EXTRACTION, NUTRITIVE VALUE, AND ACCEPTABILITY OF ALFALFA LEAF PROTEIN FRACTIONS

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ABSTRACT

EXTRACTION, NUTRITIVE VALUE, AND ACCEPTABILITY
OF ALFALFA LEAF PROTEIN FRACTIONS

By

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One of the novel supplements which has shown potential in alleviating world protein shortages is leaf protein. Unfortunately, when extracted by classical methods it possesses an undesirable green color and grassy flavor. Consequently, this study, using alfalfa, was undertaken to investigate a simple method of extraction which might yield a light colored and relatively bland protein isolate with good biological value and organoleptic acceptability. Potentials of the by-product mother liquor were also investigated.

Basically, the extraction procedure involved blending fresh alfalfa with water, pressing the juice from the pulp, removing the chloroplasts by mild heating and centrifugation, increasing the pH to 8.5 followed by centrifugation, and acidifying to pH 4.5 followed by centrifugation. This yielded the acid precipitate and acid supernatant.

The protein isolate (acid precipitate) and liquor (acid supernatant) extracted from three cuttings of field alfalfa made during the summer were analyzed for proximate composition, amino acid composition, nutritive value (using meadow voles), and acceptability.

With young and green alfalfa 60% of the nitrogen in the leaf was extracted and of the extracted nitrogen 27% appeared in the precipitate

(or 3.3 g dry protein/lb wet alfalfa) and 30% in the supernatant (or 3.7 g/lb), the remainder being a part of the chloroplastic and alkali precipitates. The precipitate contained about 72% protein, whereas the supernatant contained only about 25% protein. Both had desirable amino acid distributions, being high in threonine, lysine, and tryptophan, but limiting in methionine. The nutritive value of the precipitates varied slightly with cutting, that of the precipitates from last two cuttings close to that of casein. The supernatant fractions (shown to have high saponin levels), particularly the last two cuttings, were toxic to the meadow vole. Necropsy indicated the primary lesion to be enteritis.

Organoleptically the precipitate--fairly bland and light colored--was acceptable, the supernatant--bitter and brown--was not.

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INTRODUCTION

Work on the practical utilization of leaf protein* as a dietary protein supplement was initiated some thirty years ago. Equipment has been designed to extract the juice, with reasonably good yield, from several species of green plants. Classically, the protein is precipitated from the juice by either heat or acid. Filtration or centrifugation yields a green cake, termed leaf protein concentrate (LPC), which on a dry basis is generally about 60-70% protein, 20-30% lipid, and has small amounts of carbohydrate and ash. It has a satisfactory distribution of amino acids with the exception of methionine; and limited biological assays have shown LPC to have a nutritive value greater than oilseeds but less than casein or egg.

Problems have arisen concerning acceptability, for LPC imparts a dark green color and grassy flavor to food preparations. Also, the high lipid content (particularly of unsaturated fatty acids) severely restricts the shelf life of the product. These objections can be largely overcome by solvent extraction of the LPC. However, this is an expensive process. Therefore, it would be desirable if the extraction process could be varied slightly so that the final product would be lighter in color, blander in flavor, and contain a lower lipid content. Other desirable features would be to increase nutritive value and protein content.

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*Presently, leaf protein is understood to be that protein extracted from the green portion of the plant, i.e., to include both stem and leaves.

Therefore, investigations concerning several modifications of the extraction procedure were made. These were followed by: analyses of the protein precipitate for proximate composition (protein, carbohydrate, lipid, and ash) and amino acid composition, biological assays using the meadow vole, and an organoleptic evaluation.

A great amount of speculation has been offered concerning the potential of the by-product mother liquor, particularly as a liquid feed supplement. However, no systematic studies of this fraction have been published. Therefore, it was subjected to the same analyses for quality (by chemical and biological evaluations) and acceptability as was the protein precipitate.

Alfalfa was chosen because of its widespread availability, ease of extraction, and high production of protein and essential amino acids per acre when compared to other common forage or seed crops. This legume is readily available in the tropics where the need for protein supplements is greatest.

