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RUMEN LIQUOR AS A PROTEIN SOLVENT

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RUMEN LIQUOR AS A PROTEIN SOLVENT

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ABSTRACT

RUMEN LIQUOR AS A PROTEIN SOLVENT

By

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Rumen fluid was collected from rumen-fistulated Holstein cows fed either corn silage or alfalfa hay. The rumen fluid was either autoclaved or filtered and used as solvent on protein solubility tests for corn meal, alfalfa hay and casein.

Protein solubility was not different ($p > 0.05$) due to either autoclaved or filtered rumen fluid. Protein solubility of corn, alfalfa and casein were $32.17 \pm 11.46\%$ $43.48 \pm 11.46\%$ and $79.02 \pm 11.46\%$, respectively. The difference between corn and casein was significant ($p < 0.05$). Protein solubility of all the protein sources at pH 6.0, 6.5, and 7.0 was $54.14 \pm 1.93\%$, $53.05 \pm 1.93\%$, and $47.49 \pm 1.93\%$, respectively. The difference between pH means was significant ($p < 0.05$). Linear effect of pH was significant ($p < 0.05$), but the quadratic effect was not ($p > 0.05$). Exposure to the solvent for 60 minutes or 120 minutes gave different protein solubility ($p < 0.01$).

I would like to dedicate this work to my wife, Cristina Pohlenz de Celma, whose love and support gave me the strength to fulfill this goal and to my mother, Mrs. Estela Alvarez de Celma, for her love and understanding.

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INTRODUCTION

The ever growing human population demands the production of huge amounts of food from different sources. Milk is one of the best food sources, but its supply is becoming shorter than its demand for human consumption. While the number of dairy cows is diminishing, the production per cow has been increasing due to improved genetic potential, better management techniques and a higher rate of feeding.

Protein nutrition is critical for the dairy cow. For many years, it was believed that the supply of amino-acids (A-A) from ruminal microorganisms, which are capable of synthesizing high quality microbial protein from non-protein-nitrogen (NPN) sources such as: urea, biuret, ammonia, etc. was sufficient for fulfilling the A-A requirement of the dairy cow. Actually the demand on the high producing dairy cow to produce at the top of her genetic potential makes it necessary to supply an extra source of dietary A-A's to satisfy her requirements for milk production, growth, and foetal development.

Because milk production is the purpose of dairy cattle, the supply of nutrients to the mammary gland is vital. It follows that the quality of the protein--that is, the A-A supply reaching the lower gut and becoming available--is important.

The main sources of A-A's to the lower gut are: 1) the rumen microbial population which contributes A-A's from its own protein structure, and 2) bypass protein, i.e. that dietary true protein that will pass through the rumen without being degraded. This bypass protein will supply A-A's in addition to that provided by the microbial population. Thus, there are two main sources of A-A's for satisfying the A-A's requirements of the high producing dairy cow.

One of the main factors that influences the amount of bypass protein is the physical property of the dietary protein. This factor is protein solubility (PS) which is characteristic of each protein source that gives a measure of the disappearance of nitrogen (N) from the solid phase of a feed when incubated in an inanimate aqueous solution (Bull, et al., 1977).

The amount of dietary protein degraded in the rumen has been correlated with the solubility of the protein in the rumen fluid (Crawford et al., 1978; Bull et al., 1977; Craig et al., 1978; Henderickx et al., 1963). Different *in vitro* methods have been used for evaluating the amount of soluble nitrogen (SN) in different feedstuffs (Crooker et al., 1975; Lyman et al., 1953; Wohlt et al., 1973; Burroughs et al., 1950a) using a wide variety of aqueous media, such as autoclaved rumen fluid (ARF), Burroughs mineral mixture (BMM) solution (10%), modified Burroughs mineral mixture (MBMM), distilled water, NaCl solutions, etc.

The objective of this work was to attempt to find a more accurate method, i.e. a method that gives a closer estimate of PS before any protein degradation occurs in the rumen.

LITERATURE REVIEW

Protein Solubility

Factors That Affect the Degree of Protein Solubility

a. Chemical composition

Since the investigation done by T. B. Osborne (1924) in England with plant proteins it has been known that plant proteins are compounded principally of four main groups characterized by their solubility properties.

Those proteins that solubilize in water are albumins. Globulins will dissolve in saline solutions but are insoluble in water. The plant protein fraction that is neither soluble in water, saline solutions, or alcohol but is soluble in very dilute acids or alkalies is the glutelin fraction. Prolamines are usually soluble in relatively concentrated (70%) alcohol (Clark, 1975; Crooker et al., 1975; Wohlt et al., 1973). These plant protein fractions form most of the protein structure of the plants; there are other fractions such as albuminoids, histones, and protamines but they are rather a small portion.

Wohlt et al. (1973) demonstrated that feeds whose major protein fractions were composed of albumins and globulins had a higher solubility (32% SN in unprocessed and 42% SN in processed protein sources) than those composed primarily of prolamins and glutelins (25% SN in unprocessed and 18% SN in processed protein sources).

b. Sample size

Some of the earliest in vitro studies of PS were done without considering total nitrogen concentration in the solvent (Lyman et al., 1953; Smith et al., 1959). Saturation of a solution has been shown to be an important factor for measuring PS as demonstrated by Wohlt et al. (1973), who in order to determine the effect of protein concentration on solubility placed 25, 50, 100, 250 and 500 mg. of N of either casein or soy protein per 100 ml. of Burroughs mineral mixture (BMM) at pH 6.5 at 40° C for 60 minutes. The solutions were agitated by magnetic stirrers in a dry bacteriological incubator at minimum rate to insure the movement of a stirring bar. Large amounts of nitrogen reduced the solubility of a given protein when the saturation point was approached (see Fig. 1). In the case of casein, which is 96% soluble, a decrease of solubility was observed with concentrations above 250 mg/100 ml. When soybean meal was added at different concentrations, SN increased linearly with the amount added and the solubility remained constant. Wohlt concluded

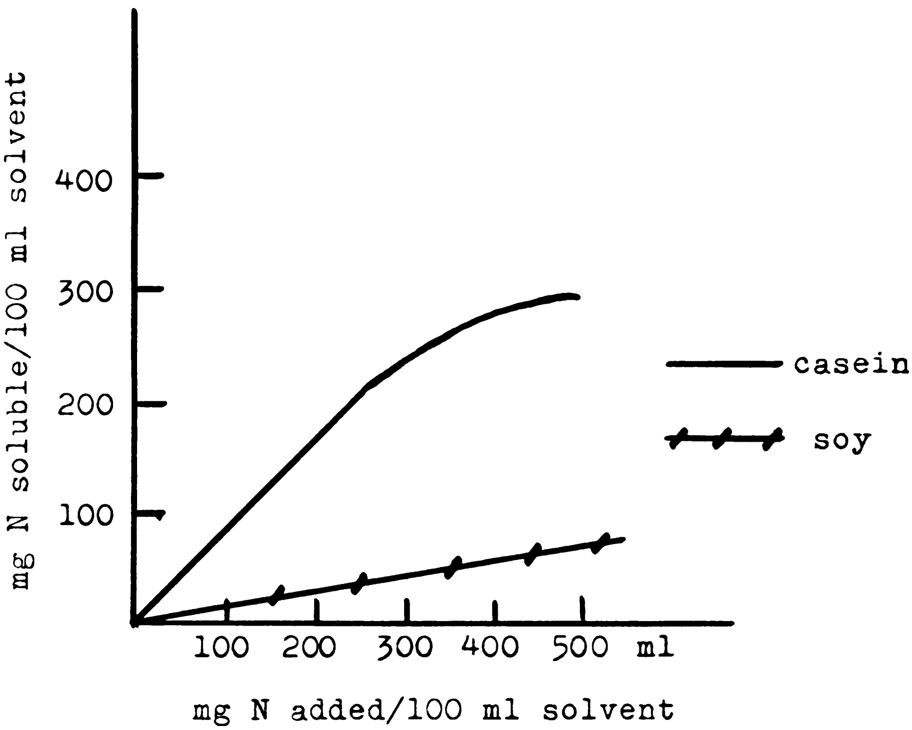

Figure 1. Effect of nitrogen concentration per 100 ml of solvent on protein solubility (Wohlt et al., 1973).

that since most of the protein sources are less soluble than casein, solubilities can be accurately measured at concentrations of 25 mg/100 ml of solvent.

Another important factor may be sample particle size as pointed out by Lyman et al., 1953; Smith et al., 1959; Wohlt et al., 1973; and Crooker et al., 1978, because of the area exposed to the action of the solvent.

c. Solvents

Differences in chemical and physical properties of different solvents used *in vitro* PS tests have resulted in distinct solubility values for a given protein source (Burroughs et al., 1950a; Little et al., 1963; Wohlt et al., 1973; Crooker et al., 1975; Crawford et al., 1978; and McDougal, 1949). If we are going to simulate the PS in the rumen, we should recognize the specific physical and chemical properties of the rumen fluid, as suggested by Jancarik et al. (1970).

