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Alternative Techniques for Measuring Nutrient Digestion in Ruminants[1,2]

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ABSTRACT: Because in vivo measurement of nutrient digestion in the rumen and small intestine requires ruminally and intestinally cannulated animals that are expensive, labor-intensive, and subject to error associated with markers and inherent animal variation, alternative techniques have been developed. Researchers have proposed various in situ or in vitro procedures for estimating ruminal and small intestinal nutrient digestion. This review summarizes these alternative techniques.

Key Words: In Vitro, Rumen, Small Intestine, Nutrients, Digestion

Introduction

In vivo measurement of nutrient digestion in the rumen and small intestine requires that animals be surgically prepared with cannulas in the rumen, abomasum or duodenum, and ileum. Use of cannulated animals requires suitable markers for calculating flow rate of digesta and differentiation between microbial and dietary nutrients flowing to the small intestine (Stern and Satter, 1982). Endogenous contributions of nutrients are difficult to measure, but they should be assessed to obtain accurate values of digestion; however, these data are limited. Because of increased public scrutiny elicited by the animal rights movement, the use of invasive surgical procedures for nutritional research has become more difficult to justify. In vivo measurement of nutrient digestion is expensive, labor-intensive, time-consuming, and subject to error associated with use of digesta flow rate markers, microbial markers, and inherent animal variation. Hence, alternative procedures for predicting nutrient digestion of dietary feed ingredients in the rumen and small intestine that are simple, reliable, and inexpensive are needed. Various in situ procedures and in vitro laboratory techniques have been used to predict ruminal digestion of feed ingredients, including the Dacron polyester bag technique, the two-stage in vitro method, gas production, continuous culture fermenters, enzymes, gel electrophoresis, ammonia N release, and near infrared reflectance spectroscopy. In addition, various techniques have been developed to estimate intestinal protein digestion, including bioassays, lysine availability, in situ mobile-bag, enzymatic methods, intestinal fluid, and a three-step in situ/in vitro procedure. This review summarizes the alternative techniques that have been used to predict ruminal and small intestinal digestion.

Methods for Measuring Ruminal Digestion

In Situ

The Dacron polyester or nylon bag technique has been used widely for estimating ruminal nutrient degradation because it is a relatively simple, low-cost method compared with methods involving intestinally cannulated animals. The technique involves suspending bags containing different feedstuffs in the rumen and measuring nutrient disappearance at various time intervals. Hence, it also may provide an advantage compared with laboratory methods because it involves digestive processes that occur in the rumen of a living animal; however, several factors affect estimates of nutrient digestion and need to be controlled for this technique to be standardized. These factors include porosity of bag material, ratio of sample weight to bag surface area, particle size of sample, method of bag placement in the rumen, diet of the animal, frequency of animal feeding, and degree of bacterial attachment to feed residues remaining in the bag (Lindberg, 1985; Nocek, 1988; Michalet-Doreau and Ould-Bah, 1992).

Several reviews recommended use of standardized in situ procedures to alleviate variation associated with these factors.

The in situ Dacron bag technique has been used to estimate ruminal DM and carbohydrate digestion, but it has been most commonly used to estimate microbial protein degradation in the rumen. Dewhurst et al. (1995) suggested that the in situ technique may not be as precise with forages as with concentrates or protein supplements because of the high proportion of water-soluble materials that can leave the bag unfermented. The Dacron bag technique was compared with the Tilley-Terry in vitro procedure, with a strong curvilinear relationship noted between carbohydrate composition of feeds and extent of overestimation of OM degradation using the Dacron bag technique (Dewhurst et al., 1995). This relationship can be partly attributed to completely unfermented material leaving the bags, which was a particular problem with highly fermentable feeds such as soybean hulls and corn germ. Dewhurst et al. (1995) concluded that the use of the Dacron bag technique to assess ruminal degradation of feeds with low ADF content, particularly those with ADF less than 25% of DM, is rather limited. When ADF content was greater than 25% of the DM, discrepancies between the in situ and in vitro techniques were less. However, lower in vitro digestibility with low ADF samples could have been related to a drop in pH of the inoculum rather than to losses of soluble substrates from the Dacron bag.

Use of the in situ Dacron bag to study protein degradation assumes that all soluble protein is completely and instantaneously degraded in the rumen; however, in some cases, rates of degradation for the soluble fraction could be slower than for the insoluble fraction (Mahadevan et al., 1980; Wallace and Kopečný, 1983). Also, all protein that disappears at zero time is assumed to be soluble protein; however, part of this protein leaves the bag because of smaller particle size relative to porosity of the bag material. Herold and Klopfenstein (1996) suggested that the in situ technique overestimates ruminal protein degradation of animal proteins compared with the ammonia release technique because of food particle losses from the bags during washing. Mansfield and Stern (our unpublished observations) found that use of recommended guidelines for particle size, ranging from .5 to 5 mm, presented problems in evaluation of heat-processed whole soybeans. Determination of protein protection from ruminal microbial degradation could be interpreted quite differently, depending on particle size. Reduction in particle size of roasted soybeans, by grinding at 2 mm, showed little protein protection compared with raw soybeans; however, grinding at 6 mm and cracking the soybeans decreased the estimates of ruminal protein degradation of the roasted soybeans by 17 and 41%, respectively, compared with that of raw soybeans. Grinding increases degradation of protein in feeds; however, the increase in degrada-

tion varies with the type of feedstuff (Michalet-Doreau and Cerneau, 1991). Further evaluation of grinding procedures and characterization of feedstuff particle size are required for this technique to be useful for a wide variety of feedstuffs. In addition, physical nature of a feedstuff may impede particle movement from the Dacron bag. Corn gluten meal is a glutinous material that has a tendency to stick together when wet, resulting in less exposure of surface area in the bag when placed in the rumen, possibly underestimating protein degradability (Stern et al., 1983a). Cozzi et al. (1993) showed that this limitation may be alleviated by mixing corn gluten meal with a fibrous material, such as sawdust, resulting in a higher degradation of corn gluten meal DM and protein.

Madsen and Hvelplund (1994) evaluated the reproducibility of CP content, N solubility, and protein degradability of measurements made in samples of the same feedstuffs in 23 laboratories using a proposed standardized in situ procedure. Mean protein degradabilities and standard deviations calculated assuming a ruminal outflow rate of 8%/h were 63 ± 11.1, 47 ± 8, 51 ± 9.1, 72 ± 8.4, and $23 \pm 5.5\%$ for soybean meal, coconut meal, cottonseed meal, barley, and fish meal, respectively. The range in mean protein degradabilities of the five feedstuffs was from 38.8 to 66.4% for the 23 laboratories, and there was an equal distribution of the estimates between the two extremes. Variation among laboratories in determination of protein degradability was mainly associated with differences in methods used for sample preparation and processing, type of filter, and nylon bag material used for incubation but was also influenced by low repeatabilities among laboratories in the determination of CP content. Madsen and Hvelplund (1994) recommended that greater emphasis be given to calibration of the CP analysis in the different laboratories and to standardization of the procedures for measuring N solubility on filter paper and protein disappearance from nylon bags. Wilkerson et al. (1995) evaluated the variation in estimates of ruminally undegraded protein of forage (smooth bromegrass hay) among eight laboratories. They found that reasonable control of error was accomplished by technician training, including the use of a demonstration videotape to clarify and standardize the procedure. However, it should be noted that their measurements were obtained at one time, 16 h, which does not account for soluble N or rate of degradation of the insoluble N fraction. The extent of protein disappearance from the bag at specific intervals of ruminal exposure does not provide a correct estimate for degradation because it does not account for protein contained in feed particles that leave the rumen with digesta flow.

Mathematical models have been proposed to combine estimates of degradation rate with outflow rates to estimate protein degradation (Ørskov and

McDonald, 1979; Mertens and Loften, 1980; Mathers and Miller, 1981; Negi, 1995; van Milgen and Baumont, 1995). Nonlinear models (Mertens and Loften, 1980) have been used more extensively to predict rate and extent of degradation of NDF than of protein, whereas the logarithmic-linear transformation has been more extensively used for protein. Huhtanen et al. (1995) reported lower NDF degradabilities using in situ values calculated by a linear model compared with results obtained in vivo and concluded that the use of a linear model can result in underestimation of NDF degradation. Consistent with this observation, Bach and Stern (our unpublished observations) noted faster rates and higher extents of protein degradation when a nonlinear model was compared with a linear model to evaluate CP degradation of several meat and bone meal samples. From these analyses, it seems that the use of nonlinear regression to predict protein degradation of meat and bone meal is more appropriate than a linear approach. Nonetheless, there were almost no differences between the two models when predicting degradation of soybean meal. Analysis of residuals vs fitted values should be done to assess whether linear or nonlinear equations are appropriate for studying degradation of a given protein source.

Broderick et al. (1991) emphasized that, although the in situ technique is imperfect in ways that cannot be fully compensated for, it is rapid, fairly reproducible, and requires minimal apparatus. However, this technique requires surgical preparation of an animal with a ruminal cannula and facilities for animal maintenance, which may be inconvenient and expensive.