LITERATURE REVIEW

The critical shortage of food, particularly that with a high protein content, has resulted in widespread malnutrition throughout the world. The consequences of failure to remedy this problem have been reiterated by numerous authors (Pirie, 1959c, 1969a; Byers, Green, and Pirie, 1965; Borgstrom, 1967; President's Science Advisory Committee [PSAC], 1967; Freeman, 1968; Brown, 1968; Munro, 1968; Altshul, 1966, 1969; Oke, 1969). Proposed solutions are generally classified as conventional or nonconventional. Conventional solutions suggest improved management of areas already under cultivation; better utilization of irrigation, drainage, and fertilization; better use of available animal breeds and crop varieties; increased disease and pest control; further technical development; and more efficient marketing, processing, and distribution of food (Pirie, 1953, 1967b; PSAC, 1967; Freeman, 1968; Buchanan, 1969a; Borlaug, 1971). However, most authors agree that the problem will not be solved without the use of some supplementary protein sources. Several sources which deserve investigation are soybean, cottonseed, peanut (groundnut), coconut, fish protein concentrate, microorganisms cultured from petroleum, synthetic nutrients, algae, and leaves (Byers *et al.*, 1965; Parpia, 1967; Borgstrom, 1967; PSAC, 1967; Wilcke, 1968; Buchanan, 1969a; Altshul, 1969; Russo, 1969a,b; Pirie, 1967b, 1969a,b,c; Anderson, 1971). One single source will apparently not solve the protein shortage problems. Therefore, as long as protein deficiency exists all potential sources must be explored and evaluated

(Oke, 1969; Pirie, 1969a,b,c; Kinsella, 1970). Green leaves are one candidate, and work on leaf protein is intended as a complement, not alternative, to work with other protein sources.

Extraction

Methods

The high fiber content of leaf requires that, for human consumption, the protein be separated from the fiber. The extraction of protein from leaves was first reported by Rouelle in 1773, who precipitated the protein of hemlock leaves. The first significant work was reported by Osborne, Wakeman, and Leavenworth (1921) and by Chibnall and Schuyver (1921). The first group extracted 44% of the leaf nitrogen from macerated alfalfa with a succession of water, alcohol, dilute alkali, and hot alkaline alcohol treatments. The second group treated disintegrated leaf tissue with water saturated with ether, a cytolyzing agent, thereby obtaining a yield of slightly less than two thirds of the total leaf nitrogen. By this procedure reasonably pure proteins were extracted from spinach (Chibnall, 1924) and alfalfa (Chibnall and Nolan, 1924). Chibnall and his associates (Chibnall, 1922a,b,c; Chibnall and Grover, 1926; Chibnall *et al.*, 1933) continued working toward improved laboratory extraction procedures. In these early years, the work was largely biochemical with little emphasis on the practical use of leaf protein.

Ereky (1926) patented the first method for the large scale processing of green leaves. He pulped the leaves with a large amount of water on a machine which was essentially a set of moving knives. Goodall (1936) used a modification of the grooved-roll sugar cane mill for extracting the leaf juice in one operation. Both procedures had been designed to extract nutrients other than protein.

Lugg (1939) macerated foliage in the presence of a mildly alkaline buffer. This acted as a protein dispersing agent, and thus extracted 92-95% of the total leaf nitrogen. Later, Lugg and Weller (1944) described the extraction of protein from pasture leaves with alkaline buffer containing lipoid solvents (ethanol and ether). These general procedures were modified by Crook (1946), who maintained mild alkalinity and a low salt concentration during grinding. Crook and Holden (1948) then reported the results of this process on 28 species of plants. On the average 75% of the total leaf nitrogen could be extracted. Smith and Agiza (1951) extracted with 90% formic acid and 95% ethanol and then, after adjusting the pH to 10.5, precipitated the protein by acid or heat. Jennings *et al.* (1968) found that leaf protein was readily extractable into a phenol-acetic acid-water mixture.

Slade (1937) was the first to consider the food potential of leaf protein which he designated as "green cheese." The following decade, World War II initiated fears of food shortages. This spurred interest in the possible use of leaf protein as a food supplement. Therefore, systematic work aimed at designing equipment for bulk leaf protein extraction was begun (Pirie, 1942a). A key proponent of the use of leaf protein then, as he is today, was Mr. N. W. Pirie of Rothamsted Experiment Station, Herts, United Kingdom. Classical studies of Pirie and his associates have led to the extraction of human consumable leaf protein on a pilot plant scale. Since World War II Pirie and his associates have continued this biochemical engineering research.