Different solvents have been used such as: autoclaved rumen fluid (ARF), cold or hot distilled water (dH_2O), 0.01 N or 0.02 N NaCl, 0.01M NaOH, 0.05M $NaPO_3$, 0.05M NaCl, 70% ethyl alcohol, 0.8M trichloroacetic acid (TCA), 0.15M NaCl, BMM, modified Burroughs mineral mixture (MBMM), McDougal's artificial saliva (MCD). See Table 1.

Table 1. Solvent Composition*

Salt	Solvent BMM[a]	Solvent MCD[b]
	(gr per 10 l of distilled water)	
$MgCl_2 \cdot 6H_2O$	....	1.3
$MgSO_4 \cdot 7H_2O$	1.13	....
Na_2HPO_4	10.41	36.80
$CaCl_2 \cdot 2H_2O$[c]	0.25	0.53
KCl	3.75	5.70
NaCl	3.75	4.70
$NaHCO_3$	26.25	98.00
$(NH_4)_2SO_4$	18.75	....
Na_2SO_4[d]	....	....
$FeSO_4$	0.04	....
$CoCl_2 \cdot 6H_2O$	0.01	....
$ZnSO_4 \cdot 7H_2O$	0.04	....
$MnSO_4 \cdot H_2O$	0.03	....
$CuSO_4 \cdot 5H_2O$	0.02	....

*Crocker et al., 1978.

[a]Burrough Mineral Mixture diluted to 10% with distilled water.

[b]McDougal's artificial saliva.

[c]Bubble CO_2 through solution after addition of $CaCl_2 \cdot 2H_2O$ until solution clears.

[d]Replaces $(NH_4)SO_4$ on an equimolar basis for Na_2SO_4 (20.155 gr) for modified Burrough's mineral mixture (MBMM).

Little et al., 1963; Crooker et al., 1978, and Henderickx and Martin, 1963, concluded that protein sources differ in soluble nitrogen; however, solubility in any one solvent was not necessarily related to the solubility in other solvents (see Table 2). Solubility of nitrogen in dH_2O and ARF was lower than in 0.02N NaOH. Heat treatment of soybean oil meal reduced NS in all the solvents. Those protein sources that were readily converted to ammonia *in vitro* at about the same rates were soybean oil meal, soy protein, linseed oil meal and casein. In contrast, heated soybean oil meal, corn gluten meal or zein were slowly converted to ammonia. Little et al. (1963) found no consistent relationship between NS and level of ammonia in incubation flasks. However, nitrogen soluble in ARF and level of free ammonia at 2 hours had the highest correlation ($r = 0.93$). The correlation for dH_2O and NaOH were $r = 0.38$ and $r = 0.52$, respectively.

Crooker et al. (1978) demonstrated that the difference among the average PS of various feedstuffs with MBMM, MCD and ARF was significant ($p < .01$)(Table 2). However, a significant difference among PS values obtained with BMM and ARF was not observed. The difference may be due to the differences in solvent composition (see Table 1). ARF was most closely simulated by BMM in extracting N from hominy, wheat and citrus pulp. ARF and BMM extracted about equal amounts of nitrogen from buckwheat. Distiller's

Table 2. Effect of Solvent on Nitrogen Solubility

Feedstuff	Solvent ARF[a]	BMM[b]	MBMM[c]	MCD[d]	0.02N NaOH	dH_2O[e]	Reference No.
	% soluble nitrogen						
Soybean oil meal	19	----	----	----	81	16	62
H. soybean oil meal[f]	10	----	----	----	30	11	62
Linseed oil meal	45	----	----	----	68	39	62
Corn gluten meal	13	----	----	----	32	11	62
Purified soy protein	7	----	----	----	99	2	62
Purified casein	81	----	----	----	98	2	62
Purified zein	3	----	----	----	99	0	62
Distillers dried grains with solubles	22.6	20.4	22.8	22.7	--	--	34
Wheat	20.8	21.7	26.4	29.2	--	--	34
Hominy	20.8	24.5	28.6	28.0	--	--	34
Citrus pulp	24.2	25.0	36.9	37.6	--	--	34
Sunflower meal	24.0	34.1	38.9	39.5	--	--	34
Buckwheat	30.3	34.1	37.1	39.8	--	--	34
Oats	18.5	36.8	43.8	43.9	--	--	34
Purified casein	78.1	79.8	----	----	--	--	106
Isolated soy protein	13.9	14.6	----	----	--	--	106

[a]ARF - Autoclaved rumen fluid.

[b]BMM - Burrough's mineral mixture.

[c]MBMM - modified BMM.

[d]MCD - McDougal's artificial saliva.

[e]dH_2O - distilled water.

[f]H. soy protein oil meal - heated on forced air oven at 110° C for 24 hours.

dried grains with solubles had similar NS measures in all solvents.

The variations among solvents in NS may be due to the various inter and intra molecular forces acting between the proteins and the various ionic species contained in each solvent (Cohn, 1943). This can be observed in the values obtained between BMM and MBMM as solvents since they only differ in two ionic species NH_4^+ and Na^+.

Among correlations between percent SN in each mineral solvent for each feed and percent SN from ARF; soluble nitrogen from NaCl were correlated (r = 0.8) while BMM, MBMM, and MCD have much less correlation with ARF (r = 0.21, 0.12 and 0.06) this higher correlation between NaCl and ARF was due to the results obtained with oats. If oats were omitted from correlations then BMM exhibited the highest correlation (r = 0.74) with NaCl, MCD and MBMM having slightly lower correlations (r = 0.71, 0.68, and 0.63)(Crooker et al., 1978).

Wohlt et al. (1973), with in vitro studies, showed that casein was more soluble ($p \angle .01$) than soy protein (79.0 vs 14.3%). They also obtained a difference ($p \angle .05$) in the percent of NS of the same nitrogen source when either ARF or BMM was used as solvent. Protein solubility in ARF was less than in mineral mixture (46.0 vs 47.2%).

Other factors that have been found to produce differences on NS are: ionic strength, temperature, pH, length of extraction, motion of stirring (Crooker et al., 1975; Crooker et al., 1978; Peter et al., 1971; Wohlt et al., 1973, and Burroughs et al., 1950a).

d. Ionic strength

Ionic strength may be defined as: $dj = \frac{1}{2}\Sigma c_i Z_i^2$; where dj = ionic strength; c = Molar concentration; Z = Valance and i = ionic species. Ionic strength is varied by appropriately changing the amount of distilled water added.

Rumen fluid has an average ionic strength of 0.15 as calculated by Salobir et al. (1970). In their work they had a range of ionic strength from 0.10 to 0.22 and identified that protein solubilities of soybean, peanut, and sunflower meal in sodium chloride solutions had similar values to those obtained with ARF at ionic strength of 0.15.

However, using other solvents (BMM, 0.15M NaCl and MBMM) ionic strength within the range of 0.11 to 0.19 had no significant effect on the amount of nitrogen extracted from various protein sources as shown by Crooker et al. (1978) (see Table 3).

e. Temperature

This factor should resemble that value usually observed in the rumen, i.e. 38° - 42° C (Burroughs et al.,

Table 3. Effect of Ionic Strength[a] of Mineral Solvents on Nitrogen Solubility*

		Feedstuff						
Solvent	Ionic strength[a] (dj).	Distillers dried grains with solubles	Wheat	Hominy	Citrus pulp	Sunflower meal	Buck-wheat	Oats
		% soluble nitrogen[b]						
BMM[c]	0.11	20.5	22.8	23.9	26.5	33.3	34.1	35.6
	0.15	18.8	21.5	25.3	25.2	34.1	39.9	39.9
	0.19	22.0	20.8	24.4	23.3	35.0	34.9	34.9
NaCl[d]	0.11	22.2	25.8	28.0	34.9	27.4	34.1	15.3
	0.15	21.8	25.0	28.0	35.2	31.1	35.7	16.4
	0.19	22.4	26.0	28.0	36.0	33.7	37.2	20.8
MBMM[e]	0.11	22.3	26.6	28.6	35.5	38.6	37.2	42.6
	0.15	24.0	27.1	29.4	37.7	29.5	37.2	43.4
	0.19	22.2	25.5	27.7	37.6	38.6	37.0	45.4

*Crooker et al., 1978.

[a]Ionic strength varied by appropriately changing the amount of distilled water added. dj = ionic strength = $\frac{1}{2}\Sigma c_i Z_i^2$. Where c = Molar concentration, Z = Valance and i = ionic species.