In Vitro

Tilley and Terry Method. The technique first described by Tilley and Terry (1963) has been the most commonly used in vitro method for predicting digestibility and as a selection tool for improving the nutritional quality of forages. Several modifications of the original procedure have been used to maximize the digestion process because in vitro systems that do not maximize digestion kinetics may not detect differences in substrate digestion (Grant and Mertens, 1992b). Maximizing in vitro digestion depends on several factors, including dilution of the ruminal inoculum, type of buffer used, particle size of the sample, type of mill used for grinding, and type of diet the donor animal is fed.

Ruminal inoculum is typically strained through several layers of cheesecloth and diluted (20:80) in saline solution, artificial saliva, or various buffers. Craig et al. (1984) suggested that strained ruminal fluid alone was not as effective as strained ruminal fluid plus an inoculum of particulate-associated bacteria for simulating ruminal fermentation of fiber from different feedstuffs. Varel and Kreikemeier (1995) compared the in situ and the Tilley and Terry techniques using alfalfa or brome grass as substrates. Differences in lag time, rate of digestion, and extent of digestion were noted between the two techniques. Lag time was shorter, rate was faster, and extent of digestion was greater with the in situ than with the in vitro technique. Differences were attributed to a lower microbial concentration with the in vitro technique compared with microbial concentrations in the rumen of the animal. Attempts to increase the microbial concentration in vitro have not been successful because of a rapid accumulation of end products and a subsequent decrease in pH. The decrease in pH might be of major concern when using the in vitro technique to study fiber digestion because cellulolytic bacteria are more sensitive to low pH than are amylolytic species (Therion et al., 1982). However, Terry et al. (1969) demonstrated a minimal decrease in cellulose digestion with an addition of 40% glucose when pH was maintained at 6.8. Grant and Mertens (1992a) developed an in vitro buffering system capable of pH control between 5.8 and 6.8 that has been successfully used to study fiber digestion in vitro (Grant and Mertens, 1992c). Starch digestion also seems to be affected by pH in vitro. Richards et al. (1995) tested different dilutions (1:1, 1:2, 1:3, and 1:4) of strained ruminal fluid and artificial saliva to study starch digestion in vitro. Rate and extent of starch digestion were similar for the 1:2, 1:3, and 1:4 inoculum dilutions but were higher than with the 1:1 dilution. The lower rate and extent of starch digestion with the 1:1 dilution could have resulted from a lower pH noted in vessels when ruminal fluid was less diluted. The authors recommended that dilutions of 1:2 or 1:3 be used when studying starch digestion of grains that are rapidly fermented.

Richards et al. (1995) showed that the type of mill used to grind the sample also affects in vitro digestibility. Several feeds were tested for starch digestion, and the ranking was different when samples were ground through a 1-mm screen using a Udy mill compared with a Wiley mill. Moreover, greater starch digestibilities were noted when samples were ground through the Udy mill than when they were ground through the Wiley mill. Screen size also might affect digestibility (Cone et al., 1989; Richards et al., 1995). Samples ground to pass 2-mm and 1-mm screens yielded similar starch digestibilities (Richards et al., 1995). However, Cone et al. (1989) reported increasing rates of starch disappearance when particle size decreased from 1 to .1 mm.

Cone et al. (1989) showed that the time of collection of ruminal fluid and the type of diet fed to the animal had an effect on the results of the in vitro technique. In vitro starch disappearance was greater when ruminal fluid was obtained from a concentrate-fed cow than when it was obtained from a hay-fed cow. Although digestion values differed, ranking among feeds was independent of the donor cow diet; Richards et al. (1995) made similar observations.

When conducting in vitro techniques, maintenance of anaerobic conditions is important. Grant and Mertens (1992b) tested several factors that could affect fiber digestibility values using the in vitro technique, including maintenance of anaerobic conditions. Purging tubes with CO_2 but not gassing continuously resulted in a 56% increase in lag time for NDF digestion and a 69% decrease in rate of NDF digestion. These authors also suggested the use of nutritional additives such as microminerals, vitamins, and tryptone to ensure that nonfiber factors did not limit fermentation, especially with substrates low in protein and microminerals.

Another approach to the Tilley and Terry technique is the use of reconstituted feces as inoculum (El Shaer et al., 1987). Nsahlai and Umunna (1996) used reconstituted sheep feces as inoculum to determine in vitro digestibility and reported a coefficient of correlation of .88 between DM digestibility obtained using reconstituted feces and DM digestibility obtained using ruminal fluid. This modification of the in vitro procedure has an advantage because it does not require a ruminally cannulated animal.

Gas Production. Anaerobic digestion of carbohydrates by ruminal microbes produces VFA, CO_2, CH_4, and traces of H_2; hence, measurement of gas production in vitro can be used to study the rate and extent of digestion of feedstuffs (Hungate, 1966). Theodorou et al. (1991) developed an in vitro method to measure head-space gas accumulation that does not require expensive glass syringes like the gas production method previously described by Menke et al. (1979). A similar procedure for measuring gas production was described by Pell and Schofield (1993) and was recently revised (Schofield and Pell, 1995). The revised procedure added a new step, which consisted of recalibration of the pressure sensors by infusing a known volume of CO_2 every time they were used. In any case, it is recommended that culture bottles be vented frequently during the rapid gas production phase to avoid accumulation of gaseous components in the head space that ultimately decrease rate of fermentation of substrate (Theodorou et al., 1994). Volume readings are subject to considerable variation in relation to altitude (Theodorou et al., 1994) and should be accounted for when comparing data from different laboratories. The gas production method requires the use of an inoculum or medium with low fermentable energy, so that gas accumulation is low in the blank control fermentation. The use of gas production to study carbohydrate digestion presents an advantage over the traditional gravimetric method because it accounts for both soluble and insoluble substrates (Pell and Schofield, 1993). High correlations between gas production and NDF disappearance, $r^2 = .99$ (Pell and Schofield, 1993) or gas production and DM disappearance, $r^2 = .95$ (Prasad et al., 1994) have been reported.

Pell and Schofield (1993) suggested that under conditions in which nutrients are not limiting, gas production is a direct measure of microbial growth, and in some respects is a better index for predicting forage ME than the indirect measure based on NDF disappearance. However, conditions in which nutrients are not limiting are difficult to achieve as fermentation in the flask proceeds and substrate is digested. Total gas production is the result of various fractions being fermented at the same time, but at different rates, which leads to complex multiple rates that are difficult to partition. Schofield and Pell (1995) suggested that plotting rates of gas production as a function of time, calculated by subtracting gas volumes at adjacent times, can be used to identify pools that are digested at different rates.

One of the most challenging problems associated with using gas production methods is that the amount of gas produced varies with different molar proportions of VFA. For example, a higher propionate concentration is associated with lower gas production because an extra carbon atom in propionate would otherwise have appeared as CO_2 (Wolin, 1960). Schofield and Pell (1995) suggested that it is important to monitor the molar proportions of VFA to correct for such differences. Several authors have proposed different mathematical approaches to handle this problem. Beuvink and Kogut (1993) tested different models (i.e., exponential, logistic, Gompertz, and Richards) to describe the gas production of grass silages as related to ruminal DM digestion and concluded that none of the models described gas production satisfactorily. They developed a modified Gompertz model that successfully described not only gas production from grass silage samples, but also from other feedstuffs such as corn silage and palm kernel expeller. Furthermore, Schofield et al. (1994) developed a model with the ability to study rate and extent of digestion of specific subfractions for a given ingredient. After testing various mathematical models, including the Gompertz model, Schofield et al. (1994) concluded that a modified dual-pool logistic equation with a single lag value was the best model to predict cellulose digestion using gas production procedures.

Prasad et al. (1994) compared digestibility values of millet straw measured in vivo with the gas production method and concluded that the best fit was obtained with 45 to 52 h of in vitro gas production. However, extent of digestion for some samples was higher in vivo compared with the gas production method. Khazaal et al. (1993) reported different correlations, depending on the length of incubation time, ranging from .58 to .84 between the volume of gas produced and in vivo apparent digestibility of several forage samples.

Most research using in vitro gas production has been conducted with forages. Further investigation is required to evaluate the ability of in vitro gas

production to predict digestibility of highly fermentable substrates.

Enzymes. Enzymatic techniques have the advantage of being completely independent of the animal, which should result in less variation, thereby making this technique relatively simple to standardize. Conversely, the biological validity of the results can be limiting as a result of incomplete enzymatic activity compared with the ruminal environment. Mahadevan et al. (1987) found large differences when comparing digestion of different protein sources using protease from *Streptomyces griseus* with an extract of ruminal microbial enzymes. They concluded that the use of nonruminal enzymes in an in vitro system for predicting dietary protein degradation may be of limited value, or even misleading because nonruminal enzymes may not have the same action as those of ruminal origin. For example, proteases from *S. griseus* and *Bacteroides amylophilus* have broad specificity for cleavage of peptide bonds; however, they may have different specificities for protein structures than enzymes from mixed ruminal bacteria. Chamberlain and Thomas (1979) reported that, although rate constants can be calculated using these proteases, results do not always rank proteins in the same order as degradabilities estimated in vivo.

