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 13 000. A molecular weight of 13 000 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% $NaDodSO_4$.

LITERATURE CITED

Fishman, M. L., *Anal. Biochem.* **74**, 41 (1976).
Fishman, M. L., Burdick, D., *J. Agric. Food Chem.* **25**, 1122 (1977).
Fishman, M. L., Evans, J. J., *J. Agric. Food Chem.* **206**, 1447 (1978).
Goering, H. K., Gordon, C. H., Hemken, R. W., Waldo, D. R., Van Soest, P. I., Smith, L. W., *J. Dairy Sci.* **55**, 1275 (1972).
Hughes, A. D., *J. Agric. Sci.* **75**, 421 (1970).
Macpherson, J. T., *J. Sci. Food Agric.* **13**, 29 (1962).
Macpherson, H. T., Violante, P., *J. Sci. Food Agric.* **17**, 125 (1966a).
Macpherson, H. T., Violante, P., *J. Sci. Food Agric.* **17**, 128 (1966b).
McDonald, P., Whittenbury, R., "Chemistry and Biochemistry of Herbage", Butler, G. W., Bailey, R. W., Ed., Academic Press, New York, 1973, Chapter 28.
McHan, F., Spencer, R., Evans, J., Burdick, D. *Proc. 74th Annual Meet. South. Assoc. Agric. Sci. (Dairy Sect.)* **54**, 10 (1977).
McKenzie, H. A., Wallace, H. S., *Aust. J. Chem.* **7**, 55 (1954).
Salisbury, F. B., Ross, C., "Plant Physiology", Wadsworth Publishing Co. Inc., Belmont, CA, 1969, Chapter 13.

Received for review February 21, 1979. Resubmitted June 21, 1979. Accepted December 6, 1979. Presented at the Division of Agricultural and Food Chemistry, 176th National Meeting of the American Chemical Society, Sept 1978. Mention of commercial items does not imply endorsement by the U.S. Department of Agriculture over others of a similar nature.

Relationship between Enzyme Levels and Extractable Proteins in Alfalfa

Marcella Camici, Ettore Balestreri,* Maria G. Tozzi, Domenico Bacciola, Isabella Saracchi, Romano Felicioli, and Piero L. Ipata

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

Laboratorio di Biochimica, Facoltà di Scienze, Università di Pisa, Via A. Volta 4, 56100 Pisa, Italy (M.C., M.G.T., P.L.I.) and Laboratorio per lo Studio delle Proprietà Fisiche di Biomolecole e Cellule, C.N.R., Via F. Buonarroti 9, 56100 Pisa, Italy (E.B., D.B., I.S., R.F.).

0021-8561/80/1428-0500$01.00/0

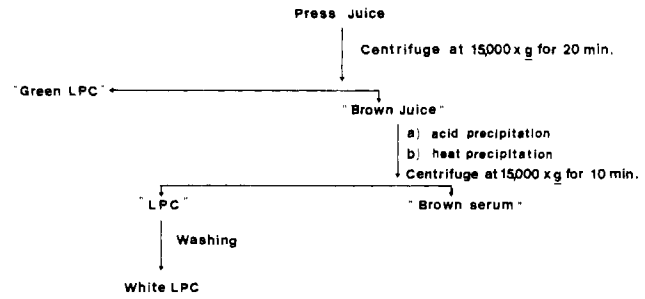

Figure 1. Fractionation of alfalfa press juice. The procedure of Free and Satterlee (1975) has been slightly modified: (a) acid precipitation. Precipitation pH of 3.5 was obtained by dropwise addition of concentrated HCl. Precipitation was allowed to proceed at 4 °C for 6 h. (b) Heat precipitation. The temperature of brown juice was adjusted at 80 °C under continuous stirring. The suspension was immediately cooled and the precipitation was allowed to proceed for 6 h at 4 °C. Washing was performed with boiling 1 mM HCl and boiling acetone according to Felicioli et al. (1978).

lowering the recovery of proteins and in progressive severe alterations of their functional properties (Singh, 1962; de Fremery et al., 1972; Pirie, 1978). Since enzyme proteins [average molecular weight 120 000; Lehninger (1972)] can be detected in the press juice of leaves (Sarkar et al., 1975; Felicioli et al., 1978; Pirie, 1978; Tozzi et al., 1979), we thought it feasible to use their levels as an indication of the content of high-molecular-weight proteins. Moreover, enzyme activities may also be useful, in principle, to estimate the degree of denaturation of proteins during the storage and during the processing of juices.

