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Extractability, Solubility, and Molecular Size Distribution of the Nitrogenous Constituents in Coastal Bermuda Grass Silage

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Ensiled Coastal Bermuda grass was extracted at pH 7.9 with 0 and 1% sodium dodecyl sulfate (NaDodSO$_4$), followed by filtration, centrifugation, and preparative chromatography on G-15 Sephadex. Two insolubles, R_I and R_{II}, and four soluble fractions, proteins (C1), polypeptides (C2), smaller polypeptides and amino acids (C3), and degradation products (C4), were obtained. At 0% NaDodSO$_4$, the percentage of total nitrogen (N_T) for R_I, R_{II}, C1, C2, C3, and C4 was 28.5, 3.41, 1.27, 22.1, 18.9, and 4.68, respectively. At 1% NaDodSO$_4$, R_I was 23.6 in N_T and C1 was 7.2; the rest were unchanged. Gel chromatography gave a broad molecular weight distribution [$(1.0 \times 10^6)-(2.5 \times 10^3)$] for C1, but a low average [$(4-8) \times 10^3$]. C2 and C3 had narrow molecular weight distributions (averages of 1800 and 950). Molecular weight averages were independent of percentage NaDodSO$_4$ in extractant (i.e., 0 and 1%) and of wavelength of detection (i.e., 254 and 206 nm). The chloroplastic proteins (i.e., most of the proteins in cut 1 from the NaDodSO$_4$ extraction) were insolubilized by lyophilization. Gel chromatography indicated that the chlorophyll-protein complex had a molecular weight in excess of 1 million.

Ensiling is a major method of preserving and storing fresh forage. Changes in the chemical composition of ensiled forages have been studied intensively over the last 30 years and are important from a nutritive standpoint. Recently, McDonald and Whittenbury (1973) reviewed thoroughly the research on the chemical changes which occur in silage.

The degradation of amino acids in silage has been well documented (e.g., Hughes, 1970; Macpherson and Violante, 1966a,b; Macpherson 1962). Perhaps the most comprehensive study of nitrogenous compounds in silage was that of Hughes (1970), who analyzed by groups the water-soluble proteins, peptides, amino acids, and amides and volatile amines in rye grass. Hughes found no water-soluble proteins in rye grass, but did not extract proteins from the silage with a buffered solution of about pH 8.0 or which also contains detergent. Such solutions will maximize protein removal from fresh grasses (Fishman and Burdick, 1977). Therefore, in this report, Coastal Bermuda grass silage was extracted with buffer and buffered detergent solutions with the intent of extracting maximum protein.

The data reported here should be useful for evaluating Bermuda grass silage as a potential source of protein concentrate. Furthermore, the data should provide basic information to those interested in the nitrogen and the actual protein content in grass silage since the molecular size distribution of nitrogenous constituents has been measured directly and quantitatively.

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EXPERIMENTAL SECTION

Coastal Bermuda Grass. Coastal Bermuda grass (*Cynodon dactylon* [L.] Pers) was obtained from Coastal Farms, Inc., Estill, SC. It was harvested and fertilized as previously described (Fishman and Burdick, 1977). A

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Table I. Distribution of 100 g of Crude (N_T)[a] Protein and Crude Protein Content (CP)[b] in Ensiled and Fresh Coastal Bermuda Grass Fractions as a Function of Detergent ($NaDodSO_4$)

	0% $NaDodSO_4$				1% $NaDodSO_4$			
	CP[c]		N_T[d]		CP[c]		N_T[d]	
fraction	ensiled	fresh[e]	ensiled	fresh[e]	ensiled	fresh[e]	ensiled	fresh[e]
residue I(R_I)	5.6	9.6	28.5	46.9	4.7	7.6	23.6	30.8
residue II (R_{II})	9.4	18.0	3.1	15.6	4.9	4.5	3.7	4.6
total insoluble (R_I + R_{II})			31.6	62.5			27.3	35.4
cut 1 (C1)	15.0	42.7	1.2	10.8	19.0	44.1	7.2	38.0
cut 2 (C2)	26.1	11.0	22.1	5.1	23.5	10.1	21.9	3.7
cut 3 (C3)	12.6	7.4	18.9	12.8	14.5	7.8	17.1	11.6
cut 4 (C4)	2.8	3.6	4.7	0.8	1.4	1.6	3.0	0.7
total soluble (C1-4)			46.9	29.5			49.2	54.0
recovery			75.5	92.1			76.5	89.4

[a] N_T = g of N in each fraction/g of N in whole grass. [b] CP = N × 6.25. [c] Triplicate analyses, standard error ±1.1. [d] Triplicate analyses, standard error ±1.7. [e] Taken from Fishman and Burdick (1977).