Pirie (1953, 1967a) and Byers, Fairclough, and Pirie (1956a) described the separation of the protein from the fibrous and strongly flavored components of leaf as follows: (1) maceration and disintegration of the leaf, (2) expression of the juice, and (3) precipitation of

the protein. The problems and requirements of large scale leaf processing have been discussed by Pirie (1952). Early work indicated that large scale addition of water was troublesome and complicated the coagulation step. Therefore, work was initiated to design machinery to pulp freshly harvested crops without the addition of large amounts of water (Pirie, 1966a). The conventional screw expeller and meat mincer proved unsatisfactory because they compressed the charge--the fiber acting as an ultrafilter to trap the protein--and generated excess heat (Pirie, 1952, 1966a, 1967a; Davys, Pirie, and Street, 1969). Sugar-cane rolls, rod-mills, edge and end-runner mills, and doughbreakers were all undesirable in this application (Pirie, 1966a). It was concluded that single stage machines are inefficient because the operations of maceration and juice expression are distinct. Thus the system developed was a pulper and a press designed specifically to handle leafy material. The pulper, developed as a modification of the hammer mill, is essentially a drum with fixed beaters on a rotating axial shaft. One to two tons of wet crop can be handled per hour. The classical description of this extractor was given by Davys and Pirie (1960). The design and operation has also been discussed in detail elsewhere (Byers *et al.*, 1956a; Pirie, 1952, 1956b, 1957a, 1961a). Hammer mills, in which the material undergoes a succession of impacts, have also been used by Smith (1940), Bickoff, Bevenue, and Williams (1947), Kohler and Graham (1951), and Raymond and Tilley (1956).

The separation of the juice from the pulp, like the pulping operation, possesses problems unique to leaves and, therefore, requires a press with definite characteristics. These have been discussed by Pirie (1959b, 1966a). All practical methods depend on the application of pressure to a constrained mass of pulp. There are several methods by which pressure

can be applied (Pirie, 1959b). Details of the earlier cam operated presses have been published by Fairclough and Pirie (1954), Byers *et al.* (1956a), Pirie (1956b, 1957a, 1959b, 1961a), and Davys and Pirie (1960). Davys and Pirie (1965) discussed their large scale continuous press, a tensioned conveyor belt on which the fibrous pulp is fed and then pressed between the belt and a perforated pulley with no differential movement between the two. It has a capacity of 3 T fresh pulp per hr. A hydraulic press was also used by Bickoff *et al.* (1947); whereas Smith (1940), Kohler and Grahm (1951) and Raymond and Tilley (1956) used a screw press for this same operation.

For the non-industrialized community a simpler and smaller "Village Unit", which pulps and presses fresh leaves in one operation, has been constructed by Davys and Pirie (1963). This unit has a capacity of up to 500 lb of leaf per operation (1-2 hr). However, it is less efficient than commercial models due to insufficient disintegration of the leaf, and extracts only 40-50% of the available protein.

Davys and Pirie (1969) recently described a laboratory scale pulper suitable for processing 2-3 kg samples of leaf. Both efficiency and yields are comparable to those of the large scale equipment. Davys *et al.* (1969) described a complementary laboratory scale press, consisting of two vertical grooved platens, which handles 1 kg quantities of leaf pulp. This unit gives results similar to those obtained with the large press.

Since its origin, the extraction equipment has undergone continued modification and improvement in both design and operational detail. This evolution is outlined in the Annual Reports of the Rothamsted Experiment Station (Fairclough and Pirie, 1954; Byers, Fairclough, and Pirie, 1955; Pirie, 1959d, 1960, 1961c, 1962, 1963, 1964b, 1965, 1966e, 1967c, 1969e).

Starting with high quality crops, about one half of the protein appears in the first extract. And, if the fiber residue is repulped with the addition of water, about one half of the remaining protein can be extracted (Davys and Pirie, 1960; Morrison and Pirie, 1961). Currently, up to 90% of the nitrogen can be separated from as much as two tons of crop per hour (Arkcoll and Festenstein, 1971).

Pirie has also designed a steel press which grinds small quantities of leaves by forcing them under extremely high pressures through a narrow slot of adjustable width. Oxygen can be removed from the system and the extraction carried out in the presence of an inert gas (Pirie, 1956a, 1959d, 1961b; Festenstein, 1961). This press has been improved slightly by McArthur and Miltimore (1964).