When enzymatic techniques are used to predict microbial fermentation in the rumen, it is crucial that the enzyme concentration is sufficient to saturate the substrate. When enzyme concentration is limiting, accumulation of end-products during incubation can lead to a progressive inhibition of the enzyme activity. Krishnamoorthy et al. (1983) attempted to simulate ruminal proteolysis in vitro by choosing a protease enzyme concentration (.066 units/mL) that would provide a proteolytic activity similar to that of whole ruminal fluid. Feed samples were subjected to proteolysis for 18 or 48 h to resemble mean retention times in the rumen of grain and roughages, respectively. Residual CP at the end of incubation was considered to estimate undegraded CP. These values and those determined in vivo for 12 mixed diets were in relatively close agreement (r = .78; *P* < .01). Krishnamoorthy et al. (1983) indicated that the technique could be used to evaluate feedstuffs on a relative basis, and that it also offered the potential to allow assessment of the influence of dynamic ruminal criteria, such as proteolytic activity and retention of feed, on the predicted undegraded CP by manipulation of enzyme concentration and incubation time.

In addition to enzyme concentration, pH also influences proteolysis. Protein conformation changes depend on the pH to which the protein is exposed; for example, a pH of 8 will favor protein solubility and increase the level of high molecular weight peptides. Such pH dependency could explain the low correlation found between the enzymatic method at pH 8 and the in situ method when using protease extracted from *S. griseus* (Assoumani et al., 1992). They found that when *S. griseus* protease was used at pH 6.5, the correlation between methods was increased. Krishnamoorthy et al. (1983) showed that the maximal activity of protease from *S. griseus* occurs at pH 8; however, they noted a higher correlation between *S. griseus* and in situ estimates with incubations at pH 6.8 vs 8. Mahadevan et al. (1987) used *S. griseus* in a solution of pH 6.8 to predict ruminal degradation of different protein sources and found improbable results, such as a higher extent of degradation from blood meal and fish meal than from soybean meal.

The presence of reducing factors also may affect enzyme activity or protein degradation because certain proteins may precipitate in a non-reducing medium (Kohn and Allen, 1992). Kohn and Allen (1995) reported increased proteolytic activity of an enzymatic mixture extracted from ruminal microbes when reducing environments were used to perform the test. However, in a similar experiment, Mahadevan et al. (1987) reported that the addition of reducing agents did not affect protein degradation.

Ruminal bacteria possess primarily serine, cysteine, and metallo-proteases (Brock et al., 1982). Various enzymes with similar activities have been evaluated for their ability to predict ruminal degradation of dietary protein. Poos-Floyd et al. (1985) examined five commercially available proteolytic enzymes for their ability to estimate ruminal protein degradation. The five enzymes were a bacterial protease (*S. griseus*), three plant proteases (i.e., papain, ficin, and bromelain), and a neutral fungal protease. All proteolytic enzymes yielded high correlations, ranging from .78 to .93, with protein degradation of various feedstuffs for incubation of 1 h. Of the enzymes evaluated, ficin and neutral fungal protease yielded the highest correlations with ruminal protein degradation. In contrast, Lindquist et al. (1989) reported low correlations between results of the ficin enzyme procedure and in situ measurements (r = .20, n = 42) and between results of ficin and continuous culture measurements (r = .44, n = 28) for estimating protein degradation of various protein sources. They concluded that the ficin assay was not reliable for predicting ruminal degradation for a wide variety of feed proteins, but results improved when feeds were classified into groups.

Kopečný et al. (1989) used different protein sources to compare the rate and extent of protein degradation determined using fromase, alcalase, papain, pronase E (endoprotease from *S. griseus*), rennin, trypsin, or trypsin plus fromase with the rate and extent of protein degradation determined using the in situ technique. The highest correlation between the in vitro and in situ results was found with papain (r = .90). When protein sources were sorted according to the absolute value of CP degradation estimated with each enzymatic technique, the order differed from the ranking from in situ results. Aufrère et al. (1991) evaluated N solubility in a phosphate buffer and the

utilization of *S. griseus* as methods for predicting in situ CP degradation and reported that both methods provided the same precision when feeds were grouped in classes. Susmel et al. (1993) used a group of 10 feeds to compare in situ and in vitro methods and found the *S. griseus* assay had the highest correlation (r = .89) with in situ values. Tománková and Kopečný (1995) compared estimates of protein degradation using papain, bromelain, and pronase E with the in situ technique. Bromelain showed the highest correlation with in situ.

Ruminal enzyme extracts have also been used to predict CP degradation. Kohn and Allen (1995) used enzymes extracted from ruminal microbial cells by using a butanol-acetone extraction to predict ruminal protein degradation, with the activity of the enzyme extract tested under various conditions. The enzyme extract seemed to provide a good estimate of protein degradation during the first 2 h of incubation, but after 2 h, protein degradation decreased dramatically. Results obtained were less than the values for the same feeds reported by NRC (1989) using in situ or in vitro techniques in the presence of microbial inhibitors (Broderick, 1987). Because the enzymes were derived directly from the rumen, time of extraction or diet fed to the animal could influence the proteolytic activity of the enzymes extracted. Moreover, low degradability could have resulted from autolysis of the mixture of enzymes, thereby decreasing enzymatic activity over time. Luchini et al. (1996a) used 15 feed proteins to compare proteolytic activities of strained ruminal fluid with a mixture of trypsin, chymotrypsin, and carboxypeptidases A and B. Rates of protein degradation using strained ruminal fluid ranged from .007 to .217/h; degradation rates using the enzyme mixture ranged from .010 to .079/h and were lower ($P = .004$) than with strained ruminal fluid. Lower rates of degradation with the enzyme mixture can be attributed to low proteolytic activity of the mixture, which might have resulted from autolysis as described above.

With enzymatic techniques, protein degradation of samples with high starch contents does not correlate with in situ results as well as samples with low starch content. The presence of starch in cereals may decrease enzyme access to the protein (Pernollet et al., 1982). Assoumani et al. (1992) suggested the use of α-amylase when studying feeds that have a starch content of 23% or more. Tománková and Kopečný (1995) showed that the addition of α-amylase to a bromelain solution does not have a negative effect on the proteolytic activity of the protease, and that it is possible to incubate both enzymes together. The correlation coefficient between in situ results and bromelain treatment without amylase was .26, whereas the addition of amylase significantly increased the correlation to .81. In contrast, Roe et al. (1991) showed that the *S. griseus*, ficin, and neutral protease with amylase methods did not yield feed protein degradation curves that had a relationship consistent with those from the in situ technique.

Moreover, neutral protease with amylase was the only method that ranked feedstuffs similarly to the in situ techniques. Similarly, neutral (*Bacillus subtilis*) and alkaline (*S. griseus*) proteases at pH 6.5 with amylase provided lower estimates of protein degradation but ranked eight feedstuffs similarly to in situ values (Assoumani et al., 1992). It also has been suggested that fibrolytic enzymes be added when studying protein digestion of forages. Abdelgadir et al. (1996) demonstrated that pretreatment of forages with 8,000 units of cellulase per gram of DM resulted in similar undegradable protein values using *S. griseus* proteases compared to in vivo results.

Enzymatic techniques also have been used to determine carbohydrate degradation in the rumen. Marten et al. (1988) used a fungal cellulase to predict fiber digestibility of a large set of forage samples (n = 499) and reported a high correlation ranging from .93 to .98 and similar ranking between the in vitro Tilley and Terry technique and the fungal cellulase method. Cone (1991) prepared a cell-free enzyme extraction from ruminal fluid for predicting degradation of starch in the rumen and observed a correlation of .96 between results obtained with the enzyme extract and those obtained with Tilley and Terry in vitro techniques. Cone (1991) also used a solution of α-amylase, and another of pancreatin, which yielded lower correlations with in vitro values using ruminal fluid (r = .77 and .65, respectively), and concluded that complete degradation of starch granules requires the presence of several non-amylolytic enzymes that were present in the cell-free extract. Lila et al. (1986) combined amylolytic, cellulolytic, and proteolytic enzymes to estimate the digestibility of forages in vitro. However, correlations between these enzymes and in vivo results were low, ranging from .11 to .49. De Boever et al. (1988) tested several modifications of two previously described enzyme procedures (Jones and Hayward, 1975; Kellner and Kirchgessner, 1976) that included cellulase and pepsin incubations. All the methods gave consistent results with the in vitro Tilley and Terry procedure when corn silage was studied; however, none of the procedures was accurate with grass silage and hay.

Electrophoretic Analysis. Rate and extent of protein degradation in the rumen is related to amino acid composition and type of protein, such as albumins, globulins, prolamins, and glutelins. Gel electrophoresis is used to separate different fractions of protein according to frictional resistance or charge of the protein particle when it is forced to migrate through a viscous medium by the action of an electrical field. The relationship between fractional protein content and digestibility has been studied in monogastrics (Eggum and Beames, 1983), but little information is available concerning the relative contribution of each fractional component to dietary protein that leaves the rumen undegraded (Rogmanolo et al., 1994). The use of SDS-PAGE allows isolation and direct quantification of feed proteins and their degradation products. Therefore, this methodology not only can be used to predict

ruminal degradation, but also to assess the type of proteins that will escape the rumen (Messman and Weiss, 1994). Van der Aar et al. (1983) demonstrated that SDS-PAGE techniques could detect differences in ruminal degradability between untreated soybean meal and alcohol-treated soybean meal. Alcohol treatment decreased the proportion of β-conglycinin. Lower contents of β-conglycinin have been related to slower rates of protein degradation (Rogmanalo et al., 1990).