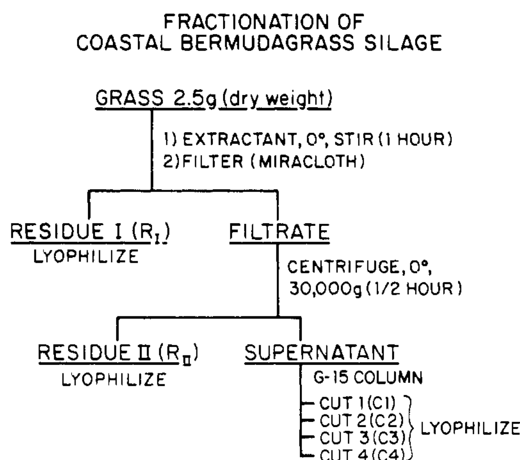

Figure 1. Flow diagram for the preparation of fractions from Coastal Bermuda grass.

portion of the grass was packed tightly into quart mason jars and capped in the field soon after harvest. The jars, which served as laboratory silos, were stored for 1 year at 33 ± 1 °C. Odor, color, pH measurements, and fatty acid analysis indicated that good silage was produced (McHan et al., 1977).

The contents of two jars were lyophilized and ground with a Wiley mill to pass a 420-μm screen and with a ball mill for 96 h at 5 °C. The powdered silage was stored at 5 °C until extracted. Micro-Kjeldahl analysis gave 13.56 ± 0.31 crude protein (N × 6.25) on a dry weight basis.

Extraction. Each of triplicate samples of the powdered silage (2.5 g of dry weight) was extracted with 50 mL of 0.2 M boric acid–sodium borate buffer (pH 7.91) which was 5 mM in sodium metabisulfite, an antioxidant. A second set of extractions had 1% w/v of sodium dodecyl sulfate ($NaDodSO_4$) added to the buffer–metabisulfite solution.

Fractions. Figure 1 is a flow sheet for the fractionation procedure. The residue, R_I, was isolated by squeezing the extraction mixture through two layers of miracloth, a quick filtration material (purchased from Calbiochem). Fractionation of the supernatant on a G-15 Sephadex column was as described by Fishman and Burdick (1977). Crude protein (micro-Kjeldahl) was determined on each fraction (McKenzie and Wallace, 1954).

Gel Chromatography. Analytical gel chromatography was performed on the automated gel filtration apparatus described earlier (Fishman and Burdick, 1977). The detector of the chromatograph measured the effluent absorbance at 206 and 254 nm. For comparison, chromatograms were replotted in terms of partition coefficient, K_{AV}. Information retrieval from the gel chromatograms and sample preparation have already been described (Fishman, 1976).

RESULTS AND DISCUSSION

Extractability and Initial Solubility of Nitrogenous Constituents. Table I shows the distribution of nitrogen in the fractions of ensiled Coastal Bermuda grass.

