THE DEVELOPMENT OF A COLORIMETRIC ASSAY TO MEASURE RUMEN DEGRADABILITY OF PROTEIN FEEDS IN VITRO

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ABSTRACT

THE DEVELOPMENT OF A COLORIMETRIC ASSAY
TO MEASURE RUMEN DEGRADABILITY
OF PROTEIN FEEDS *IN VITRO*

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Four protein feed supplements were used in developing an *in vitro* assay for rumen degradability. Degradability was determined by a synthetic bag procedure using three fistulated bovines.

The *in vitro* assay was a protease digestion followed by a colorimetric assay for solubilized protein. The five assays tested were: Lowry, Bio-Rad, A260/A280, ninhydrin, and biuret. Changes were made in the digestion in an attempt to make the digestion and colorimetry compatable. Accuracy of colorimetric methods was determined by correlation of these results with residual nitrogen and *in vivo* data.

The Lowry was too sensitive and suffered from interfering substances while Bio-Rad was insensitive to the solubilized peptides. A260/A280 tended to underestimate degradability of some feeds and overestimate others. The ninhydrin overestimated degradabilities or color development was erratic. Biuret was promising, but while correlations with soluble kjeldahl nitrogen within feeds was high (r=.97) correlations considering all feeds were low (r=.58).

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INTRODUCTION

The economic impact of the dairy cow or any ruminant for that matter, has mandated the continued investigation into its nutritional requirements. These requirements are profoundly influenced by the presence of the rumen. Two protein requirements must be considered in ruminant feeding: the needs of the animal itself and the requirements of the microbial population in the forestomach (44).

Some species of microorganisms found in the rumen require preformed peptides or free amino acids and if not provided adequately in the diet some microorganisms may disappear from the rumen. There may be a reduction in overall efficiency and protein yield (g protein per Kg fermented organic matter) if the ammonia concentration falls below 80 mg nitrogen per liter of rumen fluid (39). This critical level may be even higher when fermentation rate is rapid.

Fermentation in the rumen results in the degradation of some of the dietary protein to volatile fatty acids and ammonia, a process which in some circumstances is wasteful when the overall economy of the animal is considered (1). van't Klooster and Boekholt (46) have noted that in sheep on a low protein diet there was an apparrent net gain of nitrogen as digesta flowed to the lower gastrointestinal

tract. This increase was presumably of endogenous origin. When these same sheep were fed high protein diets, nitrogen was wasted via absorption of ammonia from the rumen after the deamination of amino acids. In fact Wright (53) was only able to recover 70% of the feedstuff nitrogen in forms useful to the host animal.

Early work by Burroughs, et al. (5) and Ganev, et al. (13) suggested that amino acids were released, during degradation, in proportions similar to those present in the feedstuff. Consequently, the quality of the undegraded protein was similar to that appearing in the feedbunk. In more recent studies, however, Craig and Broderick (8), and Santos et al. (38), showed that this is not the case. Because all proteins showed characteristic variations in amino acid release, the amino acid pattern of the undegraded protein escaping the rumen differed from that originally fed. Santos (38) has demonstrated that in some instances the residual amino acid pattern may even be improved as the essential amino acid: nonessential amino acid ratios of the duodenal digesta increased relative to the diet.

The main nitrogenous components in the digesta passing to the small intestine are α-amino nitrogen, non-α-amino nitrogen, nucleic acid nitrogen, amide nitrogen, and variable proportions of ammonia nitrogen (46). According to Smith and McAllan (41) about 50% of the total protein passing from the stomach to the intestines is of microbial origin, and half of the non-protein nitrogen is in the form

of free amino acids (48).

It must be recognized that the extent of microbial protein synthesis is also a function of the amount of energy released during fermentation, but even when energy sources are not limiting in the rumen, fermentation and microbial growth may not be sufficient to meet the animal's amino acid requirements for maximal productivity. In such cases the animal requires additional supplementation of protein or amino acids that will by-pass the rumen (15) and be further digested and absorbed in the intestines (38).

For years, feed values and protein requirements have been stated in terms of digestible crude protein (DCP). This regime has been discounted to a certain degree by the studies of Miller (27) who noted that diets of equal DCP values gave different responses in growth. Unfortunately, the DCP concept has persisted, and it has led to the belief that the animal could obtain all of its amino acid requirement from microbial protein. Recent research, however, indicate that when amino acid requirements of ruminants are high, insufficient protein is available from microbes. While the rumen microorganisms are capable of providing sufficient protein for maintenance, slow growth, and early pregnancy they cannot fulfill the needs of fast growth, late pregnancy, or early lactation (15).

The contemporary trend is to feed some ruminally undegradable or "by-pass" protein as is commonly known. Unfortunately, too great a supplementation of undegradable

protein may result in a protein deficient microbial fermentation, and ultimately decreased rumen degradative efficiency. The proper levels of by-pass protein vary according to the level of productive demands of the animal. For example, Sniffen and Chase (55) issued the following recommendation for dairy cattle:

early lactation	39-49	% by-pass of total dietary protein
mid lactation	35-41	
late lactation	34-39	

The quantitative determination of these components, degradable and undegradable protein, and how they vary with different feedstuffs and feeding regimens is central to recent proposals for evaluating the protein contribution of feedstuffs and calculating dietary protein requirements (23). Various methods, both *in vitro* and *in vivo*, have been developed to estimate rumen protein degradabilities. Unfortunately, most of these procedures are either complex, laborious, or both, and they vary in degrees of accuracy.

Perhaps the most widely used *in vivo* method is that of Mehrez and Orskov (24) which used bags of dacron parachute material to suspend feed samples in the rumen of sheep. These bags were removed at various times, depending on the type of feed used, and the residual nitrogen determined. The extent of protein degradation then, under normal conditions, is assumed to be when 90% of the digestible dry matter has disappeared from the bag (24,32).

Like all procedures this one was not without problems. Mehrez and Orskov (24) noted significant variation between

sheep and between days of incubation. In the process of accounting for the variation they found that increasing duration of incubation failed to substantially reduce variability, and Stern and Satter have concurred (43). In our lab we have seen an effect of feeding relative to the time of insertion of the bags in that there is an increased rate of degradation shortly after feeding. In the sheep studies of Mehrez and Orskov (24), the animals were fed ad lib and so the feeding effect was not studied. Mehrez and Orskov noted that neither prewashing of the bags to remove soluble feed nor location in the rumen during digestion had any effect on variability. Dry matter entering the bags or microbial attachment made no significant difference as the average increase for blank 5cm by 8cm bags was only .03g dry matter and only trace amounts of diaminopimelic acid (DAPA) was found on undigested residues. Lindberg, et al. (19) found that pore size seems to have no effect as they saw similar degradabilities of feed samples suspended in bags with pore sizes of either 10um or 40um. A critical consideration in this procedure is the preparation of the samples. Preferable, these samples should be masticated digesta collected from an esophageal canula, but a hammer-mill equipped with a 5mm recutter screen for dry feeds or a mincer for wet feeds will work quite satisfactorily (24). Zinn, et al. (54) cited other possible sources of variation in this procedure including: 1) processing of sample (particle size); 2) lag time between insertion and

initiation of normal fermentation; 3) formation of an abnormal microenvironment within the bag as a result of non-physiological moisture content, inadequate removal of end-products, preponderance of feed constituents that potentially inhibit digestion, and associative effects of feeds; and 4) lack of microbial adaptation to test feedstuff or roughage level. Weakley (49) noted a decreased rate of nitrogen disappearance with animals fed a high grain diet.

Zinn (54) also made some suggestions regarding increasing the precision of prediction estimates including: 1) greater constancy of animal, dietary, and analytical factors; and 2) use of a standardized reference feed (i.e.- SBM). In spite of the aforementioned difficulties, the dacron bag technique has the advantage of giving a very rapid, accurate estimate of the digestion of nutrients in a feedstuff (24); provided one has access to surgically prepared animals, a suitable microbial marker, and the time and labor to obtain the necessary measurements.

Another class of procedures have been developed to determine feed protein flowing to the duodenum by the difference of total nitrogen flow versus microbial and endogenous nitrogen contributions. All of these methods rely on the identification of microbial nitrogen by the organic or natural labels such as aminoethylene phophonic acid (AEPA), Diaminopimelic acid, and Ribonucleic acid, or ruminally infused isotopic markers such as ^{35}S, ^{15}N, or ^{32}P (23); all of which would be incorporated into microbial

cells during their growth. The ratio of label to microbial nitrogen is determined in isolates and the ratio can then be used to determine microbial contribution to duodenal flow (23). This value along with the proportion of endogenous nitrogen is subtracted from total non-ammonia nitrogen in the duodenum and the remainder is assumed to be the contribution of feed protein. Then this value relative to the protein level of the diet yields the percent undegradable protein.

These procedures have inherent problems. First, like the dacron bags, they requires surgically prepared animals. Secondly, action of pepsin and HCl in the abomasum may bring about the disruption of the cells resulting in a microbial isolate with a disproportionate level of marker (41). Others have circumvented this second problem, especially with ^{35}S, by measuring other parameters such as the specific activity of cystine or methionine. Unfortunately, this requires extra steps to determine the amino acid bound ^{35}S content of the microbial mass and the whole digesta (23). McAllan and Smith (29) used RNA and DAPA, and found that the two methods showed similar patterns but gave different absolute results. Moreover, without an absolute standard it is hard to determine which is more accurate. Thirdly, accuracy of measuring total flow of nitrogen and microbial protein entering the abomasum or small intestine is limited, the measuring techniques are laborious, and, as a consequence, abomasal and duodenal flow measurements are often restricted to a period of 24

hours. Reducing the variation due to non-steady state conditions in the rumen is possible through frequent feeding, but this makes the results less meaningful for practical conditions. Therefore, estimates of the proportion of dietary protein escaping microbial degradation in the forestomach are subject to high error (44). Lastly, accuracy may be improved by increasing the number of experimental animals, but according to Miller (28) this procedure would require 10 to 12 animals in order to achieve a level of significance less than 5%. Moreover, certain obvious difficulties would arise in terms of handling and disposal if the experimental models chosen were large ruminants such as beef or dairy cattle.