[b]Each value is the mean of two extractions.

[c]Burroughs mineral mixture diluted to 10% with distilled water.

[d]Sodium chloride solutions.

[e]Modified Burroughs mineral mixture - $(NH_4)_2SO_4$ replaced on equimolar basis with Na_2SO_4.

1950a; Wohlt et al., 1973; Lehninger, 1970; and Marvin, 1977).

f. pH

pH of the rumen is usually within the range of 5.8 to 7.0 (Andrew, 1977). The pH level is influenced by the diet consumed. A decreased pH after feeding has been observed which is specially associated with the ingestion of appreciable amounts of rapidly fermentable sugars. A negative correlation exists between the concentration of volatile fatty acids (VFA) and lactic acid, on the one hand, and the pH of the contents on the other (as cited in Andrew, 1977). Fasting usually decreases the concentration of VFA and the ruminal pH increases above pH 7 to a value close to that of blood (Phillipson, 1942).

Lactic acid fermentation is associated with pH values of 5.5 or less when sheep or cattle are consuming diets high in starch or sugar and low in fiber, producing acidosis that might cause death. Urea introduced in excessive quantities into the rumen can produce alkalinity due to an excessive formation of ammonia, which may also be fatal (Andrew, 1977).

The buffering capacity of the rumen does not depend entirely on the saliva secreted, as exchange across the rumen wall of unionized acid with bicarbonate accounts for about one half the acid absorbed (Ash and Dobson, 1963). Turner and Hodgetts (1955) explained the role of salivary bicarbonate and phosphate in buffering the rumen contents. Buffering capacity of rumen contents is due principally to its bicarbonate content.

Wohlt et al., 1973, demonstrated _in vitro_ that with an increase of pH from 5.5 to 7 there was an increase (p .01) in average solubility (26.7% to 57.4%). The solubility of two protein sources (purified casein and isolated soy protein) was less at pH 5.5 ($p < .01$) than at either pH 6.5 or pH 7.5. There were no significant differences in solubility between pH 6.5 and 7.5 regardless of solvent (ARF vs BMM) or protein source.

g. Time of extraction

The time that a protein is exposed to the activity of the rumen-reticulum contents depends upon the level of feed intake, physical features of the particle, and associative effects of other ration ingredients (Satter et al., 1977). The longer the residence in the reticulo-rumen the higher the amount of nitrogen solubilized and the higher the degree of degradation of dietary protein.

Wohlt et al., 1973, used two solvents (ARF and BMM) exposing different protein sources to their action. They measured the amount of NS at 30, 60, 90, 120 minutes.

Solubility at 30 minutes was less ($p < .01$) than at 60 minutes in both solvents. At 60 minutes there was no difference between solvents. In ARF, solubility decreased as time passed and slowly increased in BMM.

h. Stirring

Using different varieties of beans, Smith et al. (1952), ground them with a hammer-mill (1 mm screen) and diluted the meal with water in a relation of 40:1 (water:meal) with a pH of 6.5, different methods were used for stirring the water: meal slurry as follows:

1. Mechanical shaker (Precision Scientific Company) with a reciprocating motion at room temperature (25° C).
2. Mechanical stirrer in a beaker at room temperature (25° C). This system gives less agitation than method 1.
3. Same method as in No. 2, but at 50° C.
4. Very vigorous agitation with a stirrer which had propeller blades that were nearly equal to the diameter of the flask, and rapid rotation of the blades gave very effective shearing action, the temperature used for this method was 25° C.

The amount of nitrogen extracted from method 3 and 4 was almost the same, with method 3 being slightly greater.

Method 2 was always lower than methods 3 and 4 emphasizing the importance of controlling the shearing action.

Increasing the temperature from 25° C to 50° C was approximately equal to vigorous stirring, thus showing that the speed of stirring is an important factor for *in vitro* PS studies.

Possible explanations for this are:

1. The cell structure of the beans may not have been well enough destroyed by the hammer-mill for easy liberation of the protein.
2. A part of the protein may be attached to insoluble carbohydrate particles, and vigorous stirring or beating of the solvent is required to bring the protein into dispersion.
3. The heating (of the solvent) or stirring may be supplying the energy necessary for dispersing coarse protein particles or complexes. The action of the hammer-mill may weld some otherwise soluble protein into insoluble meal particles.
4. Another factor retarding protein dispersion and common to the first three suggestions may be the formation of a hydrated shell around each particle of protein which gives a case hardening effect that retards penetration of water and rate

of dispersion. This phenomena is observed in reverse in the drying of protein.

Other workers (Crooker et al., 1978; Wohlt et al., 1973; Lyman et al., 1953) have used different systems for stirring the solution to be utilized on PS studies such as: a) water bath in a shaker, b) magnetic stirrers, c) mechanical stirring, thus influencing on the difference given by the different authors for a given protein source.

Protein Solubility and Ammonia Concentration in the Rumen

How much dietary nitrogen is required to obtain the optimal benefit out of the rumen microbial population and their growth? This has been a question that several workers have tried to answer. For instance, Satter et al. (1975) with *in vitro* studies showed that microbial protein synthesis is highly dependent upon the amount of energy available and demonstrated that the maximum microbial protein production was obtained at a concentration of 3 to 5 mg NH_3-N/100 ml of rumen fluid, which is approximately equal to a dietary crude protein content of 12.5 to 13%. Henderson et al., 1969; Allison, 1970; Bryant et al., 1961, with *in vitro* studies found 5.0 to 6.0 mg NH_3-N/100 ml the maximum utilizable ammonia concentration in the rumen for microbial protein synthesis. Bull et al.

(1975) reported a value of approximately 20 mg NH_3-N/100 ml with *in vitro* studies.

Hume et al. (1970), using the tungstic acid precipitable nitrogen technique in sheep, obtained maximum microbial protein synthesis in the rumen with an ammonia nitrogen concentration of 13.3 mg/100 ml. No further benefit on dry matter disappearance and/or organic matter digestibility was obtained by increasing ammonia concentration in the rumen. Miller (1973), with *in vivo* studies showed that the maximum microbial protein synthesis per unit of substrate fermented was obtained with concentration of approximately 13.0 mg NH_3-N/100 ml of rumen fluid.

Other workers (Mehrez et al., 1977), with mature rumen fistulated sheep using the polyester bag technique, obtained a value of 23.5 mg/100 ml of rumen fluid as the minimum NH_3-N concentration for maximum rate of fermentation. *In vivo* studies with soybean meal vs starea (Edwards et al., 1979) demonstrated that the maximum microbial protein was produced when ammonia concentration in the rumen was 76 mg/100 ml of rumen fluid, this value was obtained with starea as the dietary nitrogen supplement.

Optimal ammonia concentration in the rumen may be defined as that which results either in the maximum production of microbial protein or in the maximum rate of fermentation. Orskov et al. (1974) showed with barley fed mature wethers that the microbial protein produced per unit of substrate fermented was not altered as a result of urea supplementation while the extent of rumen fermentation and digestibility was increased.

Production of NH_3-N depends on the degree of proteolysis in the rumen, which is influenced by proteolytic rate, rumen turnover, and PS (Isaacs et al., 1972). High correlations were noted between PS and ammonia concentration in the rumen, i.e. as protein solubility increases the amount of free NH_3-N increases (Lewis, 1957; Wohlt et al., 1976; Sniffen, 1974; Annison et al., 1954; Henderickx et al., 1963; Hudson et al., 1969; Little et al., 1963; Peter et al., 1971; Sherrod et al., 1962; Chalmers et al., 1954; El-Shasley, 1952a,b and Mangan, 1972). Hume (1970b) demonstrated that readily degraded protein is superior to non-proteic nitrogen (NPN) in supporting formation of microbial protein suggesting that ruminal microorganisms also require a supply of dietary polypeptides and/or A-A's for their growth. Little et al., 1963; Belasco, 1953; Burroughs et al., 1950b, showed that some readily available nitrogen is beneficial to rumen function while small amounts of solubilized feed protein may leave the rumen without being degraded, most of the soluble protein will be broken down. The net result of dietary protein degradation is the effect of: a) Initial period, where the highly SP is degraded and b) Slower breakdown of the less SP which is extended beyond the initial period

(Crawford et al., 1978). This was confirmed by Bull et al., 1977. They pointed out that ruminal degradation of a protein is necessarily dependent on the ability of the protein to "solubilize" in the rumen medium, solubility per se does not insure degradation and the rate of the two processes are not necessarily equal.

The NH_3-N that is not incorporated into microbial protein will be absorbed through the rumen wall and either carried out by the venous blood and excreted as urinary nitrogen (Lewis et al., 1957; McDonald, 1948; McDonald, 1952) or recycled as urea via the saliva (Marvin, 1977) or pass to the lower gut where it could be utilized by the intestine microflora (Bergen, 1978).