For ensiled Coastal Bermuda grass, $NaDodSO_4$ in the extractant did not greatly affect total nitrogen solubilized, i.e., 47–49%. In contrast, for fresh frozen Coastal Bermuda grass, $NaDodSO_4$ nearly doubled the extractable protein, 54% against 29% (see Table I). $NaDodSO_4$ apparently increases the yield of soluble proteins from Coastal Bermuda grass by disrupting chloroplastic membranes and solubilizing hydrophobic chloroplastic proteins (Fishman and Burdick, 1977; Fishman and Evans, 1978). Fishman and Burdick (1977) found that fresh frozen Coastal extracted with buffer gave a value of 15.6 for the N_T of R_{II} which was reduced to 4.6 by the addition of 1% $NaDodSO_4$ to the extraction medium. In contrast, silage had a value of 3.1 for the N_T value of R_{II} when extracted without $NaDodSO_4$. The proteins of R_{II} from fresh Coastal are predominately chloroplastic (Fishman and Burdick, 1977; Fishman and Evans, 1978). Evidently, ensilage also increased the yield of extracted and solubilized proteins from Coastal Bermuda grass by disrupting chloroplastic membranes and solubilizing hydrophobic proteins. Whereas $NaDodSO_4$ acts on Coastal strictly by solvent effects, ensilage involves microbial fermentation. The data in Table I indicate the occurrence of chloroplast destruction and resulting solubilization of the protein because the partitioning between soluble and insoluble nitrogen in the silage extractions was similar to that found for fresh Coastal Bermuda grass with $NaDodSO_4$.

The value of N_T for cut 1 was increased by the addition of $NaDodSO_4$ to the extractant (i.e., 7.2 against 1.2 for the ensiled Coastal). However, the value of N_T in ensiled Coastal was only about 11% of that extracted from fresh Coastal without $NaDodSO_4$ and about 19% of that extracted from fresh Coastal with $NaDodSO_4$. Moreover, the value of N_T for cuts 2 and 3 was not affected by $NaDodSO_4$ in the extractant, but was higher when grass was ensiled rather than fresh, i.e., 22.1 and 21.9 compared to 5.1 and 3.7 for cut 2; and 18.9 and 17.1 compared to 12.8 and 11.6 for cut 3. These results are consistent with reports of extensive protein breakdown during the ensiling of grass (McDonald and Whittenbury, 1973). Extensive disruption of chloroplasts and breakdown of proteins in silage prior to extraction would explain the greater ease of nitrogen extraction of ensiled over fresh frozen grass. Nitrogen recoveries averaged 92.1 and 89.4% for fresh Coastal compared to 75.5 and 76.5% for ensiled Coastal. These

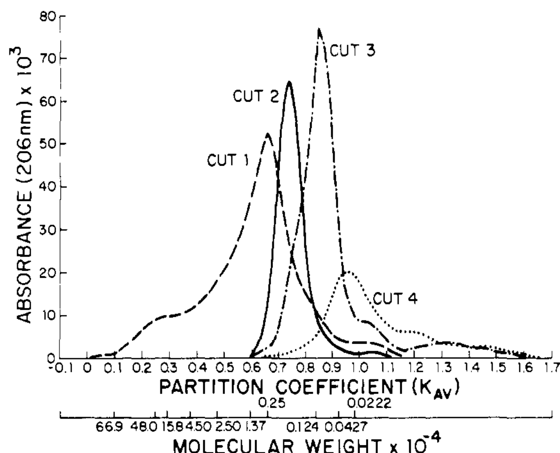

Figure 2. Gel chromatogram of cuts 1–4 was separately chromatographed on four column chromatograph. Extractant contained 1% $NaDodSO_4$.

Table II. First Moment Apparent Molecular Weight Averages ($M_A \times 10^{-3}$) of Ensiled Coastal Bermuda Grass Fractions as Determined by Gel Chromatography

fraction	0% $NaDodSO_4$		1% $NaDodSO_4$	
	206 nm	254 nm	206 nm	254 nm
cut 1 (C1)	7.3 ± 4.9[a]	4.0 ± 1.8	6.5 ± 4.6	8.6 ± 5.1
cut 2 (C2)	1.7 ± 0.4	2.0 ± 0.4	1.8 ± 0.3	1.8 ± 0.3
cut 3 (C3)	1.1 ± 0.8	1.2 ± 0.3	0.4 ± 0.3	1.1 ± 0.5

[a] Standard error, six analyses (three extractions × two chromatographs).

differences in recoveries may reflect a higher percentage of trapped volatile nitrogen in the silage as compared to fresh Coastal.