EXPERIMENTAL SECTION

Protein Determination. A direct colorimetric method (Lowry et al., 1951) and a procedure based on determination of ammonia with the Nessler reactant after acid protein digestion (Strauch, 1965) were used before and after precipitation of proteins with 7% trichloroacetic acid.

Enzyme Assays. 5'- and 3'-nucleotidase (EC 3.1.3.5, EC 3.1.3.6) were assayed at 265 nm according to Ipata (1967). The assay mixture was as follows: 0.1 M Tris-Cl buffer, pH 7.4; 5'- or 3'-adenosine monophosphate (A-5'-MP, A-3'-MP), 0.045 mM; adenosine deaminase, 2 μg; varying amounts of the sample; final volume, 2 mL.

Adenosine nucleosidase (EC 3.2.2.7) was assayed at 265 nm according to Camici et al. (1979). The assay mixture was as follows: 0.1 M Tris-Cl buffer, pH 7.4: adenosine, 0.045 mM; and varying amounts of the sample; final volume, 2 mL.

The acid phosphatase (EC 3.1.3.2) was assayed at 400 nm according to Torriani (1967). The assay mixture was as follows: 0.1 M glycine–NaOH buffer, pH 3; 1 mM *p*-nitrophenyl phosphate (PNPP); varying amounts of the sample; final volume, 2 mL.

The aldolase (EC 4.1.2.13) was assayed at 340 nm according to Racker (1947). The reaction mixture was as follows: 30 mM phosphate buffer, pH 7.2; fructose 1,6-diphosphate, 2 mM; β-nicotinamide adenine dinucleotide, reduced (NADH), 0.2 mM; a mixture of α-glycerophosphate dehydrogenase and triose phosphate isomerase, 10 μg; varying amounts of the sample; final volume, 2 mL.

Press Juice Fractionation. Freshly cut alfalfa leaves were added with sodium bisulfite according to Free and Satterlee (1975) and Bickoff et al. (1975) before pressing at 100 kg/cm^2. The press juice was centrifuged at 15000*g* for 20 min. The precipitate "green LPC" was discarded and the supernatant fluid "brown juice" was fractionated according to the following procedure (Figure 1).

Table I. Protein Content (mg/mL) of Alfalfa Brown Juice

assay method			
Lowry		Strauch	
brown juice	7% TCA precip	brown juice	7% TCA precip
13.00	0.80	24.00	1.00

a. Acid Precipitation. Concentrated HCl was added under continuous stirring to a final pH of 3.5. After 6 h at 4 °C the precipitate LPC was collected by centrifugation at 15000*g* for 10 min. The supernatant fluid constitutes the fraction "brown serum".

b. Heat Precipitation. The supernatant fluid "brown juice" was heated at 80 °C under continuous stirring and immediately cooled, then the mixture was allowed to stand for 6 h at 4°C. The precipitate LPC was collected by centrifugation at 15000*g* for 10 min. The supernatant fluid is referred to as brown serum.

Sephadex G-50 Fractionation of Brown Juice and Brown Serum. Seven-milliliter samples were applied to a column (2.8 × 88 cm) and eluted with 0.1 M phosphate–1 M NaCl buffer, pH 5.4. Flow rate was 38 mL/h and the fractions were of 3 mL. Spectrophotometric measurements were carried out by a Beckman Acta III spectrophotometer. Enzymes and substrates were purchased from Boehringer und Soehne, Mannheim (West Germany). All the other chemicals were of the highest quality available.