Fate of dietary nitrogen has been studied in vivo using nitrogen isotopes in mature sheep (Mathison et al., 1971; Nolan, 1975; and Pilgram et al., 1970) and in mature bovines (Al-Rabbat et al., 1971a, 1971b) showing that NH_3-N is indeed the central intermediate in the degradation and assimilation of nitrogen in the rumen and that NH_3-N is in the preferred or required nitrogenous nutrient of many species of rumen bacteria (Bryant, 1970). Some rumen microorganisms use peptides and some A-A's directly. Other products of the fermentation of dietary protein are VFA's (El-Shasley, 1952a, 1952b; Sherrod et al., 1964).

In order to maintain optimal conditions of the ruminal population for digestibility of dry matter (DM), organic matter (OM), crude protein (CP), nitrogen free extract (NFE) and total digestible nutrients (TDN) a minimum ammonia concentration of 5 mg/100 ml is required as shown by Wohlt et al., 1978. Satter et al. (1975) also recommend 12.5% CP in the diet for maintaining the maximum growth potential of the rumen microbiota. If dietary CP does not satisfy the minimum requirements for the rumen microflora then fermentation will be limiting and the rate of passage of feed to the lower gut will decrease (Campling et al., 1962).

Degradation of protein in the rumen increases with PS and this results in higher losses of feed nitrogen as NH_3-N. The effect of this is possible decrease of feed protein reaching the lower gut (Wohlt et al., 1976; Isaacs et al., 1972). Studies of the effects of PS on nitrogen metabolism in ruminants have shown that as PS increases the level of plasma urea nitrogen increases and the excretion via urinary nitrogen increases (Wohlt et al., 1976). Plasma urea nitrogen concentration and the amount of urinary nitrogen had a linear correlation ($r = 0.97$) (Thornton et al., 1972). Increasing PS also increased the water intake (may be due to the higher concentration of urinary nitrogen), feed intake, nitrogen intake, nitrogen absorption through the wall of the rumen and gastro-intestinal tract, fecal nitrogen excreta, and decreased gross energy digestibility of the feeds (Wohlt et al., 1976; and Blaxter et al., 1962).

Bypass Protein

Orskov (1970) demonstrated that microbial protein synthesis was able to support maintenance, slow growth and early pregnancy, but not fast growth, late pregnancy or early lactation (see Fig. 2). In order to satisfy the protein requirements of animals whose production level cannot be sustained by the out-put of the rumen microbiota, an extra source of dietary protein that will pass through the reticulo-rumen without being degraded but will be absorbed as A-A's for their absorption in the lower gut is required. This extra dietary protein source has been termed "bypass protein." It has been calculated that the normal range of bypass protein is between 20 to 60% (average 40%) of the dietary protein (Chalupa, 1975; Hogan, 1975).

Rumen microbial protein tends to remain rather constant in patterns of essential amino-acids regardless of dietary source of nitrogen (true protein or NPN) (Hatfield, 1977; Purser and Buechler, 1966) i.e., changes on microbial protein are quantitative but not qualitative. So, if any change in protein quality that reaches the lower gut is to be attained, it should be through the bypass protein. Bypass protein is influenced by solubility and degradation characteristics of dietary protein (Amos et al., 1971; Little et al., 1967; McGregor et al., 1978).

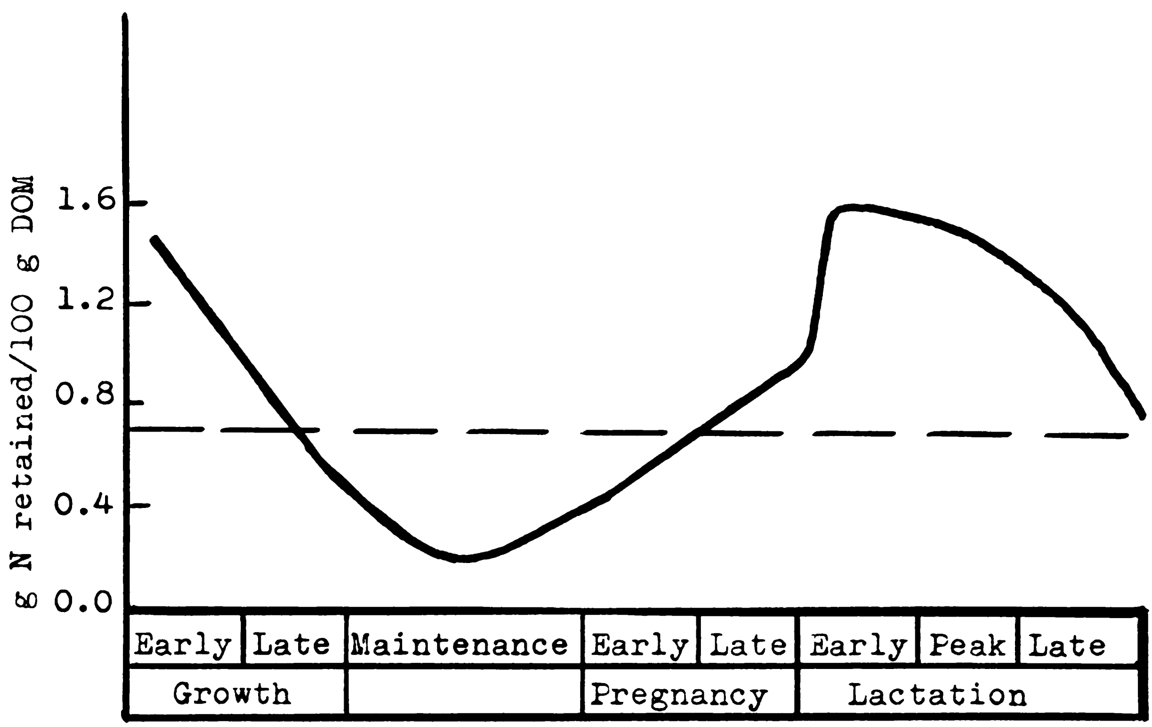

Figure 2. Effect of physiological state on potential retention of nitrogen in relation to digestible organic matter intake (Orskov, 1970).

Other factors that influence the amount of bypass protein besides resistance to ruminal degradation are the rate at which the rumen contents degrade protein and the flow rates of liquid and solid phases through the rumen because degradable fractions disappear from the rumen by degradation or passage whereas undegradable fractions disappear only by passage (Crawford et al., 1978; Waldo et al., 1972). Thus the amount of dietary protein bypassing the rumen can be depicted by the ratio Kr/(Kr+Kp) where Kr and Kp are rate constants for turnover of ruminal contents and ruminal proteolysis, respectively (Broderick, 1978).

Using in vitro methods, McGregor et al. (1978) showed that the A-A profile of the undegraded protein which bypasses the rumen may be different from the A-A profile of the dietary protein as originally ingested. In most of the feedstuffs studied there were marked differences between the A-A profile of the total protein and the A-A profile of the insoluble protein fraction. Some A-A's such as valine, leucine and iso-leucine are rather located in the soluble fraction of the feed protein. Perhaps those feedstuffs with more soluble protein may be able to support a higher rate of cellulose digestion than feedstuffs with less of these A-A's in the soluble protein fraction (McGregor et al., 1978). Other A-A's that have been identified as required for certain rumen microorganisms are methionine and cystein (Allison, 1970; Bryant, 1970; Buttery, 1976). Thus, A-A requirements for ruminants is equal to the A-A requirements at tissue level plus that of the microbial population in the rumen (McGregor et al., 1978).

The metabolizable protein (MP) concept was developed to recognize different degradability of protein sources as well as synthesis of microbial protein in rumen fermentation. These factors were used to predict the amount of amino-acids which can be absorbed postruminally and used to meet the protein requirements of the individual (Burroughs et al., 1975). It has been shown through numerous studies that changes in MP are achieved by postruminal infusion of protein or A-A's that have increased animal performance, e.g.:

Clark (1975) supplied additional high quality protein postruminally increasing both milk production by 1 to 4 kg/day/cow and milk protein by 10 to 15%.

Chalupa (1975) showed that nitrogen retention usually was increased when a mixture of methionine, lysine and threonine was supplied postruminally to growing cattle, this was duplicated by the same worker in 1976.

Different systems have been proven to increase the amount of available A-A's, such as:

a. Oesophageal groove closure reflex (Orskov et al., 1969a; Orskov et al., 1969b; Orskov, 1972; Standaert, 1979).