Table I also shows the percentage of crude protein (CP) in each fraction of ensiled Coastal. Whereas, the CP of cut 1 from ensiled Coastal was lower than Fishman and Burdick (1977) found for fresh, cuts 2 and 3 were higher. These results are further evidence of the extensive protein breakdown in ensiled grass since below we show that molecular weights decreased with increasing cut number.

Molecular Weight Distribution by Gel Chromatography. Superimposed gel chromatographs of the four soluble cuts of silage extracts from separate runs on the analytical gel chromatograph are shown in Figure 2. The first moment apparent molecular weights (Fishman, 1976) for cuts 1–3 analyzed at 206 and 254 nm are shown in Table II. The first moment apparent molecular weight of cut 4 falls below the lower end of the operating range of the gel columns; therefore it was less than 222. For ensiled Coastal, the average molecular weights of cuts 1–3 were independent of percentage $NaDodSO_4$ in the extractant and wavelength of detection. This finding is illustrated for cut 1 by typical chromatographs in Figures 3 and 4, which are similar. Fishman and Burdick (1977) reported that for fresh Coastal Bermuda grass, the average apparent molecular weights of cuts 2 and 3 unlike that of cut 1 are independent of the percentage $NaDodSO_4$ in the

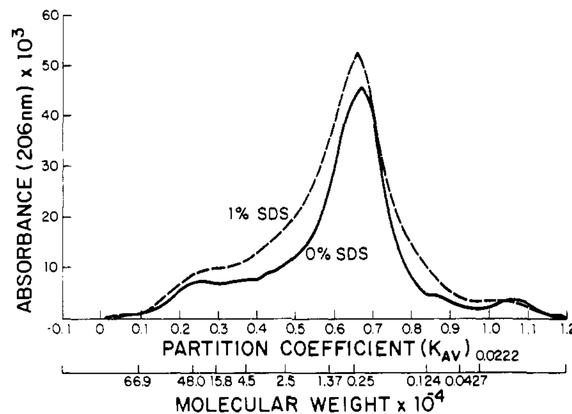

Figure 3. Gel chromatograms of lyophilized cut 1 extracted at 0 and 1% $NaDodSO_4$.

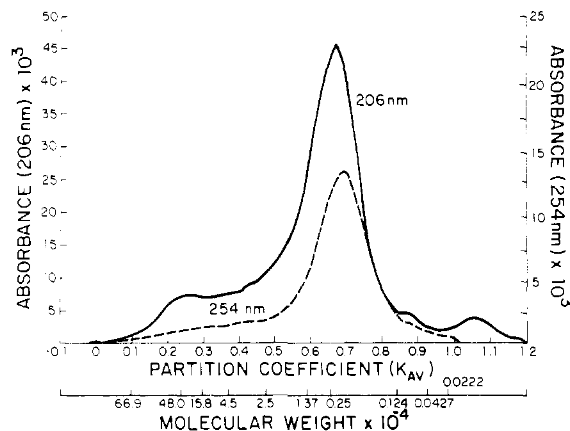

Figure 4. Gel chromatograms of lyophilized cut 1 extracted with 1% $NaDodSO_4$ and monitored at 206 and 254 nm.

extractant. The average molecular weight of cut 1 for fresh Coastal Bermuda grass was 21 200 (Fishman and Burdick, 1977) compared to 7300 for cut 1 of ensiled Coastal Bermuda grass (Table II) when each was extracted by buffer without $NaDodSO_4$. Therefore, direct evidence from gel chromatography indicates that protein undergoes degradation during the ensilage process and prior to extraction. Particularly in view of previous data which showed a substantial decrease in N_T for cut 1 and substantial increases in N_T for cuts 2 and 3 when comparing fresh with ensiled Coastal.

Protein Purity. Previously, Fishman and Burdick (1977) defined a purity parameter (F_A) as the fractional change in absorbance of functional groups at 254 nm compared to 206 nm when the grass is extracted with finite concentration of $NaDodSO_4$ rather than without $NaDodSO_4$.