Because of the complications seen *in vivo* many *in vitro* methods have been developed which avoid or reduce the problems of time, labor, and animals. These procedures are generally based on either solubility in various solvents (9,10,17,35,43,47,51), ammonia gas production after incubation with viable rumen liquor (21,25,36), or residual nitrogen after incubation with bacterial, fungal, or plant proteases (22,30,34,35,45).

The degradation of feed protein appears to be the result of 1) a rapid degradation of readily soluble protein occurring soon after the feed enters the rumen and 2) a slower break down of the less soluble protein extending beyond this initial period (9). Of the many factors that affect degradability in the rumen solubility is probably

one of the most important (47).

Many solubility/insolubility procedures, and as many solvents, have been developed through the years (9,10,17, 35,43,44,47,51). The goal of these techniques is the prediction of absorbable or digestible feed protein that escapes rumen degradation and enters the small intestine. There are, however, very few highly predictive results available in the literature (47). Of these few, Poos-Floyd, et al (35) found significant correlations between solubility and in vivo degradability (growth trials) when the feed sample was solubilized in hot water, 10% Burrough's solution, and bicarbonate-phosphate (BP) buffer. As for the poorly correlated results from other studies several factors may account for the descrepancies. These include: solvent type, pH, and ionic strength; extraction time and temperature; degree of agitation; feed patricle size; and different types and properties of soluble protein fractions in the feed (9,10, 17,35,43,44,47,51). Therefore, even though solubility may be an important determinant of rumen degradability the two characteristics should not be considered synonymous (9,17, 44). The solubility of proteins need not be related to susceptibility to enzymatic degradation (22). Thus, an in vitro procedure which could duplicate, or at least simulate, the enzymatic proteolysis of the rumen would be of great value.

The most obvious way to duplicate rumen proteolysis would be to use rumen fluid or washed bacterial suspensions.

This procedure would provide the most meaningful results because, like in the rumen, the system is complex since the bacterial population is mixed, individual organisms are at different stages of growth, and many enzymes are successively involved in the breakdown of proteins to VFA's and ammonia (1). The washing of bacteria, however, may cause a decrease or even loss of activity of some enzymes (1,21).

Menke, et al (25) and Raab and coworkers (36) have used the known relationship between the fermentation of carbohydrates and protein synthesis to determine protein degradability and metabolizable energy. Menke (25) used 150 ml syringes containing 30 ml of a rumen fluid/buffer mix plus 100-200 mg of feed sample, and placed in a rotor located in a drying oven held at 39$\pm$.5°C. The syringes were incubated for 24 hours and the final reading of gas production was taken at this time. After correcting for the blanks they noted a high correlation (r^2=.95) between this <u>in vitro</u> and <u>in vivo</u> degradability. More recently, Raab, et al. (36) saw similar correlations between gas production and ammonia nitrogen content of the fluid (r=.98), but every feedstuff had its own regression equation. Realizing that they could make use of this high correlation Raab, et al. (36) developed an equation to determine <u>in vitro</u> degradable nitrogen (IVDN).

$$\text{IVDN} = \frac{(NH_3\text{-N at zero gas production}) - (NH_3\text{-N of blank})}{\text{total N of feedstuff incubated}}$$

A major problem considered by both groups (25,36) was

that gas production reflects more the content of digestible carbohydrates than protein. Futhermore, the results may be additionally affected by atmospheric pressure, pH of the sample, sample size, and the organic acid content of the feedstuff. There is also the difficulty of keeping a standard feedstuff in order to correct for deviations caused by changes in the activity of the rumen liquor, and that feedstuffs which show significantly slower gas production may actually have a higher *in vivo* digestibility. In spite of all this the procedure does have some advantages. There is no separation step which could, depending on the feedstuff, give a bias towards digested or undigested fractions, and it requires a small sample weight-- only 20-30 mg.

Mahadevan, et al. (21) also made use of buffered viable rumen fluid (strained), but by a different method. First they linked to SBM with 7-amino-1,3-naphthalene disulfonic acid. The diazotized SBM was then incubated at 37°C for 30,60,120, and 240 minutes. At each interval perchloric acid was added to selected tubes to precipitate the undegraded SBM and the tubes centrifuged at 15,000xg for 20 minutes. Finally, the pellet was resuspended in .15M NaOH and the absorbance determined at 440nm. This method appears to be superior to the determination of amino acids with ninhydrin because the loss of amino acids by microbial deamination is avoided. Since the pellet is used, rather than supernatant, the complications stemming from reutilization of hydrolized protein by the bacteria is eliminated

(21). The ninhydrin could be made more compatable with rumen fluid incubations if bacterial deaminases are inactivated by adding 0.1mM hydrazine sulfate (6) or destroyed by repeated freezing and thawing (21). This procedure may alter the amino acid's susceptibility to rumen degradation especially the phenolic and imidazole residues. Moreover, in procedures utilizing viable rumen fluid the maintenance of anaerobic conditions throughout the digestion is essential. This is often accomplished by continued gassing with CO_2 (45), or by the inclusion of cysteine (4) or dithiothreitol (DTT) (6).

Rumen fluid activity can vary from day to day and within days due to feeding regimen. In order to provide more controlled conditions in vitro methods using standardized proteases have been developed, and this allows for a greater capability and understanding of comparisons. These proteases are of bacterial (22,30,34,35), fungal (34,35), plant (34,35), or animal origin (34,35,45).

Perhaps the most well known of these assays is that of Tilley and Terry (45). In the second stage of their assay they subject the protein to a pepsin digestion which has yielded in vitro values very similar to those found in vivo in sheep.

Mahadevan, Erfle, and Sauer (22) used the protease from Bacteriodes amylophilus to simulate rumen proteolysis. The rationale being that B. amylophilus was one of the major proteolytic bacteria of the rumen, plus it does not possess

any deaminases which would allow the use of the ninhydrin to determine the extent of degradation. Their research showed that soluble proteins are not degraded at the same rate, and in fact, they noted a six hour lag for some feed samples. They also confirmed the results of earlier workers by noting that solubility is an important factor regarding rumen degradability, but should not be considered a good measure of rumen degradability.

Nocek and coworkers (30) chose Streptomyces gresius protease for a similar study because other researchers had shown a close correlation with direct and in situ values. Poos-Floyd, et al. (34,35) also examined protease from S. gresius as well as papain (E.C.# 3.4.22.2), bromelain (3.4.22.4), ficin (3.4.22.3) and neutral fungal protease (NFP) from Aspergillus oryzae. For the most part their results were highly correlated with dacron bag and growth trial data, but the correlations decrease as the periods of incubations increased. NFP, however, did not exhibit this trend and demonstrated correlations as high as .97 at 24 hours in some cases. Ficin and NFP had the lowest coefficients of variation and were therefore selected as the enzymes best suited for in vitro prediction of degradability. Ficin was recommended over NFP because NFP only degraded small amounts of feed sample. The biggest advantages of this procedure is it does not require fistulated animals and the entire digestion can be completed in approximately four hours.

A general limitation of all *in vitro* methods is that, although they may yield a value for degradability, they do not necessarily yield data representing actual degradation *in vivo* (44). While *in vitro* methods have the advantages of being less expensive, less time consuming, and afford the opportunity to maintain more precise conditions than *in vivo* methods the application of *in vitro* data to the prediction of results *in vivo* is dependent upon how well the *in vivo* conditions are known (36). For example, rumen degradability is not only related to the kind of feed and nature of protein, but also a function of residence time in the rumen (31). Most of the aforementioned procedures measure only the fractional rate constant for degradation (Kd). There is, however, one other prime consideration and that is Kr or the fractional rate constant for rumen turnover. Thus, prediction equations have been developed to calculate rumen degradability based on the interaction of Kd and Kr.

Broderick (6) found casein degradation to be first order and from this was able to derive a very simple equation:

$$\text{Percent escape} = \frac{Kr}{Kr+Kd}$$

Unfortunately when this formula was applied to *in vitro* values it tended to overestimate the percent escape relative to *in vivo* data. Orskov and McDonald (31) and Ganev, et al. (13) derived a somewhat more complex equation which took

into account different fractions of the feed protein.

$$P = a+b\ (1-e^{-ct}\ )$$

where: P = effective digestibility
a = % rapidly disappearing protein (% of total protein)
b = % disappearing at constant rate, c, per unit of time, t

It must be understood, of course, that this is fitted to a dacron bag incubation which gives values measured under conditions which prevent any passage resulting in an over-estimation of degradation. Therefore, the rate of decrease, K, as estimated by regression analysis of Cr_2O_3 passage data can be interpreted as the fractional rate constant. So if 'f' represents the fraction (by weight) of the Cr_2O_3 treated protein which remains in the rumen at 't' hours after feeding then $f=e^{-Kt}$ (31). Then for a feed free to pass from the rumen cumulative percentage protein degradation up to time 't' can be determined by integration.

$$Pt = \int_o^t f\left(\frac{dp}{dt}\right) dt$$

$$= a+ \frac{bc}{c+K}(1-e^{-(c+k)t})$$

A problem with this equation is the lack of a lag factor to account for the time required for solubilization and establishment of feed-bacteria contact. Krishnamoorthy and others (18) also recognized the differing fractions or pools of feed protein, a and b, and the fractional passage rate Ki, and were able to derive the following equation:

$$UDN = \sum_{i=1}^{n} Bi \left[\left(\frac{Kpi}{Ksi+kpi} \right) \quad (e^{-KpiLi}) \quad + \quad (1-e^{-KpiLi}) \right]$$

where: UDN = undegraded dietary nitrogen
Bi = percent total nitrogen
Kpi = fractional turnover rate
Ksi = fractional degradation rate
Li = lag time

The correlation of this prediction equation with *in situ* data, however, was poor (r^2=.41). These results are consistent with the view that first order *in vitro* rate constants for N solubilization do not necessarily reflect rumen degradation. While the data of Broderick (6) supports first order kinetics the lack of fit of the prediction equation to *in vivo* values may indicate that rumen proteolysis is zero-, first-, or even second order. Moreover, rumen turnover rates are affected by feeding frequency, type of diet, forage to concentrate ratio, and level of intake. Therefore, it would seem necessary to include these terms in a prediction model.