RESULTS AND DISCUSSION

The leaf press juice contains protein and nonprotein nitrogen, free amino acids, and oligopeptides, but also polyphenol compounds and other molecules which interfere with the usual methods for the determination of proteins. Free and Satterlee (1975) reported a protein content ranging from 16 to 42 mg/mL using the Lowry method, Miller et al. (1972) reported 23 mg/mL using the Kjeldahl procedure, while de Fremery et al. (1972) reported 3 to 7 mg/mL using the same procedure, but subtracting the nonprotein nitrogen present in the 10% trichloroacetic soluble fraction.

A comparison of the values obtained by utilizing the Lowry and the Strauch methods in brown juices and in trichloroacetic precipitates is reported in Table I. It can be seen that both methods, when applied to the brown juices containing oligopeptides and interfering compounds, give high values, approaching those reported by Free and Satterlee (1975), while the values obtained for 7% trichloroacetic acid precipitates, probably containing only high-molecular-weight compounds, approach those reported by de Fremery et al. (1972). Even though some interfering molecules may still be retained in the precipitated material, we recommend to carry out protein estimation in the precipitates rather than in brown juices since the final preparations of concentrates and isolates contain high-molecular-weight proteins, with very low amounts of oligopeptides (Free and Satterlee 1975) and polyphenols (Howarth et al., 1973). Thus the direct evaluation of the initial amounts of high-molecular-weight proteins will be a useful indication of the quality of the starting brown juices and at the same time will allow a more accurate determination of the recovery of proteins for feed or food purpose. Two nucleotidases, the adenosine nucleosidase, the acid phosphatase, and the aldolase have been detected in alfalfa leaf juice (Felicioli et al., 1978; Tozzi et al., 1979). As shown in Figure 2, the decrease of aldolase activity of alfalfa juice, kept at 37 °C parallels the fall of proteins as determined in the 7% trichloroacetic acid precipitated material. The other enzyme proteins, mainly the 5'-nu-

Table II. Enzyme Activities[a] of Brown Juice Stored at 37 °C and Amount of High-Molecular-Weight Proteins Present in Peaks a of Figure 3

time of incubation, h	enzyme					proteins, mg
	5'-nucleotidase	3'-nucleotidase	adenosine nucleosidase	acid phosphatase	aldolase	
0	1.980	0.590	0.490	2.280	0.073	6.610
6	1.350	0.530	0.430	2.100	0.040	3.310
10	1.460	0.340	0.320	1.840	0.039	1.840

[a] Activities are given as micromoles of substrate transformed per min per milliliter of brown juice.

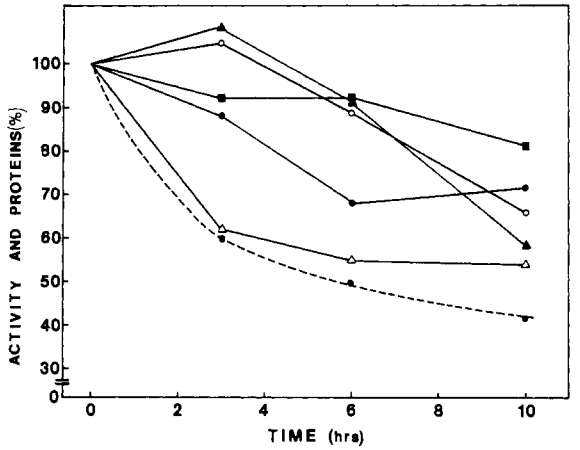

Figure 2. Decay of 7% trichloroacetic acid insoluble proteins and of enzyme activities of brown juice stored at 37 °C for the time indicated in abscissa. The 7% trichloroacetic acid insoluble proteins (--) were assayed by the method of Strauch (1965). 3'-Nucleotidase (▲–▲), 5'-nucleotidase (●–●), acid phosphatase (○–○), adenosine nucleosidase (■–■), and aldolase (△–△) activities of brown juice were tested as indicated in the Experimental Section. Activity levels and proteins at zero time were considered 100%.