For ensiled Coastal Bermuda grass, cuts 1–3 had values close to 1 at 1% $NaDodSO_4$ (Table III). Since there is extensive disruption of chloroplasts and degradation of proteins in Coastal Bermuda grass silage prior to extraction, $NaDodSO_4$ in the extractant produces small changes

Table III. Solubility and Purity Parameters from Gel Chromatography for Ensiled Coastal

fraction	0% $NaDodSO_4$		1% $NaDodSO_4$			
	$A_{206} \times 10^2$[a]	A_{206}/A_{254}	$A_{206} \times 10^2$[a]	A_{206}/A_{254}	F_A	F_S
cut 1 (C1)	650 ± 71[b]	3.91 ± 0.25	135 ± 40	3.72 ± 0.18	1.05 ± 0.12	0.21 ± 0.09
cut 2 (C2)	153 ± 7	6.40 ± 0.43	247 ± 73	6.89 ± 0.47	0.93 ± 0.13	1.61 ± 0.54
cut 3 (C3)	231 ± 100	12.1 ± 3.5	393 ± 140	13.0 ± 2.6	0.93 ± 0.45	1.7 ± 1.31
cut 4 (C4)	16000 ± 1400	2.49 ± 0.19	4368 ± 1500	1.55 ± 0.35	1.60 ± 0.48	3.7 ± 1.6

[a] Units = milliliters/milligram of protein (N × 6.25). [b] Standard error, six analyses (three extractions × two chromatographs).

Table IV. Comparison of Fresh and Ensiled Coastal Bermuda Grass by Purity Parameters

fraction	$F_A{}^0$	$F_A{}^1$
cut 1 (C1)	2.17 ± 0.54[a]	0.99 ± 0.10
cut 2 (C2)	0.79 ± 0.07	0.71 ± 0.09
cut 3 (C3)	0.70 ± 0.25	0.76 ± 0.37

[a] Standard error, six analyses (three extractions × two chromatographs) for cuts 1 and three analyses (three extractions × one chromatograph) for cuts 2 and 3.

in properties when comparing protein extracted with and without $NaDodSO_4$.

Fresh and ensiled Coastal Bermuda grass can be compared more directly by defining a new purity parameter, ($F_A{}^Y$), the fractional change in absorbing functional groups at 254 nm compared to 206 nm by extracting silage instead of fresh grass at Y concentration of $NaDodSO_4$ according to

$$F_A{}^Y = (A_{206}/A_{254})_F/(A_{206}/A_{254})_E \quad (1)$$

where $(A_{206}/A_{254})_F$ is the ratio of areas under the gel chromatography curves for fresh grass at Y concentration of $NaDodSO_4$ in extractant and $(A_{206}/A_{254})_E$ is the ratio of areas under the gel chromatography curves for ensiled grass at Y concentration of $NaDodSO_4$ in extractant. Values of $F_A{}^Y$ at 0 and 1% $NaDodSO_4$ in the extractant for cuts 1–3 are in Table IV. Cut 1 had a value of about 2.2 for $F_A{}^0$ and about 1 for $F_A{}^1$. Since cut 1 extracted without $NaDodSO_4$ from fresh Coastal Bermuda grass was the only one which contained virtually no chloroplastic proteins, only its value for $F_A{}^Y$ was appreciably above 1.

Cuts 2 and 3 have $F_A{}^Y$ values in the range 0.7–0.8 (Table IV). Possibly, many of the polypeptides in cuts 2 and 3 from the ensiled grass would have been cut 1 proteins had there been extraction of fresh rather than ensiled grass. In fresh Coastal, cut 1 is less contaminated with nonprotein substances having a high absorbence at 254 nm than cuts 2 and 3 (Fishman and Burdick, 1977).

Solubility. The solubility characteristics of cut 1 extracted from ensiled Coastal Bermuda grass were very similar to those of cut 1 from fresh Coastal at 0% $NaDodSO_4$. Both cuts were straw-colored when eluted from the G-15 Sephadex column, freeze-dried to a cream-colored powder, redissolved in 0.8 N NaCl to form a straw-colored solution, and left negligible residue on passing through a 0.40-μm membrane filter. With $NaDodSO_4$, cut 1 was a green solution, which when eluted from the G-15 Sephadex column gave a ultraviolet-visible absorption spectra similar to that found for other chlorophyll–protein complexes (Salisbury and Ross, 1969). Lyophilization yielded a green powder that partially dissolved in 0.8 N NaCl, left an oily green residue on the filter, and gave a filtrate similar to that from cut 1 for the 0% $NaDodSO_4$ extraction.