Messman and Weiss (1994) incubated several feedstuffs in ruminal fluid for 2 and 20 h. Extracts of feed proteins and particle and fluid fractions of the residue after incubations were electrophoresed and densitometrically scanned. Correlations of .94 and .95 for the 2- and 20-h incubations were noted between their technique and degradation values in NRC (1989). Rogmanolo et al. (1994) used electrophoretic analysis to estimate ruminal degradabilities of corn protein and concluded that zein from corn and distiller grains is relatively resistant to ruminal degradation, whereas albumins, globulins, and glutelins were more rapidly degraded. Messman and Weiss (1994) and Shwingel and Bates (1996) also have shown that glycinin is highly undegradable. Results obtained with SDS-PAGE are consistent with values obtained using enzymatic procedures (Aufrère, 1982) or bacterial cultures (Mahadevan et al., 1980).

Electrophoretic analysis is less labor-intensive and less expensive than determining in vivo digestibility and also allows measurement of the soluble protein fraction. However, more efficient extraction methods are needed for SDS-PAGE to be used for various feedstuffs because current techniques do not allow for extraction of proteins from certain feedstuffs such as fish meal or corn gluten meal (Messman and Weiss, 1994).

Near Infrared Reflectance Spectroscopy. Near infrared reflectance spectroscopy (**NIRS**) has proven to be a rapid, inexpensive, and fairly accurate method for estimating chemical composition of various feedstuffs. This technique also has potential for estimating DM and protein degradation of feedstuffs in the rumen. Marten et al. (1988) developed prediction equations for estimating ruminal degradation of different forages, reporting coefficients of multiple determination that ranged from .87 for birdsfoot trefoil to .97 for alfalfa. Reeves et al. (1991) attempted to predict digestibility of forages using wet and dry samples and reported superior results using dry samples. Low predictive value when using wet samples has also been reported with silages (Reeves et al., 1989). Possible reasons for inferior results with wet samples could be peak broadening or alterations in the structure of sample components at the molecular level, including changes in ionization, H-bonding, or crystallinity (Reeves et al., 1991).

Todorov et al. (1994) compared degradability of DM and CP for 34 forages, consisting of grasses, legumes, straw, haylage, and dehydrated alfalfa, using NIRS and in situ procedures. The relationship between NIRS and CP degradability was lower than between NIRS and DM digestibility. Differences were attributed to a lower accuracy using in situ determination of CP degradability of forages because of bacterial contamination. The authors concluded that NIRS has potential for predicting DM and CP degradability of forages, which could save a considerable amount of time and money.

Antoniewicz et al. (1995) used 16 grass and 38 alfalfa samples harvested in advancing maturing and growth stages to test the applicability of NIRS for estimating ruminal CP degradability. The NIRS technique predicted protein degradability of dried grass and alfalfa forage quickly and accurately, and samples from the two families could be determined simultaneously based on a common calibration equation. The NIRS technique estimates accounted for 87 to 99% of the variance in protein degradability affected by maturity and growth type of forage. Halgerson et al. (1996) reported that the ficin procedure could replace the in situ procedure for estimating ruminal CP degradability of forages, and that NIRS could be used to predict CP degradability determined by either method. Tremblay et al. (1996) evaluated NIRS as a technique for estimating ruminal CP degradability of roasted soybeans. They used scans from 121 samples to develop calibration equations and a validation set of 145 samples to evaluate the accuracy of the equations for predicting undegraded protein. The coefficient of determination between NIRS and undegraded protein estimated by the inhibitor in vitro technique was .70, and it was concluded that further research is needed to confirm that the NIRS method can be used successfully to estimate ruminal undegradable protein in roasted soybeans and other feedstuffs.

In Vitro Inhibitor Techniques. Attempts have been made to use ammonia release from protein sources incubated in vitro with mixed ruminal fluid inoculum as an index of ruminal protein degradation. However, ammonia concentration is the result not only of microbial degradation of protein but also of microbial utilization, and concentrations are influenced greatly by the amount and nature of the carbohydrates that are fermented (Miller, 1982). For example, Broderick (1982) showed that ammonia release from corn and sorghum grains was negative, which occurred because readily fermented carbohydrate stimulated greater microbial uptake than release of ammonia. In an attempt to alleviate this problem, Broderick (1987) developed an in vitro procedure for estimating ruminal protein degradation rate that uses chloramphenicol and hydrazine sulfate to inhibit protein synthesis and amino acid deamination by ruminal microbes, which otherwise would be influenced by fermentable substrates such as starch. Hydrazine sulfate is a noncompetitive inhibitor of phosphoenolpyruvate carboxykinase and, therefore, blocks gluconeogenesis, so that

microorganisms cannot use the carbon skeleton of amino acids as a source of glucose. Chloramphenicol is an antibiotic that interrupts microbial protein synthesis by blocking protein translation. Because neither of these inhibitors decreases proteolytic activity, the inhibitor method might be expected to yield useful kinetic estimates of protein degradation. This method and the in situ procedure ranked proteins similarly for degradation; however, extent of degradation estimated by the two methods differed significantly (Broderick et al., 1988).

Hristov and Broderick (1994) modified the in vitro inhibitor procedure by using $^{15}NH_3$ to correct for microbial incorporation of NH_3N. Peltekova and Broderick (1996) used this modified procedure to estimate the rate and extent of in vitro degradation of protein in alfalfa hay and silage. Use of a two-compartment model, based on soluble and insoluble CP fractions assumed to pass with the fluid and particulate phases, yielded estimates of ruminal protein degradation that were comparable to NRC (1989) values. Luchini et al. (1996b) compared frozen ruminal microbes with freshly strained ruminal fluid for proteolytic activity and as an inoculum source for determination of ruminal protein degradation rates by the inhibitor in vitro method. They concluded that after reconstitution, frozen mixed ruminal microbes required preincubation to enhance degradative activity because preincubation increased rate and extent of microbial degradation of expeller soybean meal and solvent-extracted soybean meal.

Neutze et al. (1993) described a modified inhibitor in vitro method that estimated the degraded protein fraction as trichloroacetic acid (**TCA**)-soluble N rather than the sum of α-amino N and NH_3N. They compared the modified in vitro method with in situ and found that the coefficients of variation for degradation were higher for in situ. In vitro and in situ estimates were significantly different for the same feed samples, and ranking was not the same between methods. Because of the absence of an absolute method for determining protein degradation, they could not conclude which method was more accurate; however, they suggested that their technique can be used successfully to detect heat damage in forages.

Raab et al. (1983) proposed an alternative method for determination of protein degradation based on measurements of NH_3 concentration and gas production when feedstuffs were incubated in ruminal fluid. Starch or other fermentable carbohydrates were added in graded amounts to the incubations. Gas production (CO_2 and CH_4) and NH_3 concentration at 24 h were measured. Extrapolation of NH_3 release to zero gas production was assumed to correct for NH_3 uptake because zero gas production should represent zero microbial growth when NH_3 is not incorporated into microbial protein. Broderick et al. (1991) noted that, although this method might lend itself to analysis of

the dynamics of protein degradation, the large number of incubations required for such an analysis could be a disadvantage. Another approach to determining proteolysis has been to attach a marker molecule to the test protein through diazotization (Mahadevan et al., 1979; Baintner, 1981). Proteases split off colored azopeptides, and the red color of the azopeptide can then be developed with alkali and measured by spectrophotometry. Depending on the chemical structure of the protein, diazotization may cause a change in structure, with possible effects on the rate of proteolysis.

Nitrogen Solubility and Protein Fractionation. Earlier studies by Hendrickx and Martin (1963) reported a high correlation (r = .99) between N solubility in 10% Burroughs buffer (Burroughs et al., 1950) and in vitro degradation of purified protein to NH_3 in ruminal fluid. This observation stimulated interest in the use of N solubility to predict ruminal protein degradation. More recent studies have shown that soluble proteins may be degraded rapidly or slowly and that insoluble proteins are degraded at various rates; therefore, solubility is not synonymous with degradability as previously proposed. Stern and Satter (1984) reported that the correlation between N solubility and in vivo ruminal protein degradation of 34 diets that contained various dietary N sources was .26. There also was no relationship (r = .02) between percentage of N solubility and nonammonia N (**NAN**) flow to the duodenum (grams of NAN/100 g of dietary N) for the diets used. Consistent with these observations, Madsen and Hvelplund (1985) also reported a low relationship between N solubility and in vivo or nylon bag degradation of CP when used over a wide range of feeds. However, relationships between degradation and N solubility have been observed within similar feedstuffs (Beever et al., 1976; Laycock and Miller, 1981). Therefore, N solubility seems to be a more useful indicator of protein degradation when applied to different samples within a feedstuff than when used to compare several feedstuffs differing greatly in their physical and chemical properties.

A submodel of the Cornell Net Carbohydrate and Protein System for evaluating cattle diets has been developed for estimating degradation of dietary protein (Sniffen et al., 1992). Crude protein was partitioned into five fractions (A, B_1, B_2, B_3, and C). Fraction A is TCA-soluble N. Unavailable or protein bound to cell wall (fraction C) is derived from ADIN, and slowly degraded true protein (fraction B_3) is neutral detergent insoluble N minus fraction C. Rapidly degraded true protein (fraction B_1) is TCA-precipitable protein from the buffer-soluble protein minus nonprotein N (**NPN**). True protein with an intermediate degradation rate (fraction B_2) is the remaining N. Protein degradation was calculated on the basis of pool size and ruminal degradation of protein fractions in combination with ruminal passage

rate. Estimated protein degradations were correlated (r = .93) with tabular values from NRC (1989); however, this model requires further validation.