cleotidase, appear to be more stable up to the third hour; thereafter, there is a progressive decay in their activity. The decrease of enzyme activities is probably due to endogeneous proteolysis, even though other denaturation processes cannot be excluded a priori. As shown in Figure 3, fractionation of the brown juice kept at 37 °C by Sephadex G-50 gel filtration leads to a progressive disappearance of the peak, emerging with eluant front (180 mL), and associated with high-molecular-weight proteins: the UV spectrum of this peak shows a maximum between 275 and 280 nm, in contrast with the spectra of the other major peaks, associated with low-molecular-weight molecules, showing different maxima, also in the near UV region (see inset of Figure 3A). It must be emphasized that the high-molecular-weight protein peak is absent in the elution pattern of the brown serum of Figure 1, obtained after acid and/or heat treatment of the brown juice (Figure 4). A comparison of this pattern with that of the brown juice reported in Figure 3A shows that the high-molecular-weight protein fraction concentrates in the LPC of Figure 1 and is therefore the major constitutent of the final concentrate preparation. The five enzyme activities reported above have been assayed in the G-50 gel filtration eluate of the brown juice incubated at 37 °C. The results reported in Figure 5 show that these activities are eluted in the vicinity of the minor peak containing the high-molecular-weight proteins. Again the decrease of the enzyme levels parallels the disappearance of the protein peak.

The amount of proteins of peaks a of Figure 3 (corresponding to zero time, 6 h, and 10 h of storage at 37 °C have been related (Table II) with the enzyme levels measured in the respective brown juices before gel filtration. It is evident that a clear correlation exists between enzyme

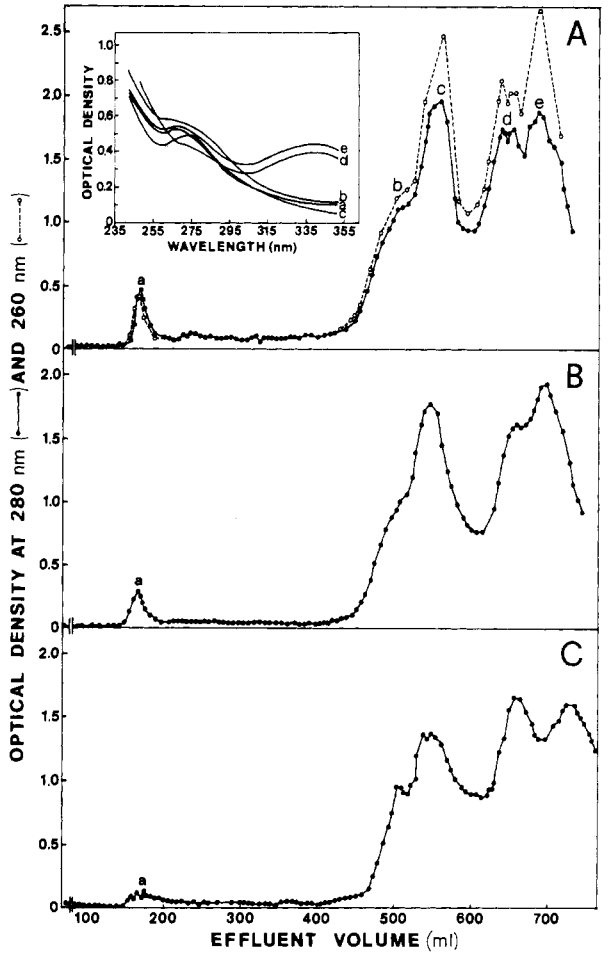

Figure 3. Sephadex G-50 elution patterns of alfalfa brown juice. The brown juice was filtered by G-50 Sephadex before (A) and after incubation at 37 °C for 6 h (B) and 10 h (C). The inset of Figure 3A reports the absorbance spectra of the indicated fractions.