The solving of such a prediction equation would appear to take a great deal of time and effort, but with the advent of micro computers such an operation would require only seconds to calculate once the data has been entered. It is the lack of relevant data that restricts progress, and in my research I have attempted to reduce the time and labor required to determine the fractional degradation constant, Kd.

The two objectives of my research were to: 1) establish *in vivo* degradabilities for four protein feed supplements

using the synthetic bag procedure of Mehrez and Orskov (24); and 2) to evaluate several colorimetric protein assay methods for possible use as a final step in an improved *in vitro* degradable protein assay which utilized the ficin protease method of Poos-Floyd, et al. (34,35). Finally, the criterion of evaluation for each of the colorimetric assays was based upon the degree of correlation with the *in vivo* and standard ficin assay degradabilities.

MATERIALS and METHODS

Four protein feed supplements-- soybean meal (SBM), canola meal (CM), linseed oil meal (LOM), and 38% Dairy N.P. (38-D) (supplied by Honnegers and Co. Fairbury, IL appendix IV)-- were used as test materials for the development of the assay. In an effort to conserve time and reagents usually only one or two of the feed samples-- SBM and CM-- were digested in the *in vitro* assay. The remaining two feeds were saved in the event a colorimetric assay proved sufficiently accurate to warrant a study of more feeds in order to further characterize the assay.

A nylon bag procedure was carried out on the samples utilizing two ruminally fistulated, nonlactating Holstein cows and one Holstein x Angus crossbred steer. Three replications were made with this procedure. The first two with the Holstein cows and the third with the steer. In the first trial these animals were fed 20 lbs. grass hay fed at 0800 and 20 lbs. at 1600. In the second the animals were on a 40% forage :60% concentrate test diet which was fed at the same amount and frequency as the hay. In the third trial the steer was fed 30 lbs. of corn silage once each day at 0730. The dacron bags measured 5cm x 10 cm and had an mean pore size of 35 um. Approximately 1 g air dried sample was placed in a bag of predetermined weight. The

bags were then lyophylized for approximately 48 hours and then transferred to a dessicator until the bag with the sample was weighed and the weight recorded. The initial amount of dry matter was determined by difference. The tops of the bags were closed first with a drawstring and second with a number 10 rubber band looped several times around the top. Following this the bags were rinsed in tap water until the effluent was clear and then further rinsed in distilled water. Finally, three bags of each feed were attached to each paddle and two empty "blank" bags were also included. Each plexiglass paddle measured 6 cm x cm and had a nylon retaining string of approximately 50cm which was tied off outside of the fistula. The drawstrings were used to tie the bags to the paddles and in a manner to leave about 5 cm of free movement. The bags were evenly distributed along both long sides of the paddles. All paddles were placed in the rumen via the fistula and removed at 4,8, and 12 hours in trials one and two, and at 2,4,8, and 12 hours in trial three. At each designated interval the paddles were taken from the rumen and submerged in an ice water bath to halt the microbial activity. The bags were rewashed in tap water to remove solubilized feed material, microbes, and other rumen particulate matter. This was followed by a final rinse of distilled water and drying in a 100^{o}C forced air oven. Again the bags were retained in a dessicator until weighed and the residual DM determined by difference. The residual was then removed from the bag,

weighed, transferred to a flask, and residual nitrogen determined by macro Kjeldahl.

The *in vitro* assay chosen was that of Poos, et al. (35) (appendix I) and is hereinafter referred to as the ficin assay. This particular assay was chosen because the procedure has provided very reliable results. The assay has been altered in an attempt to make it more compatable with the colorimetric final step and these changes will be detailed. At the end of the digestion the solute was separated from the solid phase by filtration or centrifugation and the liquid phase was saved for colorimetry. The remaining solid was analysed for residual nitrogen by macroKjeldahl, and this value subtracted from total nitrogen was the gage used to determine the accuracy of each of the colorimetric methods.

The five colorimetric assays chosen were the Lowry (20), Bio Rad (3), A260/A280 (14), ninhydrin (2), and biuret (16,37). The absorbance was measured on a Gilford 2400S spectrophotometer using a 10mm light path and quartz cuvettes.

Two ficin sources were used in the assay. The first which was the most frequently used ficin was of a crude form and had an activity of 0.35 units per mg. The second source was of a purified form and had an activity of 1-2 units per mg. These values were determined as per Sigma (11, appendix II) and a unit is defined as a A280 of 1.0 per minute at pH 7 at 37^{o}C, when measuring trichloroacetic acid soluble

products liberated from casein in a reaction volume of 10.0 ml and a light path of 10 mm. The pH of the ficin and phosphate buffer solution was about 5.5 and for the complete digestion mixture was 6.5-6.8 which are the optimum pH ranges for initial activation and maximal activity of the ficin (7,50). The enzyme, in powdered form, was stored in an air tight container and kept under refrigeration (5°C) until needed. A fresh enzyme mix was prepared for each digestion run.

RESULTS and DISCUSSION

The results of the nylon bag studies appear in table 1. These results varied over time, diet, and oil meal (coefficient of variation = 5.8%). It was determined that triplicate feed samples were adequate to determine degradability due to the lower coefficient of variation (5.8%) than that reported by Mehrez and Orskov (24) (16.5%) utilizing quadruplicate feed samples.

TABLE 1. Percent Degradability by Nylon Bag

DIET**	1		2		3	
OIL MEAL*	SBM	CM	SBM	CM	SBM	CM
TIME (Hr)						
0	13.26	14.00	28.64	30.75	23.44	27.84
4	34.51	36.71	50.85	62.86	32.01	45.38
8	79.24	76.27	63.88	76.07	41.53	59.85
12	63.29	65.91	68.50	75.44	60.16	69.46

** - 1 = 20 lbs hay; 2 = 40 lbs 40:60; 3 = 30 lbs corn silage
* - SBM = soybean meal CM = canola meal

There was also an effect between diets. In diet 1 the results show an undulation in degradability values between 4 and 12 hours. Presumanly this is an effect of a second feeding occurring 30 to 45 minutes prior to the 8 hour sampling. The mechanism for this, however, was not determined. Perhaps this problem could have been avoided had the feeding interval been changed to twelve hours and the

paddles inserted during the morning feeding. There were significant ($P < .001$) effects of time by diet and diet by oil meal, but not time by oil meal or time by diet by oil meal (appendix III).

With diet 2 the degradability of the feeds seemed to be reaching an asymptote after 8 hours. The cause for the two fold difference in the 0 hour values of diet 1 versus diet 2 is unknown, but it may have been due to a technical error such as improperly weighing the feed samples or the bags. The initially greater degradability and the lack of fluctuating values following the pre-8 hour feeding is most likely a result of the high energy 40:60 (forage : concentrate) buffered ration. The level of readily fermentable energy enabled rapid microbial growth while the buffer, sodium carbonate, maintained a stable rumen environment and fermentation. Also the rumens of the animals on this diet were observed as being considerably wetter (gross observation) and this may have been a factor in the establishment of feed-microbe contact and the removal of the end-products of degradation. The degradability values determined with the steer on diet 3 are somewhat more linear than those of diet 2, but the feeds are still ranked similarly, plus the 0 hour values of diets 2 and 3 are in closer agreement than with the values of diet 1. A 2 hour degradability estimate was also determined on this third diet, but it was not used in the comparison. Because the third degradability trial is more comprehensive, was not influenced by a second

feeding, and contains the data of two other protein supplements it was the data chosen for evaluation of the colorimetric assays. The results of this third trial are depicted in figures 1a-1d for SBM, CM, 38%-Dairy, and LOM, respectively.

The ficin assay as per Poos-Floyd, et al. (appendix I) showed similar degradability trends and levels of variation as the composite nylon bag data. The coefficients of variation of two replicates within time periods of the ficin assay for SBM and CM were 6.2, and 5.6, repectively. The absolute values, however, and even the ranking of the feeds is reversed relative to the nylon bag studies (table 2).