Continuous Culture Fermenters. Various continuous culture fermentation systems have been designed to simulate the ruminal environment, enabling the study of factors affecting microbial ecology and digestion of nutrients (Hoover et al., 1976; Czerkawski and Breckenridge, 1977; Teather and Sauer, 1988; Fuchigami et al., 1989). Advantages of these systems compared with in vivo measurements include decreased cost, time, and variation among experimental units. Furthermore, there are no complications from endogenous sources, and digesta flow rate markers are not required because passage rates are regulated and measured directly. However, similar to in vivo measurements, reliable techniques are required for isolation of microbial cells and for differentiation of effluent digesta into microbial and dietary N fractions. Most of these techniques are based on determination of a single chemical marker that is thought to characterize microbial components. Diaminopimelic acid (**DAPA**), aminoethylphosphonic acid, nucleic acids (DNA, RNA, or purines), and isotopes (^{35}S, ^{15}N, ^{32}P) incorporated into protein in the rumen have been used for this purpose. Advantages and disadvantages of these methods were discussed in reviews by Stern and Hoover (1979), Broderick and Merchen (1992), and Stern et al. (1994). Broderick and Merchen (1992) recommended the use of purines or ^{15}N as microbial markers. The technique for analyzing purines is simple, fast, and inexpensive; however, the purine:N ratio may change with time after feeding (Cecava et al., 1990), between particle- and fluid-associated bacteria (Firkins et al., 1987), or as a result of incomplete destruction of dietary purines in the rumen (Smith et al., 1978). If feeds vary in their purine content, and purine degradation is different from 100% or variable among feeds, residual feed purines reaching the duodenum could influence treatment effects on microbial N flow to the duodenum. In vitro continuous culture fermentation offers several advantages compared with in vivo studies for evaluating microbial markers, including no confounding effect of endogenous contribution of N, decreased protozoal purine contribution, and constant feed intake, outflow rates, and pH. Calsamiglia et al. (1996) used a modified dual-flow continuous culture system of Hoover et al. (1976), as described by Hannah et al. (1986), to compare purines and ^{15}N as microbial markers. Bacterial N flows obtained using purines were more variable than estimates obtained using ^{15}N. Moreover, bacterial N flows calculated using ^{15}N in bacteria isolated from fermenters were more variable than those obtained using bacteria isolated from the effluent. The use of purines as a microbial marker resulted in lower estimates of protein degradation and smaller differences among

dietary treatments than use of ^{15}N. Results suggested that escape of feed purine N seems to be a minor factor in the calculation of bacterial N flow and that the use of ^{15}N in effluent bacteria may be a more accurate procedure than use of purines when using continuous culture fermenters.

The two most commonly used in vitro continuous culture fermenter systems for measuring nutrient digestion by ruminal microbes are the Rusitec system (Czerkawski and Breckenridge, 1977) and dual-flow continuous culture (Hoover et al., 1976). The Rusitec system has a single outflow, and residence time in the rumen is simulated by placing feedstuffs into nylon bags and suspending these bags inside the reaction vessel for 48 h. Prevot et al. (1994) evaluated the Rusitec system and found that in its present form this system cannot reproduce the in vivo state of conventionally reared animals. Ciliated protozoal populations are eliminated in the fermenter and probably many bacterial species also; therefore, certain variables need to be studied and perhaps modified. Carro et al. (1995) studied the effects of bag pore size (40, 100, and 200 μm) and dilution rate (2.3 and 3.5%/h) on fermentation patterns and the ciliated protozoal population in Rusitec fermenters. The slower dilution rate decreased pH from 6.36 to 6.17, but digestion of DM and NDF was not affected. Pore size of the bags incubated in Rusitec fermenters influenced not only DM and NDF digestion of the diet, but also the microbial population in the system and, therefore, the fermentation (Carro et al., 1995).

Mansfield et al. (1995) used ruminally and duodenally cannulated lactating dairy cows and dual-flow continuous culture fermenters to compare fermentation of total-mixed diets and microbial ecology of in vivo and in vitro systems. Results of these comparisons are presented in Table 1. Organic matter truly digested (corrected for microbial OM) did not differ between the in vivo and dual-flow continuous culture methods. Partitioning OM into carbohydrate fractions revealed that digestion of total nonstructural carbohydrate was greater (74.6 vs 60.6%) and NDF was less (47.4 vs 58.3%) in continuous culture than in vivo. Most of this effect was a result of extreme values obtained from continuous culture fermentation of diets containing 40% nonfibrous carbohydrate (**NFC**). The continuous culture system seemed to have difficulty simulating nonstructural carbohydrate and NDF digestion for the 40% NFC diets, despite the fact that OM digestion was similar and pH was controlled at 6.4. The moisture, pressure, and heat applied to continuous culture diets during pelleting may have partially gelatinized the corn starch contained in the 40% NFC diets, rendering the starch more available to microbial fermentation. Amylolytic bacterial concentrations did not differ in the rumen and fermenters (Table 1), but there was a culture × NFC interaction (*P* = .02). Amylolytic bacterial concentrations were

greater in vitro than in vivo when 40% NFC diets were fed, which is consistent with the nonstructural carbohydrate digestion data, indicating that in vitro, the 40% NFC diets exhibited the greatest digestion. Cellulolytic bacterial concentrations decreased (P = .04) in vitro, as did the percentage of cellulolytic isolates (P = .002). These data paralleled the decreased NDF digestion that occurred in the fermenters. Digestion of CP was less in the rumen of cows than in the continuous culture fermenters (38.8 vs 42.9%). Correcting the in vivo value using a factor of 3.6 g of endogenous N per kilogram of duodenal DM flow (Brandt et al., 1980) produced a mean ruminal CP digestion of 47.6%. Regardless of the in vivo estimate used, the difference in CP digestion in vivo and in vitro was less than 5 percentage units. Other differences observed between fermenters and the rumen can be attributed to lack of absorptive capacity and defaunation in vitro. However, it should be noted that interpretation of results in vivo and in vitro was similar for 80% of the individual measurements that were evaluated, supporting the dual-flow continuous culture system as an excellent model for studying ruminal microbial fermentation. Because continuous culture systems are elaborate, expensive, and require inoculation with ruminal digesta, the technique is not suitable for routine analysis of microbial digestion for individual feed ingredients.

Methods for Measuring Intestinal Protein Digestion

The total amount of protein available for absorption from the small intestine depends on the flow of microbial and dietary protein to the duodenum and their respective intestinal digestibilities. Digestion of protein that leaves the rumen starts in the abomasum with acid-pepsin digestion and is completed in the small intestine with pancreatic and intestinal proteases. The NRC (1989) recognized that intestinal digestion of protein supplements may differ; however, empirical data are lacking, and as a result, a constant value of 80% is used for all feeds. Efforts have been made to incorporate more accurate estimates of intestinal protein digestion into feeding systems. The main problem with incorporation of these estimates is the lack of reliable techniques for estimating intestinal digestion of proteins. In vivo estimation of intestinal protein digestion involves expensive and labor-intensive experiments and requires the use of surgically prepared animals. Apparent digestion of protein is calculated as the disappearance of CP or amino acids between the duodenum and ileum, which is subject to considerable error associated with digesta sampling, use of digesta flow rate markers, and inherent animal variation. Therefore, several alternative procedures for estimation of intestinal protein

Table 1. Comparison of dietary component digestion and microbial ecology
in the rumen of dairy cows and in vitro continuous culture fermenters[a]

Item	Culture method			Significance of effect[bc]		
	Rumen	In vitro	SEM	Culture	Culture × NFC	Culture × NFC × DIP
Digestion, %						
True OM	47.5	46.8	1.6	NS[d]	NS	.001
Total nonstructural carbohydrate	60.6	74.6	2.9	.001	.001	.05
NDF	58.3	47.4	3.1	.001	.002	NS
CP	38.8	42.9	2.0	.02	NS	NS
Microbial ecology						
Viable count, $\log_{10}$ cells/mL	9.44	9.70	.25	.01	NS	NS
Amylolytic, $\log_{10}$ cells/mL	8.17	8.34	.30	NS	.02	NS
Percentage of viable	28.23	3.26	15.72	.001	NS	.03
Proteolytic, $\log_{10}$ cells/mL	7.70	7.71	.25	NS	NS	NS
Percentage of viable	9.40	3.26	14.85	NS	NS	NS
Cellulolytic, $\log_{10}$ cells/mL	7.79	7.61	.20	.04	NS	NS
Percentage of viable	5.40	1.71	2.73	.002	NS	NS
Protozoa, $\log_{10}$ cells/mL						
Total	5.57	.45	.35	.001	NS	NS
Holotrichs	4.01	.00	.30	.001	NS	NS
Fungi, $\log_{10}$ cells/mL	2.41	2.48	.23	NS	ND[e]	ND

[a]Adapted from Mansfield et al. (1995).
[b]NFC = nonfibrous carbohydrate; DIP = ruminally degraded intake protein.
[c]Effect of culture × DIP not significant (P > 0.1).
[d]NS = not significant (P > 0.1).
[e]ND, counts were only performed on three periods, so interaction effects could not be tested.

digestion in ruminants have been developed. An in situ mobile bag technique was devised to determine intestinal protein digestion in ruminants (Hvelplund, 1985). More recently, a three-step procedure that combines in situ and in vitro methods was developed to estimate intestinal digestion of proteins in ruminants (Calsamiglia and Stern, 1995). Other procedures have been proposed for predicting protein quality and CP digestibility, and these will also be discussed in this review.