Oesophageal groove closure is a normal function in young ruminants, passing suckled liquid from the esophagus through the reticular groove and omasal canal into the abomasum. Factors that are believed to influence this reflex are age, posture of the animal, temperature of the liquid, chemical composition of the suckled liquid and salts contained in the solution (sodium salts; copper salts; silver and zinc salts). In mature ruminants the oesophageal groove is not closed very easily. According to Wester (1926) the oesophageal groove mechanism regresses with age due to a failure of the groove to develop proportionately with the rumen and reticulum. Also, its vagal innervation regressed with age. Oesophageal groove closure requires more investigation at this moment.

b. Heat treatment of protein sources

A reduction of protein degradation has been observed when heat has been applied to the feed before consumption by ruminants. This reduction is thought to be due to a decrease in solubility of the protein (Danke et al., 1966; Gree et al., 1954; Hudson et al., 1969; Little et al., 1963). Heating could be disadvantageous. Overheating decreases the availability of A-A's in the lower gastro-intestinal tract (Goering et al., 1972; Goering and Waldo, 1974; Goering et al., 1973; Hill and Noller, 1963). The maillard reaction between free amino groups of protein and sugar aldehyde groups is responsible for the decrease in digestibility (Goering and Waldo, 1974; Waldo et al., 1973a and 1973b). Heat damage can occur without oxidizing fat or sugar (Bjarnason and Carpenter, 1969, and 1970).

Nitrogen retention and animal performance have been increased when the protein source has been exposed to heat and pressure in such a way that protein is not degraded in reticulo-rumen but the A-A's of that protein remain available for post-ruminal digestion (Goering et al., 1974; Hudson et al., 1969; Sherrod and Tillman, 1964).

c. Chemical treatment

Chemicals which form reversible cross-linkages between the amino and the amide groups make the A-A less degradable in the reticulo-rumen. Later on in the abomasum with the HCl produced in this organ, these linkages are broken down and the A-A's are available for proteolysis and intestinal absorption. Two main chemical compounds have been tested, formaldehyde and tannins. Formaldehyde has been used extensively in practical feeding (Walker, 1974; Fraenkel-Conrat et al., 1946, 1948). Formaldehyde treatment of plant protein has resulted in better feed efficiency (Chalupa, 1975). Nitrogen retention has increased as a result of formaldehyde treatment of casein. Wool production and muscle growth have also been increased (Barry, 1972, 1973; Faichney, 1971; Hemsley et al., 1973; Reis et al., 1969; and Wright, 1971). Animal performance was also enhanced when they received forages that were treated with formaldehyde at ensiling time (Waldo et al., 1973a, 1973b; Brown and Valentine, 1972).

Tannins are found in forages and seeds, they may be responsible for some of the natural protection observed in these proteins (McLeod, 1974). Chemically, tannins have been classified as either hydrolysable or condensed. Studies done by Zelter et al. (1970) indicated that complexes formed by condensed tannins may not be hydrolysed to release amino acids in the abomasum. Hydrolysable tannins have the propriety to form cross-linkages with proteins through hydrogen bonding (Ferguson, 1975). Saba, Hale and Theurer (1972) showed that sorghum varieties with high tannin content are less degradable in the rumen than sorghum varieties with low tannin content. However, other investigators (Manson et al., 1973) demonstrated that high tannin sorghum has a lower net energy and apparent protein digestibility than normal sorghum.

d. Antibiotics

Antibiotics have been studied as means for protein protection against rumen microbial degradation, the results have not been encouraging (Hogan and Weston, 1969; Schelling et al., 1972).

MATERIALS AND METHODS

Animals and Management

Two mature Holstein cows fitted with rumen fistula were kept in an individual stall and fed at 8 a.m. every day either alfalfa hay or corn silage *ad libitum*. A 15-day period was allowed for adaptation to the diets. After the adaptation period, rumen fluid was collected and utilized as explained below.

When collection of rumen fluid of the first period was over, the cows' diets were reversed and again a 15-day period for adaptation was permitted before starting the second collection period of rumen fluid.

Rumen Fluid Processes

Two l. of rumen fluid from each cow were collected 2 hours after feeding, strained through 4 layers of cheese cloth and poured into one l. flasks. The samples were transported to the laboratory where they were centrifuged: first at 1,500 X g for 10 minutes followed by a second centrifugation at 13,000 X g for 20 minutes.

After the centrifugation process was done, the rumen fluid supernatants were either autoclaved at 121° C, with

15 lbs/in^2 pressure for 45 minutes or filtered using an all glass millipore filter apparatus #4 (47mm); first with a pore size of 0.8 m and a second filtration through a filter with pore size of 0.45 m. Rumen liquor of both treatments (autoclaved or filtered) was stored overnight in a cooler room at 4° C.

Solubility Tests

The percent soluble nitrogen of three different protein sources (alfalfa hay, cornmeal and casein) was determined in autoclaved rumen fluid (ARF) and/or filtered rumen fluid (FRF). Rumen fluid was processed as described above.

The nitrogen sources were ground with a Wiley mill to pass through a 1 mm mesh screen, allowed to air equilibrate overnight, and analyzed for total nitrogen, using the Macro-Kjedahl method (Appendix A), and expressed on a dry matter basis.

The solvents were allowed to warm up until they reached room temperature (25° C) and then preheated in a water bath set at 40° C.

Solvents and nitrogen sources were mixed at a concentration of 20 mg N/80 ml of rumen fluid in 250 ml flasks. The pH was adjusted with ortho (85%) phosphoric acid or 2N sodium hydroxide to three different pH's: 6.0, 6.5 and 7.0.

The mixtures were placed in a Dubnoff shaking water bath at 40° C at a shaking rate of 50 strokes per minute.

Fifteen ml samples were withdrawn after 60 minutes and 120 minutes and then centrifuged at 1500 X g for 10 minutes. After centrifugation, a 4 ml aliquote of the supernatant was used for soluble nitrogen determination using the macro-Kjeldahl method (Appendix A). The final value of SN was the average of two determinations, after subtracting blanks (Appendix B). An example of the calculations performed is given in Appendix C.

Statistics

The experimental design was a quadruple split-plot with repeated measurement with three factors in space and one factor on time with 2 x 2 change over in whole plots. Main effects and their interactions were tested by analysis of variance (ANOVA) (Gill, 1979) and differences between means with more than one degree of freedom by the Fisher's variance ratio or F-distribution (see Table 4). The difference between two means was tested using various appropriate statistical tests (see Table 5).

Table 4. Sources of Variation and Degrees of Freedom

Sources of Variation	d.f.[a]
Cow (C)	1
Period (P)	1
Forage (Fo)	1
Error A	0
Method (M)	1
Fo x M	1
CM + PM = Error B	2
Feeds (F)	2
Fo x F	2
M x F	2
CF + PF + CMF + PMF + FoMF = Error C	10
pH (H)	2
Fo x H	2
M x H	2
F x H	4
CH + PH + FoMH + CMH + PMH = Error D	38
Time (T)	1
Fo x T	1
M x T	1
F x T	2
H x T	2
CT + PT + FoMT + CMT + PMT + FoFT + CFT + PFT + MFT + FoMFT + CMFT + PMFT + FoHT + CHT + PHT + MHT + FoMHT + CMHT + PMHT + FHT + FoFHT + CFHT + PFHT + MFHT + FoMFHT + CMFHT + PMFHT = Error E	65

[a]d.f. = degrees of freedom.

Table 5. Statistical Tests Used on the Different Traits

Trait	Test Used
Method (M)	Dunnet's
Feedstuffs (F)	Bonferroni's
pH's	polynomial orthogonals
Time (T)	Dunnet's
M x F	Bonferroni's
F x pH	Tukey's
F x T	Tukey's

RESULTS

Rumen fluid from cow A seemed to yield more soluble protein (SP) on the mean of the three protein sources examined than that from cow B (55.9% vs 47.21% soluble protein). There was also a higher protein solubility with rumen fluid from cows fed alfalfa hay (58.76% SP) compared to corn silage (44.36% SP). The mean values for soluble protein in period I was 53.1% and for period II it was 50.01% (see Table 6). These period differences could not be evaluated statistically.

Table 6. Main Effect of Cows, Forages and Periods on Percent Protein Solubility

Forage/period	Cow A I	Cow A II	Cow B I	Cow B II	Composite Mean
	% PS[a]				
Alfalfa hay	64.65[b]	-----	-----	52.86	58.76
Corn silage	-----	47.15	41.56	-----	44.36
Mean, cow	55.9		47.21		
Mean, period I	53.1				
Mean, period II	50.01				

[a]% PS: Percent protein solubility.

[b]Mean.

Autoclaved rumen fluid (ARF) was similar to filtered rumen fluid (FRF) in percent protein solubility when used as a solvent ($p > .05$), see Table 7.

Table 7. Effect of Either Autoclaved or Filtered Rumen Fluid As Solvent On Percent Protein Solubility

Method	
Autoclaved	Filtered
% PS[a]	
51.89 $\pm$ 9.06[bI]	51.22 $\pm$ 9.06[bI]

[a]% PS: percent protein solubility.