TABLE 2. Degradability Values*: Nylon Bag vs. Ficin Assay

Nylon Bags			Ficin		
hr	SBM	CM	hr	SBM	CM
0	21.78	24.20	0	34.78	33.11
4	39.12	48.32	.5	61.44	56.26
8	61.55	70.73	1	68.92	63.51
12	63.98	70.72	2	80.40	70.52

*- composite of three trials or assays

The CM determinations for water solubility and overall degradability are in reasonable agreement, but the estimates for SBM differ by almost 20 percentage units. One possible explanation for this might be that ficin is capable of cleaving SBM at more or different residues than the enzymes of whole rumen bacteria. The increasing divergence of the *in vivo* and *in vitro* values of SBM as time of digestion increases is consistent with the findings of Poos-Floyd,

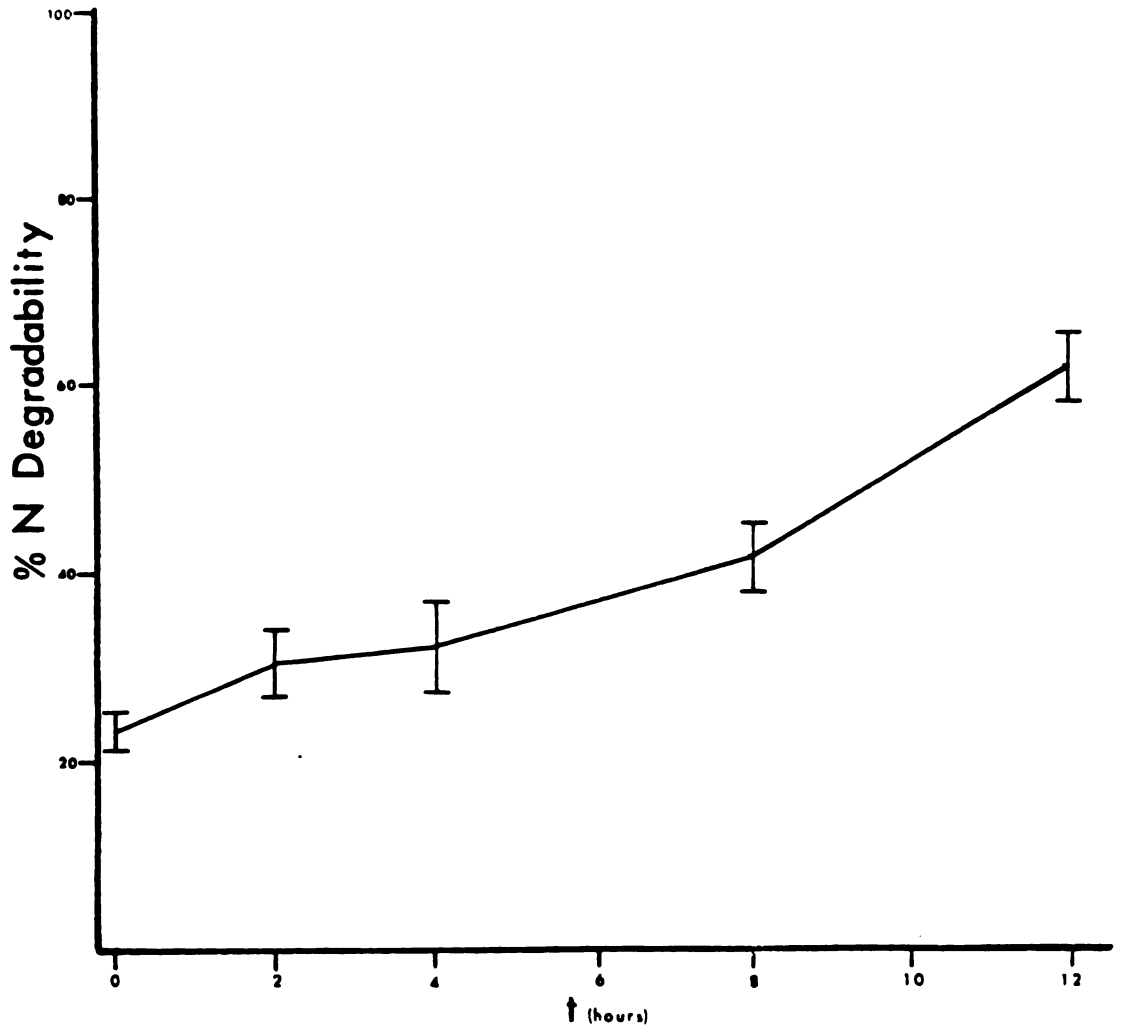

Figure 1a - Percent N degradability of SBM by nylon bag

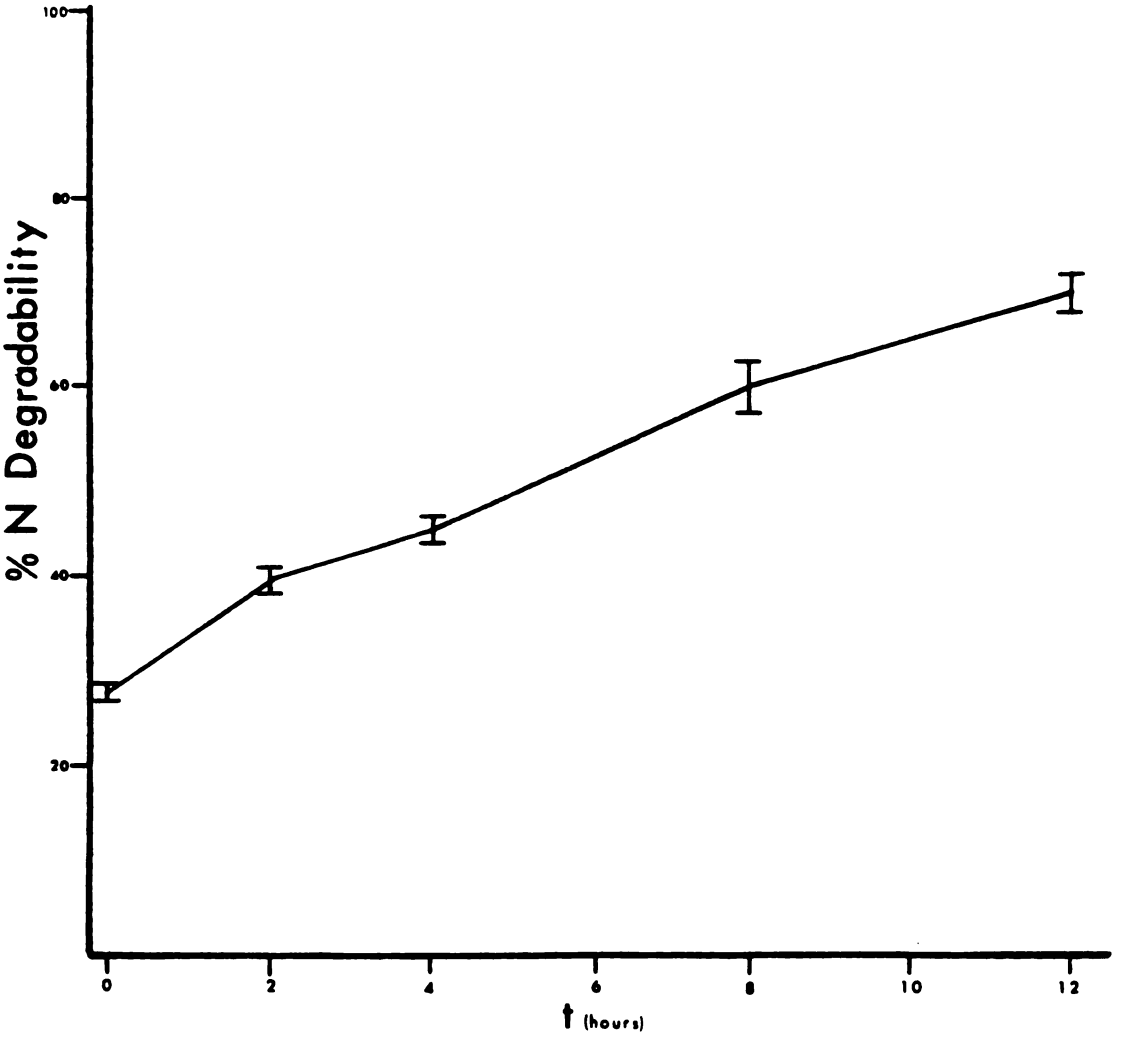

Figure 1b - Percent N degradability of CM by nylon bag

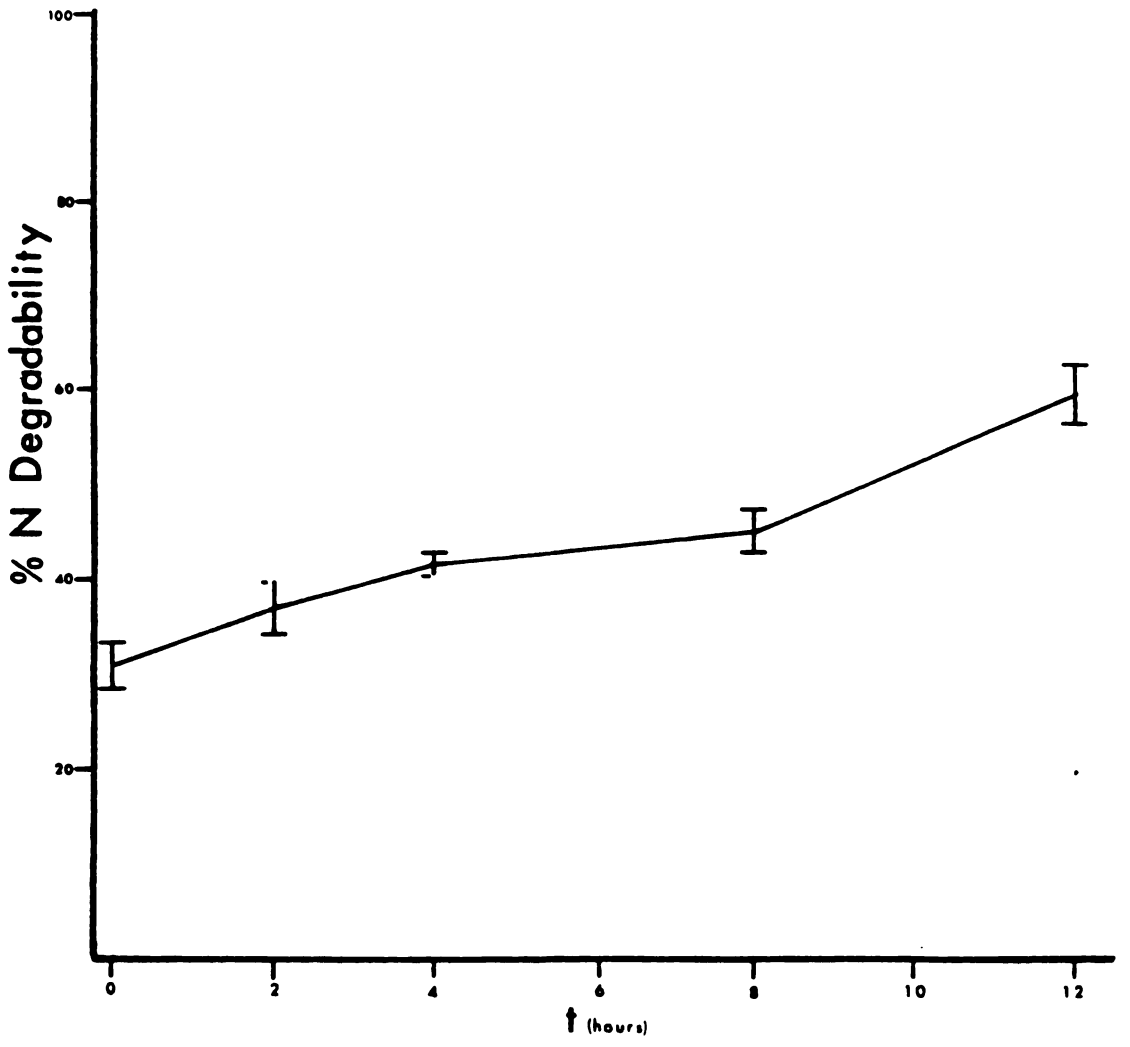

Figure 1c - Percent N Degradability of 38%-D by nylon bag

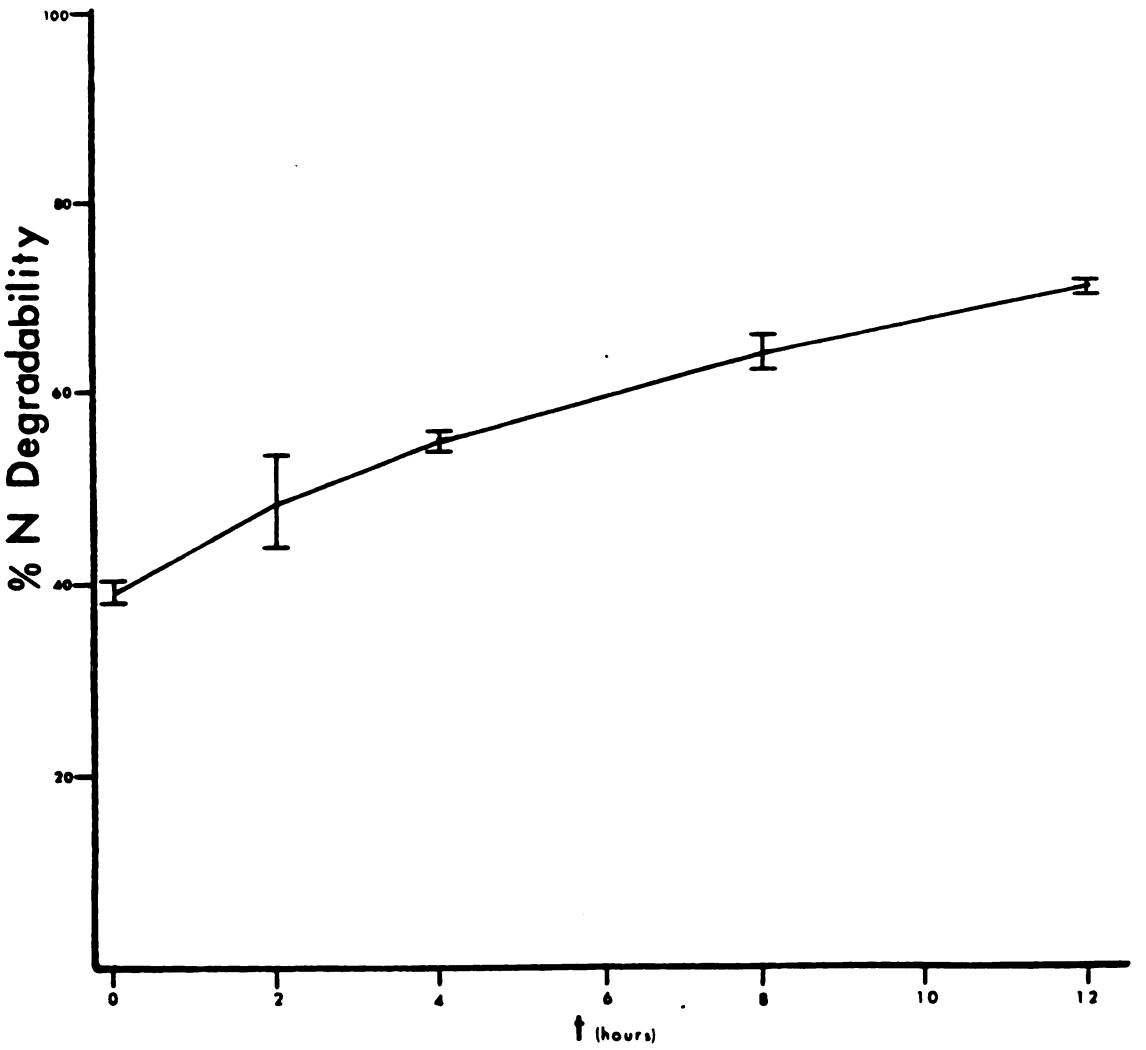

Figure 1d - Percent N Degradability of LCM by nylon bag

et al. (35) who noted a decreased correlation between the two determinations as the digestion progressed.