Of the many possible methods for separating the protein from the juice, Morrison and Pirie (1961) found that sudden heating to 80 C by steam injection was the most convenient for large scale preparation and gave the most filterable curd. According to Pirie (1969d), this heat treatment does not damage the nutritional quality of the protein but inactivates the endogenous enzymes which cause undesirable changes in the stored product. In addition, partial pasteurization is achieved. However, heating does denature the protein and it becomes insoluble. The production of a curd by acidification also has this insolubility effect (Pirie, 1961a). Therefore, the functionality of leaf protein as an ingredient is somewhat limited in that partial hydrolysis is probably the only method for getting leaf protein into solution (Pirie, 1957b).

Leaf curd can be formed by the above method in an automatic coagulation unit described by Byers *et al.* (1955) and Pirie (1957a). However, this unit is not satisfactory for use with the batch extractor. Therefore, Younus Ahmed and Singh (1969) designed one for use on a smaller scale.

On a laboratory scale coagulation can be realized by acidification to pH 3 or 4. The protein coagulum is filtered via a pump-fed filter prss. The resulting cake is crumbled, suspended, and washed in 10 to 20 times its volume of water by vigorous mechanical stirring, which removes undesirable, strongly flavored substances. Lowering the pH to 3.5 or 4.0 insures removal of alkaloids and improves the keeping quality of the final product. The suspension can then be refiltered and pressed (Morrison and Pirie, 1961; Byers *et al.*, 1956a; Pirie, 1959c, 1969d, 1970).

McDonald (1954) has shown that yield and crude protein content of the curd are dependent on the efficiency with which the mother liquor is removed from the wet protein coagulum. According to Singh (1969) the curd will contain variable protein and nonprotein constituents depending on the coagulation conditions and the nature of various constituents in the extract.

The final product is a dark green cake (termed leaf protein concentrate--LPC) containing 50-60% moisture and is only slightly soluble in neutral or alkaline water. On a dry basis it contains 9-11% nitrogen (about 50-68% protein), 5-10% starch, 5-30% lipid and 1-10% ash (Morrison and Pirie, 1961; Pirie, 1966a,b, 1969d, 1970). The consistency and texture are similar to cheese or pressed yeast (Byers *et al.*, 1956a; Pirie, 1956b, 1959c, 1966a, 1967a, 1969d; Morrison and Pirie, 1960, 1961; Buchanan, 1969a).

The fibrous residue, containing about 30-50% of the total leaf protein nitrogen, is usually about 1-3% nitrogen on a dry basis (Raymond and Tilley, 1956; Byers *et al.*, 1965; Pirie, 1953, 1966c, 1967c). The composition of the liquor depends on the species and maturity of the plant. The following ranges have been reported: dry matter, 20-47 g/l;

nitrogen, 0.4-1.5 g/l; and carbohydrate, 4-40 g/l (Pirie, 1961a, 1967c).

In summary, by the methods of Pirie and his associates, fresh green leaves are pulped and separated into juice (A) and fibrous residue (B) as shown in Figure 1 (Pirie, 1942a, 1952, 1953, 1956b, 1957a,b, 1961a, 1966a,c,e, 1968, 1969d; Byers *et al.*, 1965). Very little more than