Bioassays

Several animal models have been developed to decrease labor and cost but still maintain the physiological environment of the digestion process; however, few of these models have been developed and applied to ruminant research. Titgemeyer et al. (1990) used a precision-fed cecectomized rooster assay as a model to estimate intestinal digestion of amino acids in cattle. Freeze-dried duodenal samples obtained from steers fitted with duodenal and ileal cannulas were used to validate the technique (Titgemeyer et al., 1989). Data from the rooster assay were highly correlated (r = .94) with estimates of in vivo apparent amino acid digestion. This technique was also used by Henning et al. (1989) to determine digestion of utilizable N (total N minus nucleic acid, ammonia, urea, and uric acid N). Results were compared to those obtained in duodenally and ileally cannulated sheep. Digestion was lower and highly variable in the rooster assay, which was probably a result of the poor relationship between utilizable N and amino acid nitrogen content of the digesta. Titgemeyer et al. (1990) indicated that the rooster assay may not be appropriate for estimating CP digestion because rooster excreta contains urine and feces. However, the content of amino acids in birds' urine is small and fairly constant, and the technique may be useful in predicting amino acid digestion.

Rat growth studies also have been used to determine the quality of ruminally undegraded protein (Rooke, 1985). Changes in growth rates were highly correlated (r = .96) to estimates of intestinal protein digestion obtained using the in situ mobile bag technique. Because the biological value of proteins depends on the requirements of the animal species under study, extrapolation of data derived from rats to ruminants depends on the assumption that amino acid requirements of rats and cows are similar, an assumption that is tenuous at best. However, the technique may be useful in preliminary studies measuring digestion and biological value of ruminally undegraded protein.

In Situ Mobile-Bag Technique

The mobile-bag technique (**MBT**) was originally introduced to measure protein digestibility in pigs (Sauer et al., 1983) but has been modified to study postruminal digestion of feedstuffs by ruminants (Kirkpatrick and Kennelly, 1984; Hvelplund, 1985). Using this method, a small amount of intact feed or feed that has been preincubated in the rumen is placed in bags. The bags are then preincubated in a pepsin-HCl solution or directly introduced into the duodenum and subsequently collected from the ileum, or more typically from the feces. Bags are then washed to remove endogenous and other contaminating proteins. Therefore, total tract measurements using this technique can be considered as estimates of true, rather than apparent, digestibility (Robinson et al., 1992). However, Vanhatalo (1995) showed that contamination of feed residues by non-feed N, determined using a ^{15}N-dilution technique, resulted in somewhat lower digestion values with concentrates (barley) and forages (ryegrass), but the effect of contamination was negligible with protein concentrates (rapeseed meal). Forage rich in fiber and low in protein (straw) was markedly contaminated by non-feed N. Similar to observations with in situ measurements for estimating ruminal protein degradation, the MBT is affected by several potential sources of variation. Factors that have been addressed in recent research include porosity of bag material, sample-to-surface ratio, animal and diet effects, retention time, site of bag recovery, and microbial contamination (Hvelplund, 1985; Rooke, 1985; Voigt et al., 1985).

Porosity of bag material used in the MBT has ranged from 9 μm (Hvelplund, 1985) to 80 μm (Todorov and Girginov, 1991). Comparative data from these studies indicate that differences in protein digestion using the MBT resulting from bag pore size were rather small and of minor importance with both concentrates and forages. Vanhatalo (1995) found that with several feeds, the free surface area (percentage of total cloth area) rather than the pore size of the bag material had a significant effect on N disappearance from the mobile bags. Bag cloth with a small free surface area (2%) gave lower digestion values than cloth with a large surface area ($\geq$ 5%), especially with forages. Reduction in surface area obviously restricted digestion because the difference between the cloths was not associated with digestion in the large intestine or particle loss from the bags.

The effect of ruminal preincubation on intestinal protein digestion using the MBT seems to be important only for certain feeds. Hvelplund et al. (1992) reported negligible effects of ruminal preincubation on estimates of intestinal protein digestion for soybean meal, cottonseed cake, coconut cake, peas, grass silage, and whole-crop barley silage. In contrast, ruminal preincubation had important effects on intestinal protein digestion of heat-processed canola seed (Deacon et al., 1988), rapeseed meal, meat and bone meal (Rooke, 1985), and some forages (De Boer et al.,

1987). Using the MBT, Volden and Harstad (1995) concluded that ruminal preincubation is not required for soybean meal, fish meal, extracted rapeseed meal, corn gluten meal, or guar meal; however, peas, rapeseeds, lupin seeds, barley, and oats should be evaluated after ruminal preincubation. With forages, preincubation in the rumen was necessary to predict intestinal protein digestion from the indigestible CP fraction determined with the MBT (Mgheni et al., 1994), and at least 48 h of ruminal incubation were needed (Vanhatalo et al., 1996). Consequently, it seems that ruminal preincubation should be routinely included in the MBT protocol.

Cherian et al. (1988) reported a dramatic decrease in intestinal digestion of soybean meal, meat and bone meal, and canola meal protein for pigs when pepsin-HCl predigestion was not included in the protocol. Finlayson and Armstrong (1986) indicated that in formaldehyde- or heat-processed proteins, the acidic environment in the stomach may be important for predicting intestinal protein digestion. In contrast, the effect of pepsin-HCl preincubation on intestinal digestion of protein has been considered negligible (Graham et al., 1985; Voigt et al., 1985). Recently, Vanhatalo et al. (1995) observed that pepsin-HCl preincubation did not affect intestinal CP digestion values, and pepsin-HCl preincubation is not necessary when the MBT includes preincubation of feeds in the rumen.

Average retention time of bags in the digestive tract values vary widely within and among experiments, but this variance seems to have minimal effects on estimates of protein digestion in the small intestine (Graham et al., 1985; Hvelplund, 1985; Voight et al., 1985; van Straalen et al., 1993). The proteolytic activity of the small intestine is substantial, and ideally bags should be recovered at the end of the ileum using re-entrant cannulas. However, for practical purposes, fecal collection is more convenient. Estimates obtained from fecal collection of bags assume that bags and feed residues are not contaminated with microbial protein from large intestinal fermentation and that protein leaving the ileum is not further digested by microbes in the large intestine. Because remaining N after digestion is often very small, the contribution of potentially contaminating microbial protein may be of greater concern. Hvelplund (1985) and Kohn and Allen (1992) suggested that microbial contamination was small enough to be ignored. In contrast, Varvikko and Vanhatalo (1990) reported that microbial contamination decreased estimates of intestinal digestion between 6 and 38 percentage units for ryegrass and rapeseed straw, respectively. Voigt et al. (1985) found a correlation of .92 between ileal and fecal collection of bags and concluded that fecal collection of bags was appropriate. Jarosz et al. (1994) used the MBT to estimate intestinal digestibility in 10 samples of whole plant and straw from pea, barley, and ryegrass hay at different stages of maturity. Nitrogen disappearance was greater in bags recovered from feces than in those collected from the ileum for most feeds tested; however, differences were significant only in pea and barley straw. Collecting bags from feces instead of the ileum led to a slight increase in the CP digestion values of vegetable concentrates (rapeseed meals, soybean meal), but those of animal origin (meat and bone meal) or forages (grass silages) were usually not affected by bag recovery site (Vanhatalo and Ketoja, 1995).

Despite the potential sources of variation that have been observed, several studies have shown that the MBT may be useful in predicting intestinal digestibility of protein. Hvelplund (1985) regressed in vivo intestinal protein digestion values for seven feeds measured in intestinally cannulated cows on estimates of the same feeds obtained using the MBT with fecal collection of bags and found a correlation of .81. De Boever et al. (1995) concluded that a reasonable relationship existed between intestinal protein digestibility of compound feeds based on tabular values for the ingredients and those calculated using the mobile-bag technique. Masoero et al. (1994) tested intestinal CP disappearance using the MBT and compared these values with true intestinal digestibility of proteins according to tabular values for 29 feeds. Tabular values were found to be generally higher than those estimated using the MBT (89.8 vs 84.4%). With both evaluation methods, feeds of plant origin seemed more digestible than those of animal origin. For 18 of the 29 feeds tested, intestinal protein digestibilities were similar between the two procedures, whereas differences were observed in feeds with high fiber content. Some differences could be attributed to non-feed contamination, which is consistent with Vanhatalo and Varvikko (1995), who reported that fibrous feeds with low N content, such as rapeseed straw, can be seriously contaminated by non-feed N, which affects CP digestion values using the mobile-bag method.

Intestinal digestibility of ruminally undegraded feed CP varies considerably, depending on the feed type (Vanhatalo, 1995). Protein-rich concentrates (soybean meal) were characterized by high undegradable CP digestibility, whereas protein concentrates relatively high in fiber (rapeseed meal) or ash (meat and bone meal) were characterized by lower digestibility. The undegradable CP digestibility of concentrates was usually greater than that of forages. However, the ranking of forages is likely to be affected by non-feed N. Because digestibility of undegraded CP is affected by both the extent of ruminal degradation and feed type, measured values rather than a constant factor should be preferred in modern protein evaluation systems.