[b]No statistical difference ($p > .05$).

[I]Mean $\pm$ SE.

Protein solubility of corn, alfalfa hay and casein were 32.17 $\pm$ 11.46, 43.48 $\pm$ 11.46 and 79.02 $\pm$ 11.46, respectively. The difference between corn and casein was significant ($p < .05$), see Table 8.

The large size of the SE's of the main effect on feedstuff sources (Table 8) may be due to the degrees of freedom used to divide the sum of squares (SS) of the main effect of feedstuffs plus the SS of the interactions considered within this block.

Table 8. Percent Protein Solubility of Corn, Alfalfa Hay And Casein

Feedstuff		
Corn	Alfalfa Hay	Casein
	% PS[a]	
32.17 ± 11.46^{bI}	$43.48 \pm 11.46^{b,c}$	79.02 ± 11.46^{c}

[a]% PS: percent protein solubility.

[b,c]Different subscript shows significant difference ($p < .05$).

[I]Mean ± SE.

pH level affected protein solubility, pH at 6.0, 6.5 and 7.0 yielded 54.14 ± 1.93%, 53.05 ± 1.93%, and 47.49 ± 1.93%, respectively. There was a significant difference ($p < .05$) between the mean values. Increasing the pH decreases ($p < .05$) the percent of soluble protein (see Table 9 and Figure 3).

Table 9. pH Effect on Percent Soluble Protein

pH		
6.0	6.5	7.0
	% PS[a]	
54.14 ± 1.93^{bI}	53.05 ± 1.93^{c}	47.49 ± 1.93^{d}

[a]% PS: percent protein solubility.

[b,c,d]Different subscripts $p < .05$; Linear response $p < .05$; Quadratic response $p > .05$.

[I]Mean ± SE.

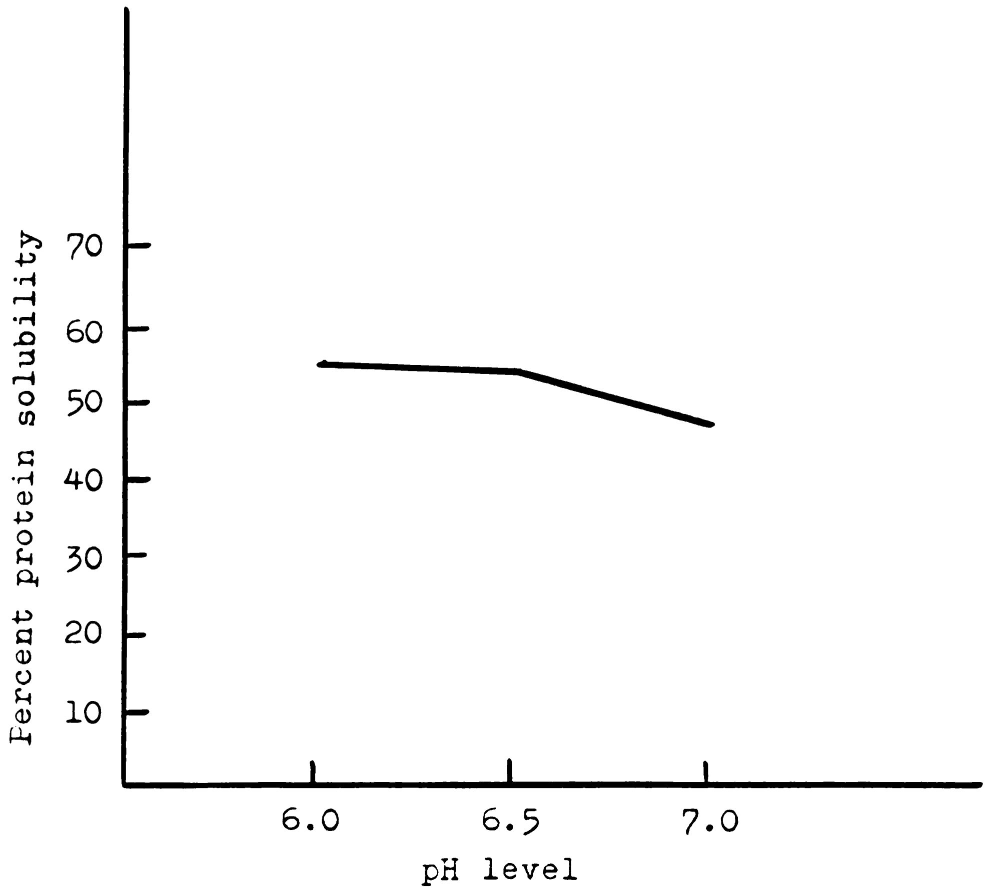

Figure 3. Protein solubility of three different pH values.

Time of exposure to the action of the solvent gave 50.19 $\pm$ 0.7% and 52.92 $\pm$ 0.7% protein solubility at 60 minutes and 120 minutes, respectively, which was significantly different ($p < .01$). See Table 10.

Table 10. Protein Solubility When Protein Sources Were Incubated for Two Intervals

Time	
60 minutes	120 minutes
------ % PS[a] ------	
50.19 $\pm$ 0.7[b]	52.92 $\pm$ 0.7[c]

[a]% PS: percent protein solubility.

[b,c]Means with different subscript are different ($p < .01$).

PS of corn, alfalfa hay and casein dissolved in either autoclaved or filtered rumen fluid was not different to those results obtained in feedstuff alone, i.e., corn was different to casein ($p < .05$) but corn was similar to alfalfa ($p > .05$) and alfalfa was similar to casein ($p > .05$), see Table 11.

Table 11. Protein Solubility for 3 Protein Sources Using Two Different Rumen Fluid Preparations

Feedstuff	Method Autoclaved	Method Filtered	Mean
	------- % PS[a] -------		
Corn	33.54 ± 16.21[b][I]	30.8 ± 16.21[b]	32.17 ± 11.46[b]
Alfalfa Hay	40.10 ± 16.21[b,c]	46.85 ± 16.21[b,c]	43.48 ± 11.46[b,c]
Casein	82.03 ± 16.21[c]	76.01 ± 16.21[c]	79.02 ± 11.46[c]
Mean	51.89 ± 9.06[d]	51.22 ± 9.06[d]	

[a]% PS: percent protein solubility.

[b,c,d]Different subscript show significant difference ($p < .05$).

[I]Mean ± SE.

The interaction between feedstuff and pH gave the following results with respect to PS (see Table 12):

a. The PS for corn meal, alfalfa hay or casein were not significantly different ($p > .05$) when each one was at either pH 6.0, 6.5, or 7.0.

b. There was no difference ($p > .05$) between corn and alfalfa hay when compared at pH 6.0, 6.5, or 7.0.

c. Alfalfa hay and casein were different ($p < .05$) at each pH level (pH 6.0, 6.5, or 7.0).

d. Difference between corn and casein was significant ($p < .05$) when compared at the same pH (6.0, 6.5, or 7.0) (see Figure 4).

Table 12. PS for Corn, Alfalfa, and Casein at Three Different pH Levels

Feedstuff	pH 6.0	pH 6.5	pH 7.0
	% PS[a]		
Corn	37.73 ± 3.34^{b}	30.9 ± 3.34^{b}	27.89 ± 3.34^{b}
Alfalfa	50.86 ± 3.34^{b}	42.11 ± 3.34^{b}	37.46 ± 3.34^{b}
Casein	73.83 ± 3.34^{c}	86.13 ± 3.34^{c}	77.11 ± 3.34^{c}

[a]% PS: percent protein solubility.

[b,c]Different subscripts show $p < .05$.

Feedstuff-time interaction showed that the percent protein solubility of corn and alfalfa hay at either 60 minutes or 120 minutes of exposure to the action of the solvent was not different ($p > .05$) for each feedstuff. However, protein solubility of casein at 60 minutes and 120 minutes was different ($p < .005$), see Table 13 and Figure 5.

