would ensure that this would not happen (Yu, 1978a). Differences were very small between direct cut dehydrated alfalfa and alfalfa field wilted to 65, 60 or 40% moisture before dehydration (Klopfenstein et al., 1978).

Goering and others (1974) dried alfalfa at 60, 130, 160 and 180 C and fed it to lambs in a nitrogen retention study. Nitrogen retentions were 6.0, 7.4, 6.9 and 3.9 g/day respectively. Acid detergent insoluble nitrogen, an indication of heat damage, increased to 20.4% of the total nitrogen at the higher temperature.

Formaldehyde has been studied extensively as a chemical which will inhibit protein degradation of feedstuffs. Formaldehyde forms acid-reversible cross-linkages with amino and amide groups rendering the protein insoluble at rumen pH (Macrae et al., 1972).

Early work with formaldehyde treatment concentrated on comparisons of treated and untreated casein. Casein is degraded very rapidly by the rumen microbes. In diets which contained 16 g N/day supplied by casein, lambs which received treated supplements gained more than those receiving an untreated supplement and equally as well as lambs receiving casein per abomasum (Reis and Tunks, 1969). This would indicate that more treated casein reached the abomasum, resulting in more efficient utilization. In other work, lambs receiving treated casein in 10% crude protein rations gained faster and had increased protein and decreased fat in body gains (estimated with tritiated water) (Faichney, 1971). Barry (1972) found nitrogen balance in sheep to improve when treated casein was fed. He also found fecal nitrogen to increase and urinary nitrogen to decrease. Wool growth was stimulated with the addition of a treated casein in these rations.

Weight gain and wool growth responses are due to increased protein reaching the lower tract. Apparent absorption of non-ammonia nitrogen from the small intestine was greater with a treated casein diet when compared to untreated controls (Macrae et al., 1972).

With soybean meal, formaldehyde treatment (.5%) increased nitrogen retention in lambs (Amos et al., 1974). Levels of .4 to .8% decreased rumen degradation of soybean meal but did not affect post ruminal availability (Thomas et al., 1979a). In feeding trials, an increase in gain of 35% and a 22% improvement in feed efficiency was observed when compared to controls (Thomas et al., 1979b). Addition of treated soybean meal in rations for finishing lambs resulted in non-significant improvements in average daily gain and feed efficiency (Martinez et al., 1975). Examples of other feedstuffs which have been treated with formaldehyde are: alfalfa leaves (Yu, 1978b), bermuda grass (Amos et al., 1976), clover grass hay (Sharkley, 1972), groundnut meal (Miller, 1971) and peanut meal (Faichney and Davies, 1972).

Overtreatment of protein sources (soybean meal and sunflower meal) resulted in decreased urinary nitrogen, increased fecal nitrogen and a decrease in the amount of nitrogen retained (Amos et al., 1974), with decreased animal performance (Schmidt et al., 1974). A combination of heat and formaldehyde treatment resulted in increased acid detergent insoluble nitrogen and evidence of non-enzymatic browning (Dinius et al., 1975). Lamb performance was also decreased by this combination (Reynolds, 1978).

Driedger and Hatfield (1972) treated soybean meal with tannin compounds at 5, 10, 15, 20 and 25% of the dry matter. All levels depressed in vitro ammonia production with no effect on pepsin degradation. Ten percent was considered the optimum level for tannin treatment. Tannin treatment also increased growth and nitrogen retention of lambs. There was some evidence of an adaptation response to tannin treatment. Nishimuta and coworkers (1973) observed no significant differences between normal and tannin-treated soybean meal. The reaction of

tannins is dependent on the original physio-chemical properties and heat treatment applied to the protein (Zelter et al., 1970).

Masonex is a molasses-like by-product of the hardboard industry (Hartnell and Satter, 1978). Masonex contains primarily carbohydrates, water and a small non-carbohydrate factor having a variety of phenolic compounds. Tannins also are phenolic compounds. Fifteen percent Masonex was shown to complex nitrogen, but those results were variable due to Masonex batch differences. Soybean meal has been shown to react to some degree with Masonex (Thomas et al., 1979a,b).

Complexing proteins with compounds to decrease rumen degradation has been considered as a means of bypassing more protein to the lower tract. Sodium bentonite has been shown to be such a compound. Britton and coworkers (1978) demonstrated that sodium bentonite decreased the in vitro ammonia release of soybean meal and casein. Bentonite complexes were not affected by pH. A ratio of 3 parts soybean meal and 1 part bentonite was considered optimum to reduce rumen ammonia levels and maintain animal performance. Encapsulation and polymerization of amino acids to facilitate bypass has been investigated (Broderick et al., 1970; Amos and Evans, 1978).

The use of deaminase inhibitors to reduce rumen proteolytic activity was summarized by Chalupa (1975). Diaryl iodonium chemicals have been found to suppress fermentation of amino acids both in vitro and in vivo. Decreased acetate and methane production with increased production of propionate resulted in an increased fermentation efficiency with diaryl iodonium chemicals.

During suckling, the esophageal groove of young ruminants closes, allowing milk to pass directly to the abomasum to avoid rumen fermentation. Ørskov and Fraser (1969) used this feature to bypass protein in liquid diets. However, this