Work in this laboratory (Fishman and Burdick, 1977) on fresh Coastal Bermuda grass showed that the areas under the gel chromatography curves analyzed at 206 nm and normalized to unit protein (N × 6.25) concentration could be used to measure the quantity of green protein powder which redissolves in 0.8 N NaCl. The fraction solubilized (F_S) was computed from the ratio of areas for the 206-nm curves at finite and 0 concentrations of $NaDodSO_4$ in the extractant. The values of F_S for the various cuts from ensiled Coastal extracted with 1% $NaDodSO_4$ are in Table III.

The F_S value of 0.21 ± 0.09 for cut 1 of ensiled Coastal Bermuda grass does not differ greatly from the value of 0.28 ± 0.08 for cut 1 of fresh Coastal (Fishman and Burdick, 1977), i.e., in both cases only 20–30% of the protein originally soluble redissolved in 0.8 N NaCl after lyophilization. Apparently, the additional 6% of N_T in cut 1 from the detergent extracted silage compared to the nondetergent-extracted silage (Table I) arose from chloroplasts which were not degraded by the microbes in the silage. Moreover, the product $F_S \times N_T$ for cut 1 of the detergent extracted silage is 1.5% which is very close to the 1.2% obtained for N_T of cut 1 which was extracted without $NaDodSO_4$. Apparently, the chlorophyll–protein complex from Coastal silage is similar in solubility behavior to the chlorophyll–protein complex extracted from fresh Coastal because neither dissolves in 0.8 N NaCl after lyophilization. Also, the insolubility of the chlorophyll–protein complex from silage is confirmed by the similarity of gel chromatographs in Figures 3 and 4, and by the molecular weight data for cut 1 in Table III.

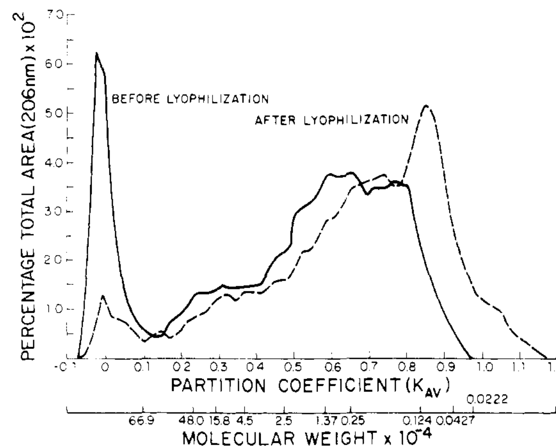

Figure 5. Gel chromatograms of cut 1 before and after lyophilization. Chromatograms have been normalized with respect to total area.

Figure 5 is the gel chromatograms of cut 1 before and after lyophilization. The chromatograms have been normalized by plotting percentage of total chromatogram area against partition coefficient or molecular weight equivalent. A large peak was present at the void volume in the chromatogram of cut 1 before lyophilization but was absent after lyophilization. The molecular weight(s) of the molecules corresponding to this peak was in excess of 1×10^6 since the molecules eluted as a peak at the void volume. After lyophilization, solubilization in 0.8 N NaCl, and filtration, the entire distribution of molecular weights was shifted to lower values. The apparent molecular weight average of the extract was 24.2×10^3 prior to lyophilization but 2.4×10^3 after lyophilization. Apparently, the peak for the material which was eluted at the void volume prior to lyophilization, i.e., the chlorophyll–protein complex, was absent in the chromatogram after lyophilization because it failed to dissolve.

The F_S values in Table IV for cuts 2–4 are above 1. All of the fractions, regardless of the percentage of detergent in the extractant were completely resoluble in 0.8 N NaCl. Equation 1 was derived on the assumption that should changes in functional groups affect the absorption at 254 nm, they would only negligibly affect absorption at 206 nm. This assumption appears to have been valid for all cuts from fresh Coastal Bermuda grass and cut 1 of Coastal silage, but not for cuts 2–4 of Coastal silage.