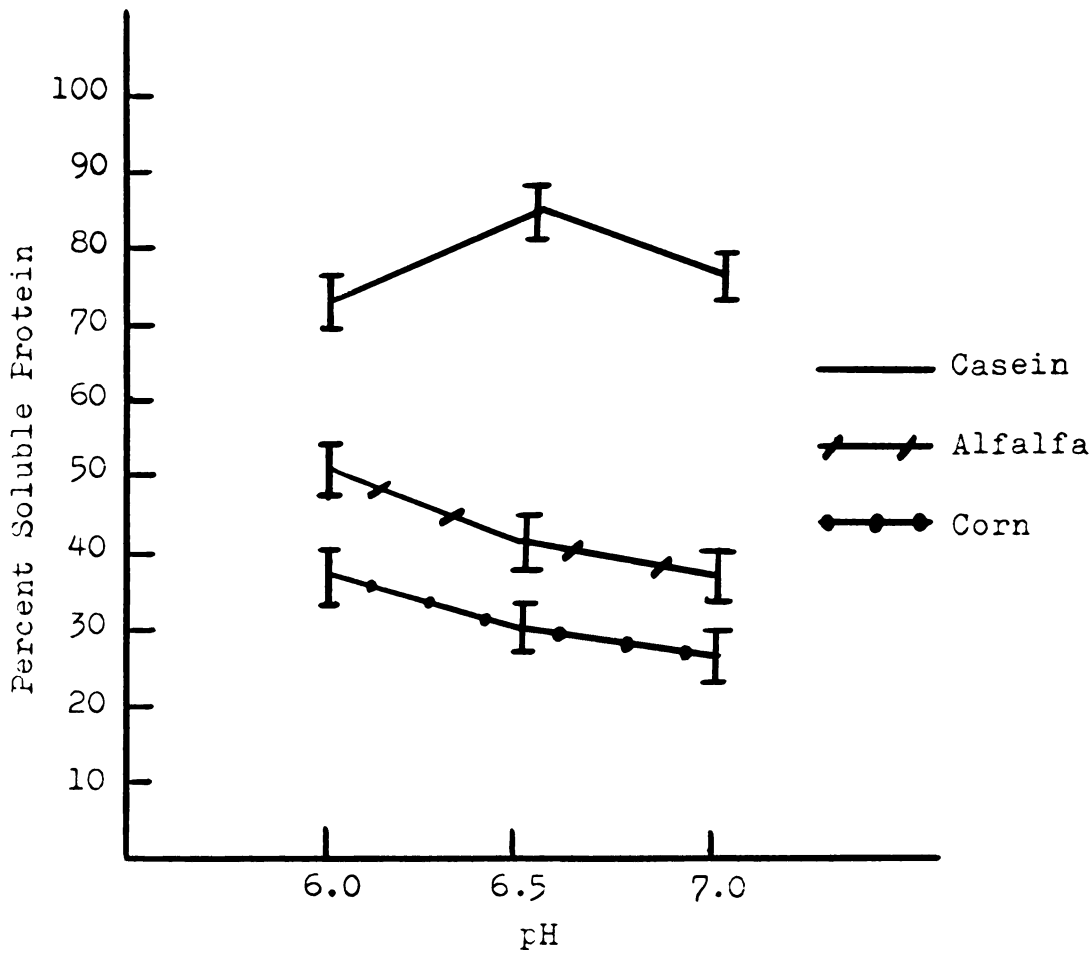

Figure 4. Protein solubility for three different protein sources at three different pH's.

Casein: $y = 1735.01 + 557.08x - 42.6x^2$

Corn: $y = 418.31 - 109.39x + 7.66x^2$

Alfalfa: $y = 475.86 - 120.08x + 8.203x^2$

$x = pH$

Table 13. PS for Corn, Alfalfa, and Casein at Two Intervals

Feedstuff	Time: 60 Minutes	120 Minutes
	------ % PS[a] ------	
Corn	31.35 ± 1.22^{b}	32.99 ± 1.22^{b}
Alfalfa	43.20 ± 1.22^{c}	43.75 ± 1.22^{c}
Casein	76.02 ± 1.22^{d}	82.03 ± 1.22^{e}

[a]% PS: percent protein solubility.

[b,c]Mean with different subscripts are different ($p < .05$).

[d,e]Mean with different subscripts are different ($p < .005$).

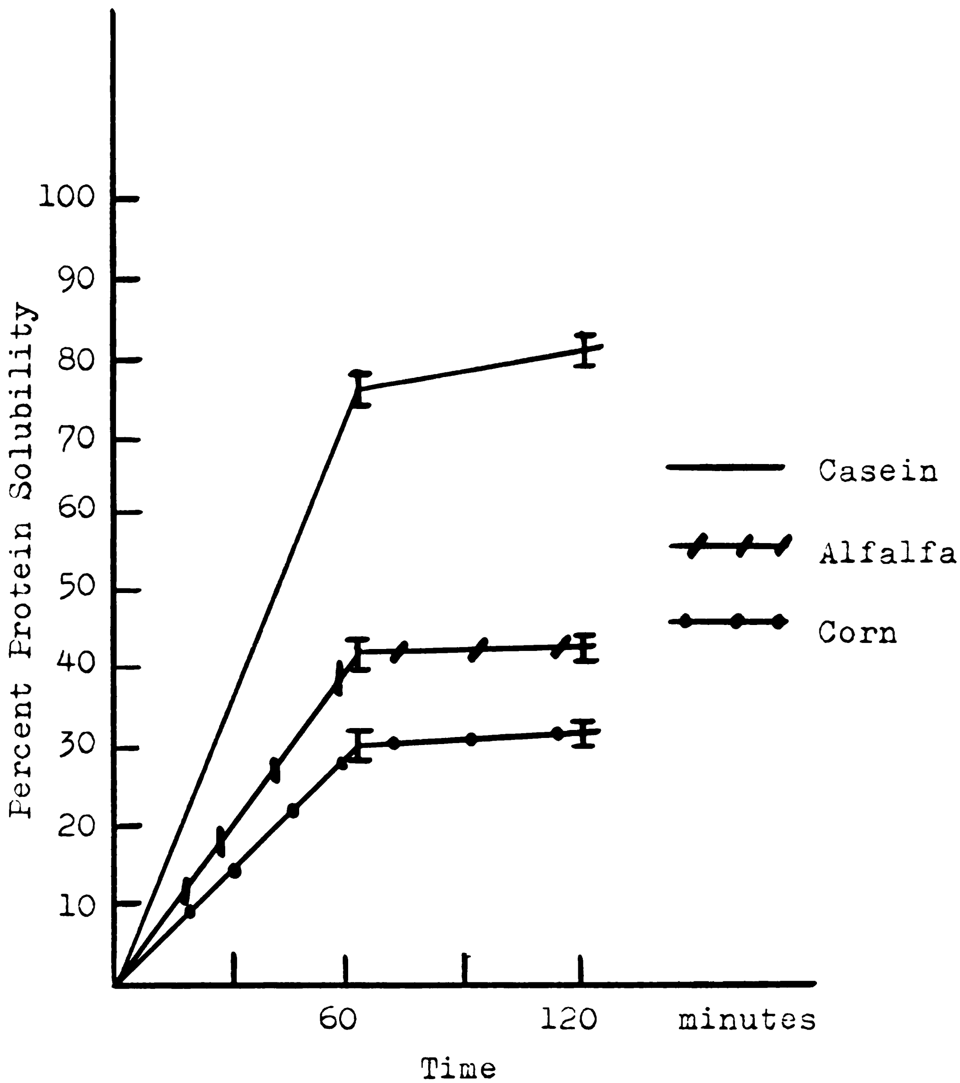

Figure 5. Protein solubility for three protein sources at two intervals.

DISCUSSION

Cow's Effect

Protein solubility of corn, alfalfa hay and casein was greater with rumen fluid from cow A than that from cow B (see Table 6). This was consistent throughout the entire experiment, i.e., when cow A was fed either alfalfa hay or corn silage as forage, its rumen fluid yielded higher protein solubility values. Since rumen fluid samples from both cows were treated equally, i.e., they had the same standardized pH, temperature, degree of agitation, length of extraction time, sample and particle size of the protein material tested; none of these factors could have been responsible for the difference in protein solubility between rumen fluid from different cows. Another possible factor is ionic strength but, according to Crooker et al., 1975; Crooker et al., 1978; and Salobir et al., 1970, protein solubility was not affected by an ionic strength from .10 to .22. They also calculated that rumen fluid has an average ionic strength of .15, therefore, it is possible to assume that ionic strength is not the factor responsible. Thus, the difference in protein solubility may be the result of other factors that are unknown now.

Forage Effect

Protein solubility of the various protein sources was greater when they were incubated in rumen fluid from cows fed alfalfa hay than that from cows fed corn silage (see Table 6). Rumen fluid from cows fed corn silage had a higher density due to high soluble starch content which form gels that do not filter easily. Rumen fluid from cows fed alfalfa hay was easier to filter than that from cows fed corn silage. Thus the difference in protein solubility may be due to differences in solvent composition, as it is with the values obtained between Burrough's mineral mixture (10% solution) and modified Burrough's mineral mixture (10% solution) as solvents since they only differ in two ionic species NH_4^+ and Na^+.

Periods

Protein solubility mean during period I was 53.1% and that in period II was 50.01% (see Table 6). These mean values are close enough to assume no difference. If one considers this a difference then it may be due to changes in the chemical composition of both forages through time. This change in chemical composition may similarly change rumen fluid composition.