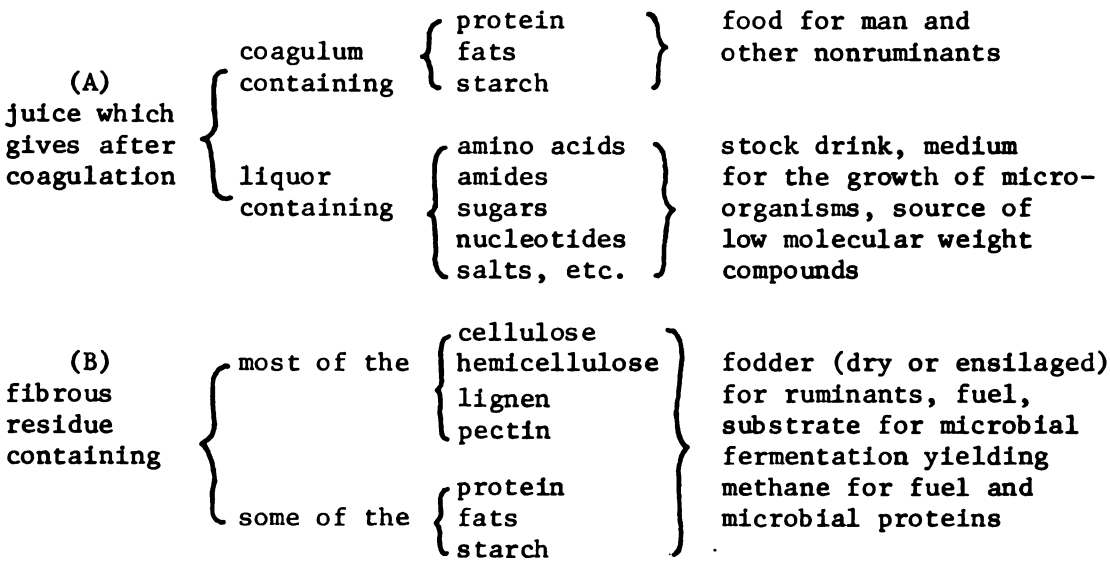

Figure 1. Product utilization distribution scheme.

speculation has been offered on the potentialities of the by-products of leaf protein extraction. Ferguson and Armitage (1944) reported experiments on the growth of bakers yeast on the mother liquor from bracken juice. Bickoff *et al.* (1968) reported that an aqueous extract of fresh alfalfa was shown to stimulate the growth of microorganisms. Preliminary results of feeding trials with cattle suggested that the liquor may be a valuable additive to liquid supplements (Perry, Peterson, and Beeson, 1969). Studies by Oelshlegel, Schroeder, and Stahmann (1969b) showed that the fibrous residue produced a good silage which was readily eaten

by cows. The residue from lucerne* is unsuitable for most papermaking processes (Raymond and Tilley, 1956).

Other British innovators, Slade, Branscombe, and McGowan (1945), employed the screw press to both shear the cells and to exert sufficient pressure to express the juice. Protein was coagulated by heat under slightly alkaline conditions. This produced a green curd devoid of fiber and containing about 50% protein. In a large scale operation the input was half a ton of grass per hour. This yielded about 33% of the total protein or about 25 lb crude protein per ton of fresh crop (Raymond and Tilley, 1956). Many workers followed the lead of Slade *et al.* in extracting the juice from fresh leaves with screw expellers of varying designs and modifications (Nebraska Chemurgy Project, 1947; Ayala and Johnson, 1951; Tallarico, 1952; Miller, 1952; Isajev, 1956). Powlings (Anon., 1953) patented his screw expeller--"Protessor"--for producing a protein concentrate from green vegetation that was suitable for feeding farmstock. It yielded 60-80 lb crude protein per ton of fresh crop (Raymond and Tilley, 1956). In the Netherlands, Deijs and Sprenger (1952) concluded that the Powling screw press was the most promising type expeller for large scale applications; and it has been used by Akinrele (1963) in Nigeria. Tilley *et al.* (1954) at the Grassland Research Institute in England used a modified Christy and Norris "coir sifter" and a screw expeller, sequentially, to separate the leaf juice from the fiber. Steam heat yielded a dark green curd and a clear brown liquor which were separated by a filter press. The curd (50% crude protein) was dried on a twin roll film dryer. Lucerne yielded 31% and

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*Both lucerne and alfalfa are common names for *Medicago sativa*. Alfalfa is the North American reference whereas lucerne is used in other regions of the world. This text will use that name found in the paper of the particular investigator being discussed.

23% of the crop crude protein as protein concentrate for the first and second cuttings, respectively.

Chayen, another British worker, took a different approach. He used the impulse rendering (IR) process (see Figure 2) originally designed for the extraction of fat from cellular material (Chayen and Ashworth, 1953; Anon., 1959; Chayen *et al.*, 1961; Smith, 1966). Protein is