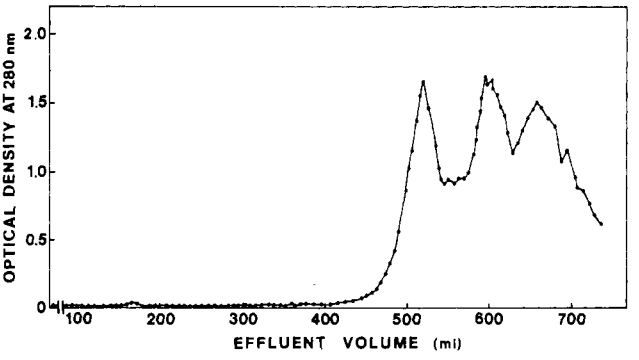

Figure 4. Sephadex G-50 elution pattern of alfalfa brown serum. The brown serum was obtained by a combination of acid and heat treatment.

activities and extractable proteins. Surprisingly, four out of five activities tested are still detectable in the LPC

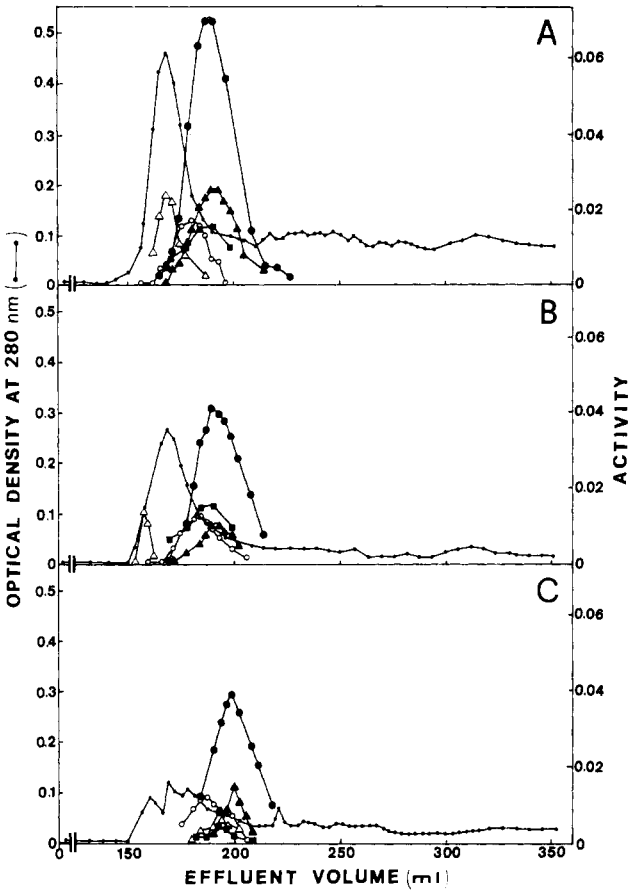

Figure 5. Sephadex G-50 elution patterns of brown juice enzymes. The brown juice was filtered by G-50 Sephadex before (A) and after incubation at 37 °C for 6 h (B) and 10 h (C). Enzyme activities are expressed as absorbance per min at the wavelengths used for the assay of each enzyme activity.

Table III. Specific Activities[a] of Different Enzymes in LPC and in the Brown Serum after pH 3.5 Precipitation

enzyme	LPC	brown serum
5'-nucleotidase	0.840	0.039
3'-nucleotidase	0.320	0.016
adenosine nucleosidase	0.136	0.123
acid phosphatase	0.367	0.008
aldolase	0.000	0.000

[a] Specific activities are given as micromoles of substrate transformed per minute per milligram of protein. No activity was detectable in LPC and in brown serum obtained by heat treatment.

obtained by acid treatment (Table III), indicating that the procedure outlined does not lead to complete denaturation of proteins.