Methods

Protein solubility was similar in both solvents, autoclaved rumen fluid (ARF) vs filtered rumen fluid (FRF). This should be interpreted to mean that physical and chemical characteristics of both solvents were similar. Thus, ARF and FRF may be used as solvents for protein solubility tests although it is suggested by the author of this work to keep using ARF because it is easier to prepare. Burrough's mineral mixture is another alternative as solvent. This mineral mixture behaves similarly to ARF as solvent when pH is 6.5 and incubated for 60 minutes at 40° C (Wohlt et al., 1973; and Crooker et al., 1978) but, Burrough's mineral mixture has no obnoxious odor as does ARF and it is more available in most laboratories and locations since no ruminants are required to obtain it.

Feedstuffs

Amount of soluble protein of corn, alfalfa hay and casein was 32.17%, 43.48%, and 79.02%, respectively. There was a significant difference between corn and casein (see Table 8). Casein solubility was similar to that obtained in ARF by Wohlt et al., 1973. However, corn and alfalfa hay had 13.5% soluble protein (SP) and 24.35% SP when incubated in Burrough's mineral mixture (10% solution) (Wohlt et al., 1973; Crooker et al., 1978, and Crawford et al.,

1978). The difference between the results obtained in this work and those of other investigators for corn and alfalfa may be due to type of protein of various batches of the same feedstuff and/or to the effect of different solvent. Wohlt et al., 1973, and Crooker et al., 1978, compared Burrough's mineral mixture (10% solution) vs ARF (obtained from cows fed timothy hay) and found that percent soluble protein yield was about the same in both solvents at a pH of 6.5 incubated for 60 minutes at 40° C. If pH, temperature or time were different, ARF and Burrough's mineral mixture would yield different protein solubility ($p \angle .05$). Since rumen fluid from cows fed either alfalfa hay or corn silage was used in this work and, corn and alfalfa hay were exposed to pH 6.0, 6.5 and 7.0, and, 60 minutes and 120 minutes of exposure to the solvent action, the difference between the quoted works and this one might be explained.

pH

The effect of pH in protein solubility as noted in Table 9 may be due to the type of protein that is being tested, i.e., its amino-acid (AA) composition. Since pH influences the acid-ionization or dissociation constant (Ka) of each AA, any pH change in the solvent will affect the titration curve proper of each AA. Thus, the nitrogen (protein) sources would have different solubilities at different pH's (Lehninger, 1970).

Time

Time of exposure of the various protein sources in the solvent at 60 minutes yielded 50.19% and at 120 minutes released 52.92% SP. The difference due to time was significant (see Table 10). This may be due to the solvent had more opportunity to dissolve that protein fraction that is attached to other structures of the feedstuff such as carbohydrates, lipids, etc.

Method-Feedstuff

Protein solubility in the method-feedstuff interaction was as expected (see Table 11), i.e., protein solubility was similar to that obtained in the main effect of feedstuff alone. Hence, ARF and FRF were similar as solvent.

Feedstuff-pH

Corn and alfalfa hay released more protein at pH 6.0 and less protein at pH 7.0. Casein yielded more protein at pH 6.5 and less protein at pH 6.0 (see Table 12). This may be due to the same reasons explained before on the main effects of pH.

Feedstuff-time

Corn and alfalfa hay yielded about the same amount of soluble protein when each one was exposed for either 60 minutes or 120 minutes to the solvent. Casein yielded more protein at 120 minutes than at 60 minutes ($p < .005$) of exposure to the solvent (see Table 13). This may be due to: a) Most of the soluble protein from corn or that from alfalfa hay was readily soluble, and the other protein was either attached to other structures, such as: carbohydrates, lipids, etc. or, it required more time to solubilize. b) Most of the casein was readily soluble in the media during the first 60 minutes, but it required more time to solubilize completely.

RECOMMENDATION

It is suggested to continue using autoclaved rumen fluid or Burrough's mineral mixture (10% solution) as solvents for quantifying protein solubility. Latter research may find better laboratory systems to quantitate protein solubility.

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APPENDIX A

Macro-Kjedahl Procedure
N- Determination

Weighing		K_2SO_4	H_2SO_4
Wet Feces	3 g	5 g	25 ml
Dry Feces	1-2 g	5 g	25 ml
Urine	5 cc	4.5 g	20 ml
Wet silage	4-5 g		25 ml
Blank (put also in the blank a piece of filter paper)			15 ml
Pro-Sil	0.5 g	5 g	18 ml
Molasses	1 g	4 g	18 ml

DIGESTION

1. Onto Whatman filter paper weigh proper amount of sample.

2. Fold filter paper; put paper and contents into Kjeldahl Flask.

3. To each Flask add:

   a) Proper amounts of K_2SO_4 - 5 g. 6-6.5 g of mixture in each flask
   b) Slightly less than 1 g of $CuSO_4$.
   c) Proper amounts of H_2SO_4 - 25 ml
   d) 3 boiling beads to all flasks

4. Digest on #2 position on burners:

   Digest until turns a blueish-green color; boil until solution only fills inside ring of the burner; then cool:

5. Add 250 cc of water to each flask:
   (Keep flask pointed towards burner when pouring water, protects self from vapors; also water may boil in acid-salt dilution reaction)

6. Let sit until cool.

Distillation:

1. Put 25 cc 4% Boric Acid in bottom of each beaker.
2. Place each flask under condenser outlet.
3. To each Kjeldahl flask add 60 cc of 50% NaOH solution. (Pour on a slant, so it settles on bottom)
4. Add pinch of zinc.
5. Attach flask on condensor: Turn burner to #3 until boiling then #2 and finally to #1 position.

   STAND BEHIND DOOR AND WAIT FOR REACTION.

6. Distill 200 cc of solution (Remove distilled flasks and substitute flasks with 200 cc water; set Kjeldhals off burner, then turn off.

TITRATE with .10 N HCl.

CALCULATE (Using .1 N HCl)

1. Solids (silage, feces)

$$\frac{\text{ml} - .6\ \text{ml} \times .14}{\text{sample weight} \times \text{DM decimal}} = \text{g N \% dry Matter}$$

2. Weighed liquids (Pro-Sil, molasses)

$$\frac{\text{ml} \times .14}{\text{sample weight}} = \text{g N \% wet}$$

3. Liquids by volume (urine)

$$\frac{\text{ml} \times .14}{\text{sample volume in ml}} = \text{g N/100 ml}$$

4. The factor .14 is N(.1) x .014 x 100). If normality is other than .1 the factor must be recalculated.

APPENDIX B

Total Nitrogen Contained in Control (Blank) Solutions

Rumen Fluid From	Method: Autoclaved	Method: Filtered
	---- g. N/100 ml ----	
Alfalfa Hay	0.035	0.0472
Corn Silage	0.385	0.0367

APPENDIX C

Example of Calculations to Obtain the Amount of Soluble Nitrogen

I. Obtain 20 mg of nitrogen from casein.

DATA

Feedstuff: casein
Percent dry matter (DM): 90.70%
Total nitrogen (% DM): 15.07%
Total nitrogen (% as fed): 13.668%

Solution

$$\frac{.02}{13.668} \times 100 = 0.1463$$ g of casein will provide 20 mg of N

II. Obtain amount of SN in rumen fluid.

DATA

Total N: 20 mg of N from casein
Total solvent: 80 ml
Temperature: 40° C
Time of incubation: 60 minutes
Amount of supernatant after last centrifugation: 4 ml
Method used for determination of total SN: macro-Kjeldahl
Titration with .1N HCl for the casein solution: 1.5 ml
Titration with .1N HCl for the blank solution: 1.0 ml
Equation for total N determination:

$$\frac{(\text{ml of .1N HCl for problem} - \text{ml of .1N HCl for blank}) \times .14}{\text{sample volume in ml}}$$

$= \text{g N/100 ml}$

Solution:

$$\frac{(1.5 - 1.0).14}{4 \text{ ml}} = 0.0175 \text{ g N/100 ml}$$

Calculate percent SN:

$$\frac{0.0175}{.02} \times 100 = 87.5\% \text{ SN}$$

APPENDIX D

Curriculum Vitae

Name: Ricardo Antonio Celma Alvarez

Nationality: Mexican

Languages: Spanish and English

B.S.; D.V.M. at the National University of Mexico (March, 1975)

M.S.: Michigan State University

Works	Place	Activity	Year
Research Assistant	National Institute of Animal Research	Physiopathology	1969-1971
Private vet. Clinic	Private	Small Species	1975-1979
Professor	National University of Mexico	Teaching--Animal Nutrition	1974-1977
Professor	National School ENEP for Professional Education	Teaching--Animal Nutrition	1975-1976