Initially the residual and solute were separated by filtering through #54 Whatman paper into a 500 ml Erlenmeyer vacuum flask. This solute plus 27.5 ml of distilled water used to rinse the tube were transferred to a clean tube for storage until assayed. This procedure soon proved to be too cumbersome, and the difficulties associated with a limited number of vacuum flasks and a large number of samples could be forseen. Thus, a new method was devised. A #6 rubber stopper with two 5 mm holes was placed atop a 100 ml (30 mm x 160 mm) centrifuge tube. In one hole a stainless steel elbow was inserted and a vacuum hose connected to the free end. A long stemmed, 60^{o} funnel was inserted into the other hole. This assembly (figure 2) was moved from tube to tube as each digestion was filtered. To minimize any contamination between tubes the funnel was thoroughly rinsed and shaken dry after each filtration.

This apparatus proved to be light, rapid, and simple, but one problem arose while filtering digests containing cycsteine hydrochloride. There was often considerable air inclusion during filtration leading to excessive foaming, and from time to time a small amount of foam would be drawn up into the vacuum line. How this affected the results is unknown, but this may have altered the final values if the foam was from the ficin mixture. The foaming did not occur when dithiothreitol was substituted for cysteine

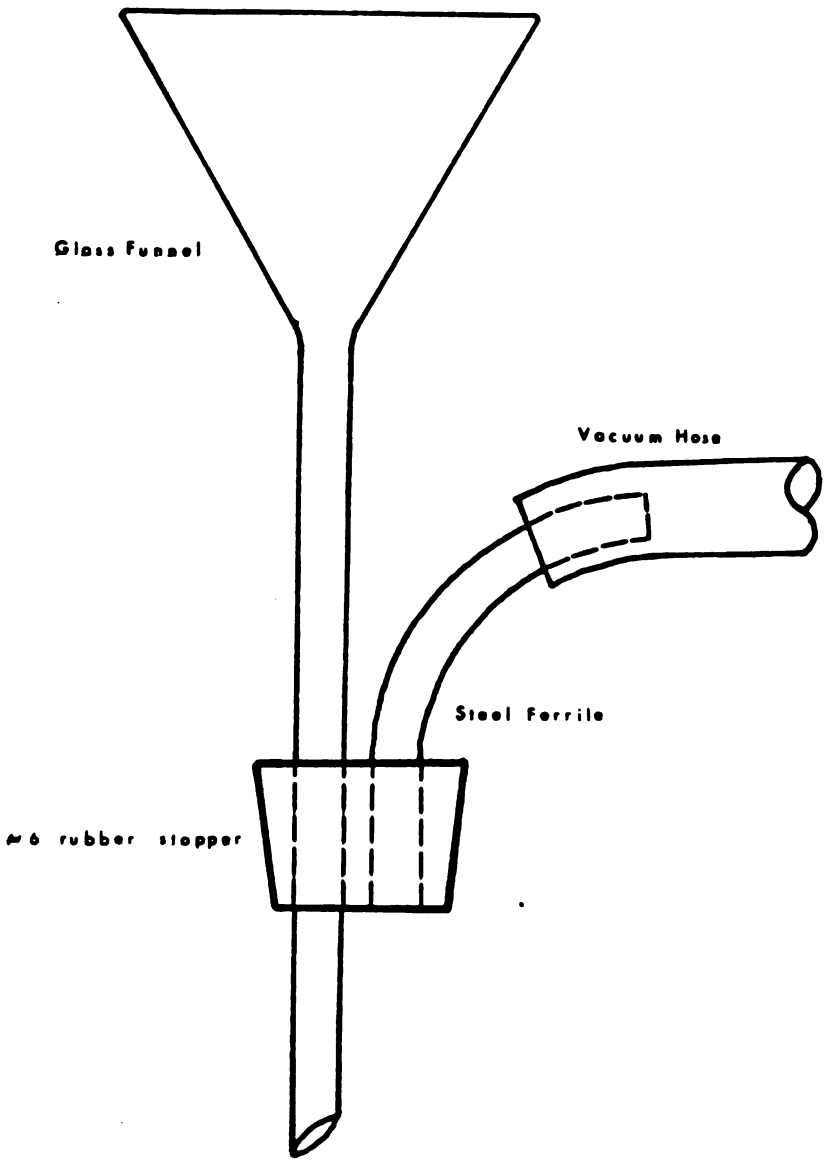

Figure 2. Funnel Assembly

hydrochloride, or it was no longer a factor once the solute volume was reduced.