The MBT provides an easy and fast way to determine intestinal digestion of proteins. However, it

is necessary to standardize and validate the procedure to provide consistent and reliable results so that a database can be generated for intestinal CP digestibility of supplements commonly fed to ruminants.

In Vitro

Lysine Availability Test. The lysine availability test (AOAC, 1984) has been used as an indicator of heat-processed or damaged protein. The technique measures total lysine and lysine remaining after treatment with 1-fluro-2,4-dinitrobenzene, with the difference between these two measurements representing available lysine. Faldet et al. (1992) evaluated heat-processed soybeans using lysine availability and rat growth studies and found a high correlation (r = .99, n = 20) between these techniques. However, intestinal digestion of protein may be affected by chemical reactions other than bound lysine, including amide bonds (Bjarnason and Carpenter, 1969), racemization and crosslinking of amino acyl residues (Maga, 1984; Schwass and Finnley, 1984), disulfide crosslinking (Opstvedt, 1984), and phenolic-hydroxy crosslinking with protein (Kumar and Singh, 1984). Therefore, measuring lysine availability alone does not necessarily determine potential decreases in intestinal protein digestion. Although the technique may be appropriate for comparing different samples within the same feeds, it is not recommended for use among various feeds.

Intestinal Fluid. Furuya et al. (1979) indicated that in vitro incubation of protein samples with jejunal fluid could be used to estimate protein digestion in the small intestine. Feed samples were preincubated with an acid-pepsin solution to simulate protein digestion in the stomach, followed by incubation with jejunal fluid. Estimates of intestinal CP digestion of seven diets were highly correlated (r = .98, n = 7) to intestinal digestion measured in duodenally and ileally cannulated pigs. In contrast, more recent studies (Graham et al., 1989; Lowgren et al., 1989) noted low correlations between intestinal fluid incubation and CP digestion estimated in vivo, most probably as a result of build-up of microbial matter in the media. Although the technique decreases cost and labor compared with in vivo methods, results do not seem to provide accurate measurements of intestinal protein digestion, and it requires animal resources to obtain jejunal fluid. To date, no similar experiments have been conducted with ruminants.

Enzymatic Methods. A large variety of enzymatic digestion systems for estimating intestinal digestion of protein have been tested (Akeson and Stahmann, 1964; Buchanan, 1969; Rhinehart, 1975; Hsu et al., 1977). Most enzymatic techniques follow a similar protocol; feed samples are incubated at optimal temperature and pH with an enzyme or combination of enzymes. After the incubation period, protein digestion is measured as the amount of amino acids and small peptides released from the protein, divided by the amount of protein in the original sample. End products of digestion in the intestine are a mixture of free amino acids and short peptides. Determination of protein digestion requires that the method used will estimate amounts of these amino acids and peptides that are released. Various techniques differ in the enzyme or combination of enzymes used and the method selected for determining the digested protein.

Single enzyme systems have been used with variable success (Buchanan, 1969; Saunders et al., 1973; Rhinehart, 1975). The AOAC (1984) approved a standardized pepsin digestion procedure for estimating total tract unavailable N. Pepsin-insoluble N (**PIN**) was strongly correlated to total tract unavailable N in forages (Goering et al., 1972; Yu and Veira, 1977; Shelford et al., 1980) and other protein supplements (Loerch et al., 1983). In contrast, Zinn and Owens (1982) and Britton et al. (1986) indicated that PIN was a poor predictor of total tract CP digestibility. Zinn and Owens (1982) reported that about 20% of the N leaving the ileum was soluble in acid-pepsin solution, suggesting that PIN may underestimate unavailable protein in feeds. The lack of accuracy reported with single enzyme techniques is likely the result of a limited spectrum of specificity for any single enzyme. Because most enzymes have specificity for individual peptide bonds, protein digestion may be more dependent on the number of bonds sensitive to that enzyme in the protein than to the actual intestinal digestibility. Although the use of a combination of enzymes improved correlations with in vivo methods, estimates were not accurate in predicting intestinal digestion of protein in a wide variety of feeds (Saunders et al., 1973; Rhinehart, 1975; Rich, 1978), probably as a result of differences in activity and specificity of the combination of enzymes compared with those found in the small intestine. It was evident that an in vitro technique to estimate protein digestion should include enzymes with activity and specificity similar to those found in the digestive tract of the animal.

The most commonly used enzymatic technique was developed by Akeson and Stahmann (1964). The system was designed to simulate abomasal (pepsin) and intestinal (pancreatin) digestion. Results were highly correlated (r = .99, n = 12) with rat growth studies, and the technique was successfully tested by other researchers (Buchanan, 1969; Saunders et al., 1973; Stahmann and Woldegiorgis, 1975; Gabilois and Savoie, 1987). This system provides a more physiological environment for digestion and contains proteolytic enzymes with specificity and activity similar to those found in the small intestine.

One of the most important factors affecting estimates of intestinal digestion of proteins is the method used to determine digested protein. Different methods have been developed, including filtration (Sheffner et

al., 1956), precipitation with strong acid followed by centrifugation (Akeson and Stahmann, 1964), centrifugation followed by filtration (Saunders et al., 1973), chromatography (Ford and Salter, 1966), and dialysis (Mauron et al., 1955). Assumptions involved in each of the methods differ, and interpretation of results may be affected by the method used. Gauthier et al. (1982) developed a digestion cell designed to closely simulate in vivo conditions. A two-step digestion (pepsin-pancreatin) was performed inside a dialysis bag. End products of digestion could be carefully separated based on molecular weight by selecting the appropriate pore size of the dialysis membrane. The continuous flow of buffer through the digestion cell removed end products of digestion, avoiding end product inhibition of proteases and stabilizing pH, a situation very similar to the physiological environment in the small intestine. The few experiments conducted using this technique seem to provide good agreement in regard to protein and amino acid digestion in the small intestine (Gabilois and Savoie, 1987; Savoie et al., 1988). Design and assumptions underlying this technique offer the potential for this procedure to be an excellent tool for studying protein digestion in the small intestine; however, few feeds have been tested, and the technique is more expensive and slower than the other enzyme techniques previously discussed.

A different approach was used by Hsu et al. (1977), who speculated that amino acids released from enzymatic digestion in a non-buffered solution will result in pH changes of the solution. This hypothesis was tested by incubating 23 different feed samples in a pH 8 solution that contained a mixture of purified enzymes (trypsin-chymotrypsin-pepsin). Changes in pH were recorded after 10 min and values correlated to estimates of digestion obtained in rats. The pH at 10 min was highly correlated (r = .90) with in vivo estimates of intestinal CP digestion. Satterlee et al. (1979) modified the technique by including an additional protease and measuring pH after 20 min of incubation; however, the correlation coefficient obtained was less (r = .77) than noted by Hsu et al. (1977). Several authors suggested that the correlation could be improved if different equations were used for each feed type (Rich, 1978; Marshall et al., 1979; Bodwell et al., 1980). In contrast, Satterlee et al. (1981) indicated that the use of a general equation would result in only a minor deviation in the estimates of intestinal CP digestion. Jewell et al. (1980) devised an unbiased method to determine protein type based on its amino acid composition. Correlation of in vivo intestinal digestion vs changes in pH using this feed-type classification was high (r = .92). The pH technique may provide a fast, inexpensive method to estimate intestinal digestion of protein; however, an equation needs to be developed for each feed type. Furthermore, Hung et al. (1984) indicated that the buffer capacity of tested feeds can interfere

with estimates of digestion. This may be particularly important in ruminants because the buffer capacity of feeds may be affected by ruminal preincubation.

Application of a pepsin-pancreatin digestion technique in animal nutrition has been limited. Van der Poel et al. (1991) reported a low correlation between intestinal CP digestion in pigs and pepsin-pancreatin digestion. However, pancreatin digestion was conducted for only 1 h, which is not consistent with the 24 h recommended by Akeson and Stahmann (1964). Britton et al. (1986) also reported a weak correlation between pepsin-pancreatin insoluble N and total tract unavailable N; however, results from the pepsin-pancreatin digestion should be correlated to intestinal digestion and not to total tract digestion. Van Straalen et al. (1993) used the pepsin-pancreatin digestion procedure of Antoniewicz et al. (1992) and reported a strong correlation (r = .91, n = 28) between the MBT used in dairy cows and the pepsin-pancreatin digestion test. A pepsin-pancreatin digestion technique provides a physiological environment similar to that found in the small intestine, and results that have been obtained seem promising. Nonetheless, it has been necessary to conduct experiments to optimize the technique and to validate the results with in vivo estimates of intestinal digestion. The pepsin-pancreatin technique may provide an inexpensive, rapid, and efficient means of estimating intestinal digestion of protein supplements commonly fed to cattle.