CONCLUSIONS

The ensilage process disrupts chloroplasts and degrades proteins. Hence maximum yields of proteins, polypeptides, and amino acids should be obtainable with milder conditions of extraction than those used to obtain comparable yields from fresh Coastal. Since about 75% of the chlo-

roplastic protein already has been degraded during ensiling, it should be more stable toward degradation than the same protein extracted from fresh grass. On the negative side, much of the extractable true protein is degraded. Hence, methods of protein isolation based on denaturation such as heat precipitation will not work. Furthermore, without additional isolation steps, the highest concentrations of CP isolated was 26% compared to about 45% in fresh Coastal. Finally, about 15% of the CP recovered from fresh grass is not recovered from silage.

From a nutritional point of view, the N_T value of 23.6% which was observed for R_I from the detergent extraction (see Table I) is a maximum value of bound N in Coastal silage. Conventionally more drastic chemical methods than those employed here are used to extract bound N in forages and hence lower values than 23.6% are obtained usually for N_T (Goering et al., 1972). Some of R_I may be true protein which is covalently bound to the cell walls of the plant. The N_T value of soluble true protein is 0.6%. This value was obtained from the product of $N_T \times F_S \times 0.4$. The factor 0.4 was chosen in that about 40% of the area under the 206-nm curve in Figure 4 represents solute with molecular weights in excess of 13000. A molecular weight of 13000 was arbitrarily selected as the lower limit for true protein. The values of N_T (Table I) and F_S (Table II) are for cut 1 from the 1% $NaDodSO_4$ extraction. The N_T value for true protein which is initially soluble but becomes insoluble because of denaturations is about 5.7%. This value is the product of N_T and $(1 - F_S)$. The soluble nitrogen in cuts 2 and 3 is NPN and includes polypeptides and amino acids. Nonprotein nitrogen is 41% of N_T for the 0% $NaDodSO_4$ extraction and 42% of N_T for the extraction with 1% $NaDodSO_4$.

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Relationship between Enzyme Levels and Extractable Proteins in Alfalfa

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Some enzyme levels (3′-nucleotidase, 5′-nucleotidase, acid phosphatase, adenosine nucleosidase, and aldolase) have been determined in alfalfa (*Medicago sativa*) extracts stored at 37 °C for different time intervals and in various fractions obtained during preparation of leaf protein concentrate (LPC). It was noted that the decay of aldolase activity parallels the decrease of 7% trichloroacetic acid precipitable proteins and the disappearance of the high-molecular-weight proteins peak emerging with the eluant front when the alfalfa "brown juice" is eluted on Sephadex G-50. Since these proteins are retained in final LPC preparations, the determination of aldolase levels in brown juices may be useful to determine the amount of protein which is extractable for feed and food utilization. The other enzyme activities such as 3′- and 5′-nucleotidases are more stable and are still detectable in LPC. The determination of their levels may therefore be useful to estimate the degree of denaturation of proteins in final preparations.

Preparations of feed grade leaf protein concentrates (LPC) and isolates from alfalfa (*Medicago sativa*) press juices contain high-molecular-weight polypeptides; oligopeptides and amino acids are removed during the fractionation steps. The maximum amount of protein which can be extracted ranges between 5 and 12% of the dry weight of alfalfa (Chayen et al., 1961; Morrison and Pirie, 1961; Lazar et al., 1971; Free and Satterlee, 1975; Felicioli et al., 1978). Obviously the protein yield depends on the extraction procedures used; however, the values obtained may be influenced by the different procedures for protein determination. Several discrepancies have been reported because of the presence of interfering compounds present in leaf juices, such as free amino acids, oligopeptides, nitrogen-containing molecules, polyphenols, and added reducing agents (de Fremery et al., 1972; Miller et al., 1972; Howarth et al., 1973; Free and Satterlee, 1975). High temperature, pH, and solvent precipitations are generally used to prepare concentrates or isolates and may lead to alterations of the functional structure of the protein molecules. Also, endogenous proteolysis might result in

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