The solute was held in the 100 ml centrifuge tube which was stoppered and refrigerated (5°C) until assayed colorimetrically. A small amount of a whitish-tan substance would precipitate out of solution after 24 hours, but quickly went into solution when the tube was shaken or vortexed. The solute usually remained usable for 14 days. After this time a mold often developed and the solute would have to be discarded.

The first colorimetric procedure tested was the folin-phenol method of Lowry, Rosebrough, Farr, and Randall (20). This assay was suggested primarily for its capability of detecting protein concentrations as low as 2 ug per ml. Unfortunately, the concentration of these solutes was uncertain so four dilution rates were tested: 500ul, 100ul, and 50ul all diluted to 1 ml. The standard was composed of bovine serum albumin (BSA) at a concentration of 3.2 mg N/ml diluted to 0.4, 0.8, 1.6, and 2.4 mg N/ml. A solution of the various components of the ficin digestion (triton x-100, buffers, ficin, etc.) was prepared in a ratio similar to the assay but at double the concentration. This was then distributed among labelled tubes at the four rates previously described and diluted to 0.5 ml with distilled water. To this 0.5 ml was added 0.5 ml of the respective BSA solutions bringing the final concentrations of the standard to .2, .4, .8, 1.2, and 1.6 mg N/ml. The analysis then

proceeded as per Lowry, et al. (20). All tubes were of approximately the same absorbance, but were too dark to read on the spectrophotometer so no quantitative data is available. While there were slight differences between dilutions the absorbance within dilutions was roughly the same. A white particulate matter was noted which could be precipitated by centrifugation, but this did not change the absorbance. Perhaps the $MgSO_4$ or NH_4 bicarbonate was the interfering substance, but according to Peterson (33) these, and all other constituents, were at acceptable levels in the final dilution. L-cysteine hydrochloride, however, was very near the upper limit in the final dilution so β-OH mercaptoethanol (ME) was substituted at a rate of 0.1 moles per mole of L-cysteine hydrochloride. This switch, unfortunately, only served to make the assay unwieldy because ME slowed down the filtration and the acrid odor permeated the entire laboratory. Due to these complications, the procedure of Lowry, et al. (20) was no longer used.

The second colorimetric method chosen was the dye binding Bio-Rad assay. This assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595nm when binding to a protein (3). The Bio-Rad concentrate was diluted 1 to 4 with distilled water and filtered through #54 Whatman filter paper to remove any coagulated material. Following this, one ml of standard (triplicate) or sample (duplicate) was placed in 16mm x 125mm test tubes with 4ml

of the diluted Bio-Rad solution. Each tube was vortexed for for 5 seconds, allowed to react for a minimum of 5 minutes, and the absorbance determined at 595nm. All tubes were of the same deep blue color and it was reasoned that the ficin solution was overloading the dye binding reaction. Therefore, a precipitation using 25% trichloroacetic acid (TCA) diluted 1 to 5 with a sample solution was tested. The solution of TCA and sample was vortexed, centrifuged at 2,000xg for 15 minutes, and then 1ml of supernatant was processed through the Bio-Rad procedure as previously outlined. There was a noticeable difference in color development this time -- all tubes were reddish brown. This is a result of the limited sensitivity of Bio-Rad which can only detect proteins of a molecular weight of 3,000 or greater, but TCA precipitates proteins having a molecular weight of greater than 2,500. This is the major reason this assay was discontinued, but another factor was the staining of the cuvettes. In order to maintain any reproducibility (even if it was all the same absorbance), the cuvettes had to be rinsed after every other sample with 95% ethyl alcohol to remove the blue tint.

The third method of choice was a A260/A280 analysis. This colorimetric procedure is based on an absorbance differential of 260nm versus 280nm and detects aromatic amino acid residues. The protein concentration (mg/ml) is then estimated by the equation:

mg protein/ml = (1.45 x A280) - (0.75 x A260) (14).

For this assay the samples had to be diluted 1 to 20 in order for the absorbance to fall within the ranges of the spectrophotometer. The degradability values derived from this procedure were quite varied demonstrating a composite coefficient of variation of 43%. Furthermore, the assay failed to correctly estimate the degradabilities as the mean degradability for SBM was only 30% and over 100% for CM. Because of the inaccuracies and variability this assay was no longer used.

The last two methods chosen -- ninhydrin and biuret-- were also the most frequently tested. Therefore, there are several changes in the ficin assay which are common between them.

First, 5ml of 25% TCA was substituted for the 2ml of t-butyl alcohol to halt the enzyme activity, as Krishnamoorthy, et al (18) showed that t-butyl alcohol had no significant effect on enzyme activity plus the TCA would also precipitate the ficin. This was followed by a centrifugation at 27,000xg for 20 minutes. It was this centrifugal force that produced the clearest supernatant (gross observation) in the shortest duration of time.

The second procedural change was to use a more purified ficin (1-2 units activity/mg) on an equal activity basis (1.7g ficin /l). Unfortunately, the degradation, as measured by Kjeldahl of the residual material, was severely decreased (table 3). The reason for this decreased degradative activity was not determined, but perhaps there is

something that is lost during the purification that aids in contacting the feed sample and/or binding the ficin to the feed. Due to this unfavorable result the purified ficin was no longer used.

TABLE 3. Degradation by Purified Ficin Enzyme

	hour	% degradability*	CV
SBM	0	11.01	18.1%
	1	16.63	22.0
	2	18.84	3.1
CM	0	24.45	2.3%
	1	37.00	2.9
	2	42.08	4.1

* - means of duplicate samples

Increasing sample size from .25g to .30g was the next alteration in procedure. The rationale being that if the enzyme could be saturated with substrate then only the feed sample would be undergoing degradation. This would eliminate self-degradation of the ficin and further degradation of liberated peptides. The majority of the liberated proteins would thus be of one fraction -- small peptides -- which would allow the use of the biuret while reducing the variation caused by protein existing in other undetectable forms such as amino acids or large precipitated peptides.

In order to minimize variability and interference an attempt was made to reduce the background nitrogen in the assay. There are only three reagents in the digestion that contain any nitrogen: ammonium bicarbonate, ficin, and

cysteine hydrochloride. The ammonium bicarbonate is in a very low concentration (<.8g/l), and, as demonstrated with the Lowry, the activity of the ficin was reduced when ammonium bicarbonate was omitted. Ficin is the enzyme that simulates microbial proteases, and without it the procedure becomes a solubility trial. The cysteine hydrochloride cannot be deleted as it is necessary to maintain a reduced state in the digestion under aerobic conditions (4). Moreover, cysteine acts not only as a reducing agent, but also as a coenzyme and an activator of ficin which involves the formation of dissociable compounds between the activator and the enzyme (50). Therefore cysteine cannot be deleted, but only substituted. The most logical substitute at this time appeared to be dithiothreitol (DTT). This dithioanologue of the reduced sugar threitol is a white powder with little, if any, odor, and the oxidation thereof results in an inactive cyclic disulfide which does not react with protein disulfides as β-OH mercaptoethanol has been known to do (40). The most effective concentration appears to be 15mM (2.313g/l phosphate buffer) as determined by the Sigma procedure (11). The enzyme and phosphate buffer solution seem to foam much less than when cysteine was used and it remained stable under refrigeration (5^{o}C) for periods of up to a week whereas the cysteine solution was usable for only 48 hours, maximum.

The fifth and final change was the substitution of 2ml of 50% sulfasalicylic acid (SSA) for TCA or PCA. SSA does not interfere with the Kjeldahl as does TCA, and it

precipitates large peptides (2,000 m.w.) more thoroughly than PCA resulting in a clearer and more uniform supernatant (gross observation).

The ninhydrin procedure was first tested with the solute from the digestion using the purified ficin enzyme. The assay was carried out as per AOAC (2) with a sample of the 27,000xg supernatant. The results showed a difference only between the 0 hour and 1 hour samples for canola meal, and even then the values severely underestimated the degree of degradation (table 4).

TABLE 4. Percent Degradation by Ninhydrin

oil meal hour	SBM	CM
0	1.04	1.29
1	2.97	3.11
2	3.88	3.00

The ninhydrin method was also tested with the solute from the digestion using DTT instead of L-cysteine hydrochloride primarily because of increased hydrolysis seen with other assays and now that cysteine was no longer a part of the assay perhaps the overloading previously encountered would be eliminated. Unfortunately, this was not the case. The assay was attempted twice and both times were unsuccessful. The color development was erratic and very unstable even though all reagents were made up fresh before each attempt. The only explanation to offer for this occurance is that possibly the 1,2,3 Triketohydrindene was

no longer stable as it was the only organic compound in the assay other than the digestion supernatant itself.

The final test of the ninhydrin procedure was made using the supernatant from the precipitation using 50% SSA. Unfortunately, similar problems were encountered with the ninhydrin so the results are still inconclusive.