Three-Step In Situ/In Vitro Procedure. A three-step procedure was developed by Calsamiglia and Stern (1995) to estimate intestinal digestion of proteins in ruminants. The technique was developed to 1) closely simulate physiological conditions of ruminants, including potential effects of ruminal fermentation; 2) be rapid, reliable, and inexpensive; 3) be applicable to a wide variety of protein supplements; and 4) accurately reflect differences in protein digestion. Dacron bags containing feed samples were suspended in the rumen for 16 h. Residue was incubated for 1 h in a 1 N HCl solution containing 1 g/L of pepsin. After incubation, pH was neutralized with 1 N NaOH, and a pH 7.8 phosphate buffer containing 3 g/L of pancreatin was added to the solution followed by incubation at 38°C. After a 24-h incubation, a 100% (wt/vol) TCA solution was added to precipitate undigested proteins. Preincubation of samples in the rumen did not affect pepsin-pancreatin digestion of residual CP in soybean meal, corn gluten meal, and blood meal but decreased pepsin-pancreatin digestion of residual CP in hydrolyzed feather meal, fish meal, and meat and bone meal (80 vs 70, 88 vs 81, and 82 vs 56%, respectively, for nonruminal vs ruminal preincubation). Pepsin digestion before pancreatin digestion increased CP digestion of all proteins tested by a mean of 23 percentage units. The pancreatin digestion step was validated using 34 duodenal samples from which small intestinal CP digestion was determined in vivo. The regression equation of in vivo estimates on pancreatin

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Table 2. Effect of method used on digestion of nitrogen in
solvent-extracted or lignosulfonate-treated soybean meal

| | Method | | | | |
| | MBT[a] | | Three-step procedure[b] | | |
CP digestion, %	SBM[c]	LSBM[d]	SBM	LSBM	SEM
Ruminal	73.1[f]	29.1[g]	80.6[f]	31.3[g]	5.3
Intestinal	99.3[f]	99.5[f]	77.6[g]	77.9[g]	2.2
Total tract[e]	99.8[f]	99.9[f]	95.9[g]	85.1[h]	1.0

[a]In situ mobile-bag technique.
[b]Calsamiglia and Stern (1995).
[c]SBM = soybean meal.
[d]LSBM = lignosulfonate-treated soybean meal.
[e]Ruminally digested fraction + (ruminally undigested fraction × intestinally digested fraction).
[f,g,h]Means within a row lacking a common superscript differ (*P* < .05).

digestion had a coefficient of determination of .91. Intestinal CP digestion of soybean meal and lignosulfonate-treated soybean meal was similar within method using the MBT and three-step procedure (Table 2; our unpublished observations). However, there was a substantial difference between the two procedures for estimating intestinal digestion. Estimates differed by almost 22 percentage units (99.4 vs 77.8% between MBT and the three-step procedure, respectively), with almost complete disappearance of CP in the mobile-bags. Differences between methods may be a result of protein digestion that occurs in the large intestine. Hvelplund (1985) indicated that 50% of soybean meal protein that left the ileum was digested in the large intestine and observed a significant site of collection (ileum vs feces) × feed (soybean meal vs canola meal) interaction.

Application of the Technique. Estimates of intestinal digestion of different protein supplements using the three-step procedure are presented in Table 3. Values in this table clearly indicate that large variation exists among and within protein supplements. Low intestinal protein digestion was found for meat and bone meal (55%), batch-dried blood meal (63%), and hydrolyzed feather meal (67%), whereas all remaining protein supplements averaged greater than 70%. To evaluate the effects of different processing procedures on intestinal protein digestion of various animal proteins, Howie et al. (1996) obtained seven samples each of several animal byproducts. Estimates of intestinal digestion of ruminally undegraded CP, determined using the three-step procedure, ranged from 40.9 to 70.1% ($\overline{x}$ = 56.0% ± 4.0), 59.2 to 75.2% ($\overline{x}$ = 65.3% ± 2.1), 72.0 to 90.3% ($\overline{x}$ = 79.6% ± 2.5), and 28.8 to 79.2% ($\overline{x}$ = 61.4% ± 6.8) for meat and bone meal, hydrolyzed feather meal, ring-dried blood meal, and batch-dried blood meal, respectively. Yoon et al. (1994) used 18 menhaden fish meal samples from various processing plants to evaluate the effects of processing on intestinal digestion of protein and noted values (Figure 1) ranging from 72.8 to 86.4% ($\overline{x}$ = 77.7 ± 3.5). These results demonstrate that considerable variation exists in intestinal digestion of protein

among and within different protein sources, and this variation can possibly be influenced by source of raw material, quality of raw material (storage time and temperature), drying conditions, and various other factors. The three-step in vitro technique can be a useful procedure for evaluating quality control of protein.

Intestinally absorbable dietary protein (**IADP**) is defined as the amount of protein from a specific feed that is available for absorption in the small intestine and is calculated as ruminally undegraded protein multiplied by intestinal protein digestion. The IADP value provides an index of the quality of protein supplements as sources of undegraded protein for ruminants. Because there is a large amount of variation associated with measurements of ruminal degradability and intestinal digestibility among and within protein supplements, and there seems to be no correlation between ruminally undegraded protein and intestinal protein digestibility (Yoon et al., 1994), the use of published values for ruminally undegraded protein and intestinal digestion to calculate IADP is not adequate. Therefore, only data from experiments in which ruminally undegraded protein and intestinal
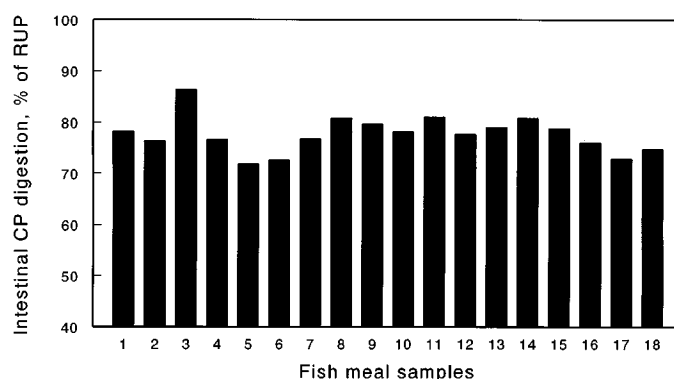

Figure 1. Intestinal CP digestion (percentage of ruminally undegraded protein [RUP]) determined using the three-step procedure for 18 menhaden fish meal samples from five processing plants (Yoon et al., 1994).

Table 3. Ruminally undegraded protein (RUP), intestinal CP digestion (ID), and intestinally absorbable dietary protein (IADP) of various protein supplements

Protein source	n	RUP (% of CP), Avg ± SD (range)	ID (% of RUP), Avg ± SD (range)	IADP[a], Avg ± SD (range)
Plant proteins				
Cottonseed meal, solvent	1	46	71	33
Cottonseed meal, mechanical	1	55	80	43
Soybean meal	5	25 ± 3 (22–29)	90 ± 4 (86–93)	22 ± 2 (20–25)
Soybean meal, expeller	6	47 ± 6 (38–53)	93 ± 7 (83–100)	44 ± 3 (38–53)
Soybean meal, lignosulfonate	6	66 ± 8 (57–77)	88 ± 4 (82–92)	58 ± 7 (49–67)
Grain byproducts				
Brewers grains, dried	5	57 ± 5 (50–63)	77 ± 2 (73–79)	44 ± 5 (37–49)
Corn gluten meal	2	83 ± 2 (82–85)	89 ± 4 (86–91)	74 ± 5[b] (70–77)
Distillers grains, dried	5	56 ± 8 (47–64)	81 ± 5 (72–85)	46 ± 8 (36–53)
Animal proteins				
Blood meal, batch-dried	12	88 ± 6 (78–98)	63 ± 17 (29–86)	55 ± 14 (25–75)
Blood meal, ring-dried	10	83 ± 4 (76–89)	81 ± 6 (72–90)	67 ± 7 (58–76)
Feather meal, hydrolyzed	12	76 ± 11 (50–88)	67 ± 6 (58–75)	51 ± 9 (36–64)
Fish meal, menhaden	13	65 ± 4 (59–73)	80 ± 5 (73–88)	52 ± 4 (43–57)
Meat and bone meal	11	59 ± 13 (40–88)	55 ± 10 (41–70)	33 ± 10 (21–56)

[a]IADP (% of CP) = RUP (% of CP) × ID (% of RUP).
[b]Using in vivo estimate of RUP of 57% (Stern et al., 1983b), IADP = 51%.

protein digestion were measured simultaneously were included in Table 3. Results indicate that corn gluten meal (74%) and ring-dried blood meal (67%) provided the largest amount of IADP, followed by lignosulfonate-treated soybean meal, batch-dried blood meal, menhaden fish meal, and hydrolyzed feather meal (51 to 58%). Because corn gluten meal tends to stick together when wet, exposure of the surface area in the Dacron bag is decreased as previously described (Stern et al., 1983a). Using an undegraded protein value of 57% as determined in vivo by Stern et al. (1983b), instead of 83%, as determined in situ, and assuming that intestinal digestion is 89%, the IADP for corn gluten meal would be approximately 51%. The IADP provided by each protein supplement is an indication of its value as a source of ruminally undegraded protein and can be used as a criterion for selecting sources of ruminally undegraded protein for ruminants.

Implications

Obtaining absolute values of ruminal and intestinal nutrient digestion for a wide variety of feedstuffs or diets by in vitro or in situ means is difficult. It is even more difficult to obtain quantitative in vivo measurements against which the in vitro and in situ methods can be sufficiently tested. Differences among studies in animal species, cannula placement, digestibility markers, microbial markers, and animal variation introduce considerable variation into what is designed to be the standard for comparison. Because of these issues, it is more realistic to obtain relative measurements of ruminal and intestinal nutrient digestion among feedstuffs and(or) diets rather than absolute values.

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