Among the enzymes tested by us, aldolase is the more labile, while nucleotidases appear to be very resistant to storage (see Figures 2 and 5 and Table II). This different degree of protein lability may be useful to estimate the yield and the state of denaturation of proteins during pilot plant testing and deserves brief discussion. The direct determination of enzyme levels (in particular aldolase) in brown juices may be of some help in judging the amount of high-molecular-weight proteins which will be recovered in the final LPC preparations. And, in fact, the amount of high-molecular-weight proteins and of TCA extractable proteins of brown juices (Figure 3 and Table II) parallels the decay of enzyme activities (Figure 5) with a close correspondence between aldolase activity and extractable proteins (Figure 2). Therefore it is conceivable that a situation in which enzyme activities are not detectable in brown juices would indicate a very low yield of proteins for food and/or feed utilization, even though it cannot be excluded a priori that proteins are present in a denatured state. On the other hand, the estimation of the levels of 3'- and 5'-nucleotidase activities during different stages of LPC preparation can be a useful tool to judge the state of protein denaturation. And, in fact, as shown in Table III, 3'- and 5'-nucleotidases are still detectable in final LPC preparation, where aldolase activity is absent.

The spectrophotometric methods used to determine enzyme levels are very rapid and simple and require a minimum amount of protein (e.g., the amount of protein present in 5–10 μL of brown juice).

It is evident that this technique is much more convenient with respect to procedures based on gel filtration or even on TCA precipitation. However, more work is needed to estimate the error involved in using enzyme activities to determine the yield and the state of denaturation of protein isolates.

LITERATURE CITED

Bickoff, E. M., Booth, A. N., de Fremery, D., Edwards, R. H., Knuckles, B. E., Miller, R. E., Kohler, G. O., Proceedings of the 12th Technical Alfalfa Conference, Berkeley, CA, 1975.
Camici, M., Cercignani, G., Mura, U., Ipata, P. L., *Boll. Soc., It. Biol. Sper.* **55**, 1001 (1979).
Chayen, I. H., Smith, R. H., Tristran, G. R., Thirkell, D., Webb, T., *J. Sci. Food Agric.* **12**, 502 (1961).
de Fremery, D., Bickoff, E. M., Kohler, G. O., *J. Agric. Food Chem.* **20**, 1155 (1972).
Felicioli, R., Balestreri, E., Ipata, P. L., Camici, M., Galoppini, C., Fiorentini, R., Proceedings of the 2nd International Green Crop Drying Congress, Saskatoon, 1978.
Free, B. L., Satterlee, L. D., *J. Food Sci.* **40**, 85 (1975).
Howarth, R. E., McArthur, J. M., Hikichi, M., Sarkar, S. K., *Can. J. Anim. Sci.* **53**, 439 (1973).
Ipata, P. L., *Anal. Biochem.* **20**, 30 (1967).
Lazar, M. E., Spencer, R. R., Knuckles, B. E., Bickoff, E. M., *J. Agric. Food Chem.* **19**, 944 (1971).
Lehninger, A. L., "Biochemistry", Worth Publishers Inc., New York, 1972, p 57
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193**, 265 (1951).
Miller, R. E., Edwards, R. H., Lazar, M. E., Bickoff, E. M., Kohler, G. O., *J. Agric. Food Chem.* **20**, 1151 (1972).
Morrison, J. E., Pirie, N. W., *J. Sci. Food Agric.* **12**, 1 (1961).
Pirie, N. W., "Leaf Protein and Other Aspects of Fodder Fractionation", Cambridge University Press, Cambridge, 1978, p 20.
Racker, E., *J. Biol. Chem.* **167**, 843 (1947).
Sarkar, S. K., Howarth, R. E., Hikichi, M., McArthur, M. J., *J. Agric. Food Chem.* **23**, 626 (1975).
Singh, N. J., *J. Sci. Food Agric.* **13**, 325 (1962).
Strauch, L., *Z. Chlin. Chem. Chlin. Biochem.* **3**, 165 (1965).
Torriani, A., *Biochim. Biophys. Acta* **38**, 460 (1960).
Tozzi, M. G., Camici, M., Saracchi, I., Cercignani, G., Balestreri, E., *Ital. J. Biochem.* **28**, 141 (1979).

Received for review July 18, 1979. Accepted November 6, 1979. This work was supported by grants of the Italian C.N.R. (Progetti Finalizzati).