The fifth and most promising method tested was the biuret assay of Koch and Putman (16) and Robinson and Hogden (37). Biuret appears to have two absorbance maxima at 275nm and 600nm (fig. 3). Koch and Putman (16) used this assay at 330nm for assaying whole bacteria with success, and I found similar success with standards and samples using their procedure. Regression coefficients for standards were often 0.999 or greater, and there was a noticeable difference between time periods of digestion. Similar results were seen with the method of Robinson and Hogden (37) who determined the absorbance at 560nm, and it was this wavelength that was chosen. At 560nm the area under the absorbance curve is greater which should allow one to discern differences between samples with greater accuracy, and the slope of the line at this point in the graph is low so any inaccuracies in wavelength will affect the results only moderately. Plus, at this wavelength there is much less variability due to electronic interferences.

There were, however, discrepancies within time periods. Within each time period the absorbance of the smaller sample was consistently greater than that of the larger sample.

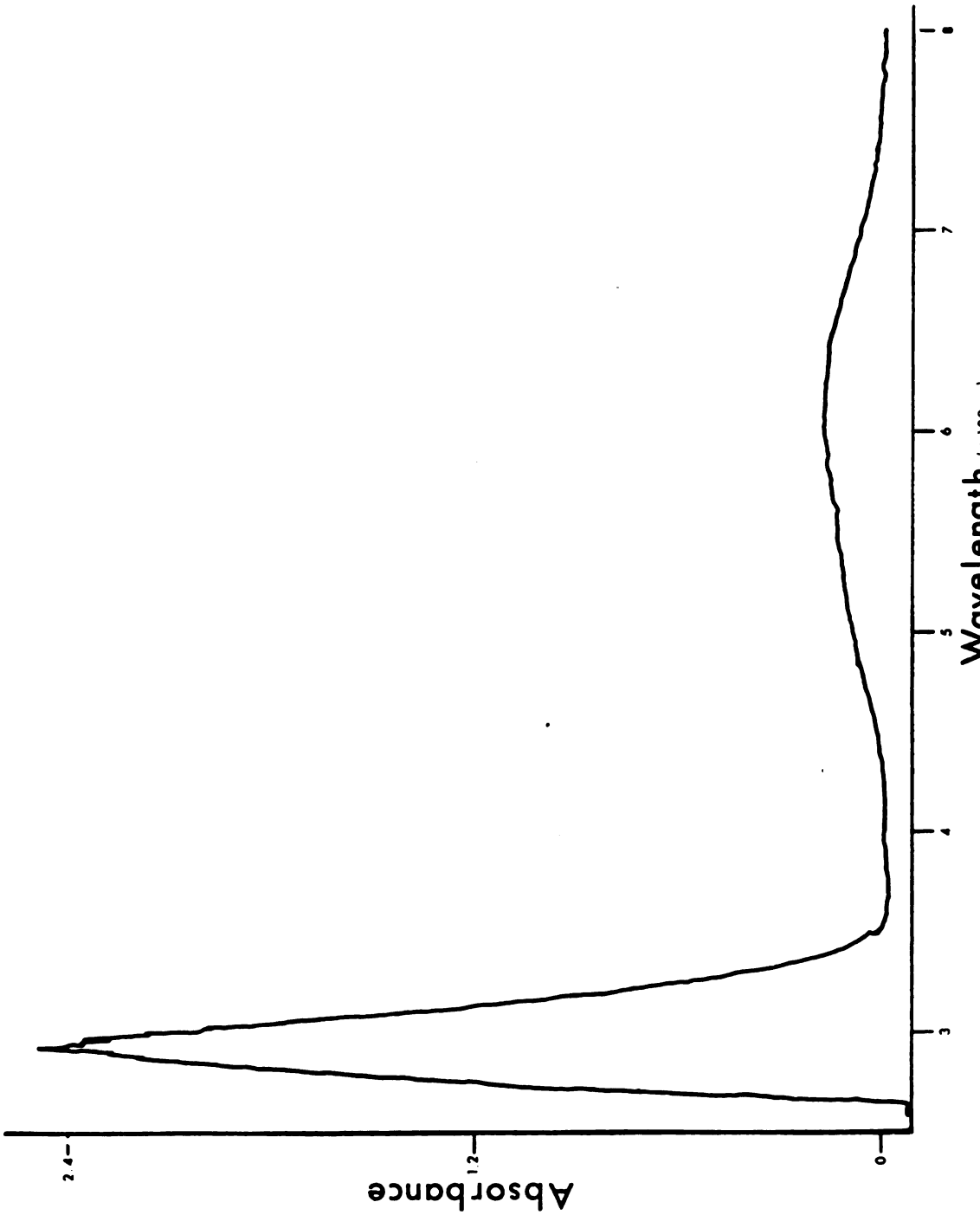

Figure 3 - Absorbance Maxima of Biuret

It was presumed that this was the result of the larger feed sample being able to bind more ficin thereby reducing the amount in the filtrate. Proceeding on the assumption that the ficin molecule was of a greater molecular weight than any of the digestion products an aliquot of filtrate was centrifuged at 2,000xg for 15 minutes without the aid of a precipitating agent. The gross appearance of the solute was not changed, but a dark brown precipitate was noted. This additional step served to sufficiently alter the absorbance such that the relationship with the original sample weights fell within expected values (table 5).

TABLE 5. Absorbance* Before and After Centrifugation

Hour	Sample weight (g)	Before	After
0	.2502	5.375	4.508
	.2519	5.240	4.720
1/2	.2502	5.308	4.500
	.2506	5.243	4.717
1	.2536	5.272	5.000
	.2546	5.161	4.899

* - Absorbance per gram SBM, n=2

In an attempt to further reduce variation the working volume was reduced in order to concentrate the soluble protein so that the aliquot used in the biuret assay would be of a greater proportion of the whole digestion solute and the ratio of soluble protein to background nitrogen (ficin, NH_4 bicarb, etc.) would be increased. The ficin solution was concentrated two fold so that only half of the original

amount need be used, and the volume of the rinse was reduced to 15 ml. Thus the final volume was now 38.5ml as originally used. This did not significantly alter the variation between samples within time periods, but did increase the differences between time periods (table 6).

TABLE 6 - Mean Absorbances* at Two Working Volumes

	55 ml		38.5 ml	
hour	Absorbance	CV	Absorbance	CV
0	1.281 1.377	5.2%	1.131 1.199	4.0%
1/2	1.582 1.481	4.8	1.308 1.241	5.2
1	1.528 1.523	5.6	1.535 1.581	2.1

*Absorbance: n=2, Coefficient of Variation: n=4

The biuret was also tested with the samples from the purified ficin digestion. It showed very favorable results with SBM in that trends with time were clearly visible, but the degradability was always ten percentage units below that which was expected. The canola meal, however, proved to be different. The only difference lay between 0 and 1 hour, plus the degradability was significantly overestimated -- 186% degradable.

Because of the poor results demonstrated with the purified ficin the procedure returned to using the crude grade ficin and the results of the Kjeldahls were more normal. When the biuret was tested with the two different

sample sizes there were no differences between sets of samples sizes in terms of degradability. The supernatant (27,000xg, 20 minutes) also underwent a Kjeldahl analysis in an attempt to correlate, or at least establish a ratio between, mg nitrogen per ml as determined by Kjeldahl versus mg nitrogen per ml as per biuret. The results of this comparison were varied, showed no trends, and in some cases were quite extreme. Part of the discrepancy may be due to TCA changing to chloroform during distillation of the Kjeldahl leading to a shift in the titration point (26). Substitution of 3ml of 3M perchloric acid (PCA) for TCA followed by centrifugation and neutralization with 3M $K_2 CO_3$ seemed to remedy this problem. The correlations of N content by Kjeldahl and biuret were .9794 and .9748 for SBM and CM, respectively. The graphs of the regression equations are depicted in figure 4. The slopes were not significantly different, but the combined regression of the biuret nitrogen determination on the values determined by Kjeldahl of SBM and CM was only .5841. The reason for the significantly different y-intercept of SBM cannot be explained.

The results of the biuret analysis on the solute with DTT showed that while the activity of the ficin was still the same when DTT was used, it appeared to be more efficient at hydrolyzing liberated peptides. The values, as indicated by the biuret, reached a maximum at the half hour interval, but the residual N values (Kjeldahl) continued to decrease

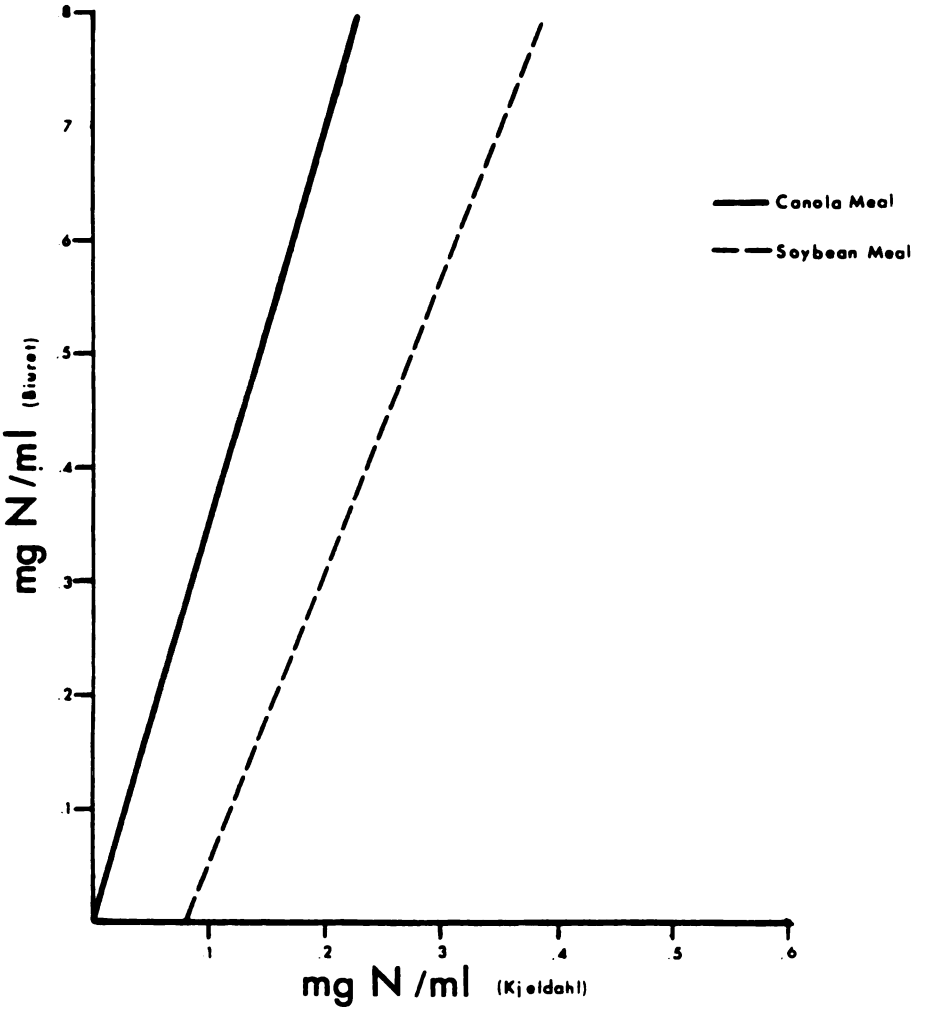

Figure 4 - N Determination of Solute: Biuret vs Kjeldahl

through two hours of digestion.

The final test of the biuret procedure was made using the supernatant from the precipitation using 50% SSA. Unfortunately, similar problems were encountered with this procedure so the results are still inconclusive.

CONCLUSION

The digestion and colorimetric assays are incompatable as they exist at the present time. Further changes will need to be made in either the digestion, colorimetry, or both in order for the procedure to be successful. Perhaps some of these changes include replacing ficin with a strict endopeptidase or increasing sample size even more so that the net effect would be more peptides and less free amino acids, and a greater compatability with the biuret assay. Perhaps using a different colorimetric procedure, but the biuret does seem to have the most promise of the five assays tested. This assay is simple, rapid, does not seem to be as adversely affected by interfering substances, and, other than the spectrophotometer, it requires no highly specialized equipment.

APPENDICES

APPENDIX I

Procedure for Determining Proteolytic Enzyme Degradation of Protein Feeds

Mary Poos-Floyd, Terry Klopfenstein, and R.A. Britton

Materials:

50 ml test tubes, #6 rubbers stoppers w/ small holes
Ficin
Incubator at 39-40°C

Reagents:

A. Stock phosphate buffer 0.5M
   10.54g K_2HPO_4 / litre

   59.66g KH_2PO_4 / litre

B. Working phosphate buffer 0.1M

   200ml stock phosphate buffer/litre
   6.1g cysteine hydrochloride/litre
   Ficin enzyme is dissolved in working phosphate buffer at a concentration of 4.3g/litre

C. 1% sodium azide solution (w/v)

D. 1% triton X-100 solution (v/v)

E. 80% t-butyl alcohol

F. In vitro rumen buffer and Macromineral solutions

In vitro rumen buffer	g/litre
Ammonium bicarbonate	4
Sodium bicarbonate	35
Macromineral solution	
Na_2HPO_4 (anhy.)	5.7
KH_2PO_4 (anhy.)	6.2
$MgSO_4$ $7H_2O$	0.6

Procedure:

A. Weigh out enough sample to provide 15mg N per tube.
   1. Prepare duplicate tubes for each sampling time
   2. Use 0, .5, 1, 2 hour samples

B. Prewet samples with 5ml distilled water 12 hours before next step.

C. Add 10ml of 1:1 solution of in vitro rumen buffer and macromineral solution to each tube.

D. Add 1ml sodium azide solution and .5ml Triton X-100 solution to each tube. Stopper and swirl and incubate at 39°C for two hours.

E. After two hours, remove tubes from the incubator and add 10ml of prewarmed enzyme solution to all except 0 hour test tubes. Stopper, swirl, and return to incubator.

   1. 0 hr tubes serve as an estimate of protein solubility.
   2. 0 hr samples should be filtered and washed through Whatman #541 filter paper.

F. At the appropriate time, remove tubes and halt incubation be adding 2ml of 80% t-butyl alcohol. Filter and wash samples on Whatman #541 filter paper and perform Kjeldahl analysis of the filter paper plus residue.

Calculations:

$$\% \text{ Residual N} = \frac{\% \text{ N Remaining}}{\% \text{ Total N}}$$

$$\text{Residual N as \% of insoluble N} = \frac{\% \text{ N Remaining}}{\% \text{ N in 0 hr sample}}$$

APPENDIX II

ENZYMATIC ASSAY of FICIN
(Sigma Chemical Co., February 1973)

I. Principle

Ficin hydrolyzes peptides, amides, and esters; its specificity is similar to that of Papain

II. Reagents and Procedure

Into two suitable vials labelled "Blank" and "Test" pipette the following reagents:

Reagents	BLANK	TEST
a. 2% casein in 0.1M KH_2PO_4 buffer pH 7.6, heat to dissolve, but DO NOT BOIL, cool to 37°C for assay	2.0ml	2.0ml
b. .25M L-cysteine, adjust to pH 7 with solid sodium bicarbonate	0.2	0.2
c. .25M EDTA	0.2	0.2
d. 25 N NaOH	0.2	0.2
e. 1.0M KH_2PO_4 buffer, adjust to pH 7.0 with NaOH	0.6	0.6
f. distilled water	0.8	0.4
Equilibrate at 37°C then, at zero time, add;		
g. enzyme solution for a crude powder prepare a solution in reagent 'e' containing 0.1 mg enzyme/ml	---	0.4

h. after adding enzyme solution mix well and incubate at 37°C for exactly 20 minutes.

i. add 6.0 ml of 5% TCA and let stand at 37°C for 60 minutes

j. filter through Whatman #50 or equivalent filter paper

k. read A280 of "Test" versus "Blank"

III. Calculation

$$\frac{A280}{20 \times \text{mg enzyme} / 10 \text{ ml reaction mix}} = \text{Units/mg}$$

IV. Unit Definition

one unit is equivalent to a Δ A280 of 1.0 per minute at pH 7 at 37°C when measuring TCA soluble products liberated from casein in a reaction volume of 10 ml and 1 cm lightpath.

APPENDIX III

STATISTICAL ANALYSES

ANALYSIS OF VARIANCE: SOYBEAN MEAL (ficin assay)

TABLES OF MEANS

TIME (MIN)	0	30	60	120
% DEGRADABILITY	34.78	61.44	68.92	80.40
REPS	6	6	6	4
SE		3.672		

ANALYSIS OF VARIANCE: CANOLA MEAL (ficin assay)

TABLES OF MEANS

TIME (MIN)	0	30	60	120
% DEGRADABILITY	33.11	56.26	63.52	70.52
REPLICATIONS	6	6	6	4
SE		3.042		

ANALYSIS OF VARIANCE: PERCENT NITROGEN DEGRADABILITY (ficin assay)

TABLES OF MEANS

TIME (MIN)	0	30	60	120
% DEGRADABILITY	33.94	58.85	66.22	75.46
REPLICATES	12	12	12	8

OIL MEAL	SBM	CM
	59.66	54.52

TIME		SBM	CM
0		34.78	33.11
	REPS	6	6
30		61.44	56.26
	REPS	6	6
60		68.92	63.51
	REPS	6	6
120		80.40	70.52
	REPS	4	4
SE	3.372		

ANALYSIS OF VARIANCE: SOYBEAN MEAL (nylon bags)

TABLES OF MEANS

TIME (HR)	0	4	8	12
% DEGRADABILITY	21.78	39.12	61.55	63.98

DIET	1	2	3
% DEGRADABILITY	47.58	52.97	39.28

DIET TIME (HR)	1	2	3
0	13.26	28.64	23.44
4	34.51	50.85	32.01
8	79.24	63.88	41.53
12	63.29	68.50	60.16

SE 3.082

ANALYSIS OF VARIANCE: CANOLA MEAL (nylon bag)

TABLES OF MEANS

TIME (HR)	0	4	8	12
% DEGRADABILITY	24.20	48.32	70.73	70.27

DIET	1	2	3
% DEGRADABILITY	48.22	61.28	50.63

DIET	1	2	3
TIME			
0	14.00	30.75	27.84
4	36.71	62.86	45.38
8	76.27	76.07	59.85
12	65.91	75.44	69.46

SE 2.736

APPENDIX IV

38% Dairy - N.P. - 337

GUARANTEED ANALYSIS

Crude Protein, not less than	38.0%
Crude fat, not less than	0.5%
Crude fiber, not more than	9.0%
Calcium, maximum	3.0%
Calcium, minimum	2.0%
Phosphorus, minimum	0.7%
Iodine, minimum	0.00009%
Selenium, maximum	0.0001%
NaCl, maximum	2.5%
NaCl, minimum	1.5%
Vitamin A	20,000 USP units per lb

INGREDIENTS

Animal protein products, plant protein products, processed grain by-products, grain screenings, cane molasses, Vitamin A supplement, D-Activated animal sterol, Vitamin E supplement, salt, calcium carbonate dicalcium phosphate, iron carbonate, manganous oxide, copper oxide, cobalt carbonate, calcium iodate, zinc oxide, zinc sulfate, magnesium oxide, natural and artificial flavors, sodium selenite

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