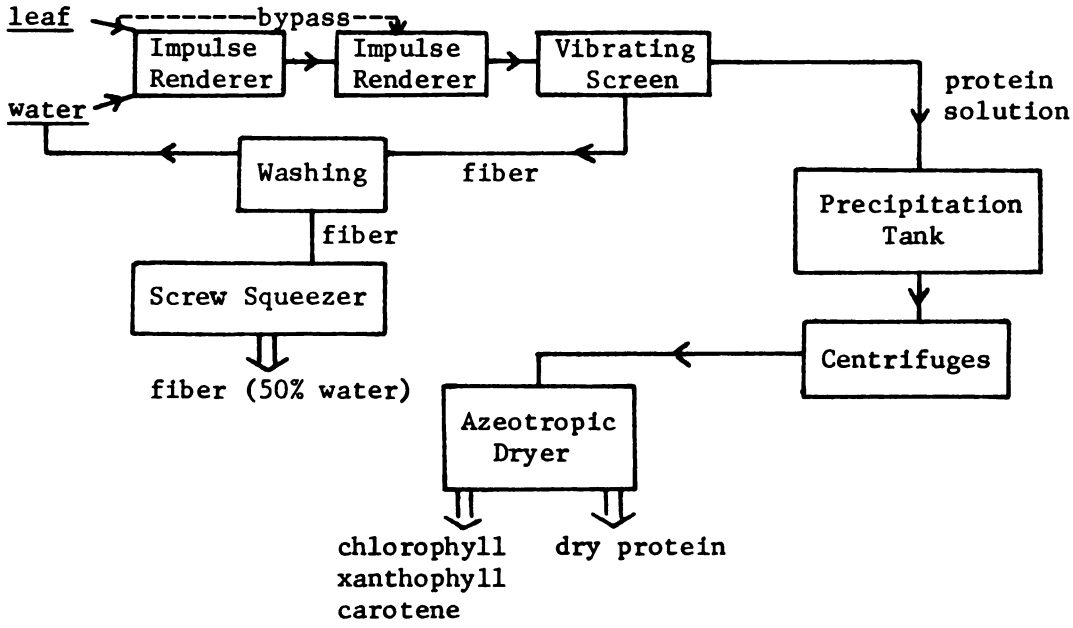

Figure 2. Diagram of the impulse rendering process.

released in the IR process by a mechanical rupturing of leaf cells by rapidly delivered impulses generated by the rotating beaters of a modified hammer mill and transmitted through a liquid medium (water or dilute solution of Na_2CO_3 which may contain an antioxidant). The effluent is freed of fiber by passage over a vibrating screen. The protein is precipitated by acidification, heated to 60-70 C, and centrifuged. The product is a green cake of about 80% moisture which may be subjected to azeotropic distillation with toluene or spray dried to yield an intensely green powder with a characteristic, grassy odor. This continuous process

with a throughput of one ton of leaves per hour can separate 75% to 85% of the nitrogen from good quality leaves (Anon., 1959; Chayen *et al.*, 1961). The composition of the isolate can be varied by modifying the IR processing conditions (Smith, 1966).

In 1943 Guha and his associates (Pal and Guha, 1953; Chakrovorthy and Guha, 1960) made the first Indian attempt to obtain protein from leaves in a form suitable for human consumption. They elucidated conditions for maximum extraction of protein and special features of protein technology. More recently on a small scale, Guha (1960) extracted 60% of the protein from various indigenous leaves by maceration with 2% Na_2CO_3 and precipitation by acidification and heat.

A concerted effort to extract leaf protein is in progress at the Central Food Technological Research Institute (CFTRI), Mysore. Singh (1964), attempting to identify suitable plants for bulk extraction, used a meat mincer, hand squeezing, trichloroacetic acid precipitation and centrifugation to extract protein from several species of local plants. Using a batch extractor and heat precipitation, he followed this first study with an investigation of lucerne to standardize the conditions of extraction and processing (Singh, 1967). The batch extractability of the 24 plant species studied at CFTRI varied from 25-60% (Singh, 1969).

Valli Devi, Rao, and Vijayarahaven (1965) used the simple method of Pirie (1957b) to extract protein from the leaves of eighteen species of plants. Also, on a laboratory scale, Sentheshanmuganathan and Durand (1969) extracted the protein from the leaves of the Ceylon rain forest by the following procedure: homogenization of the chopped leaves in a blender, filtration through cloth, increasing the pH, acidification, heat, filtration and acetone extraction.

In this country, most of the work on extraction processes has been done at the USDA Western Utilization Research Laboratory at Albany. The primary objective has been to make products useful as animal feeds. Products useable for human consumption have been only a secondary objective. However, Spencer *et al.* (1969) admitted that this latter objective could be the more important contribution of their wet processing technique. In this technique, freshly chopped alfalfa is treated with ammonia to raise the pH and the juice is expressed with sugar cane juicing rolls capable of exerting high pressures. This process, now named the PRO-XAN process, yields a pressed cake and a green juice devoid of fiber. Each of these fractions is further processed into the marketable commodities illustrated in Figure 3 (Kohler *et al.*, 1968; Anon., 1969; Spencer *et al.*, 1969; Browning, 1970; Knuckles *et al.*, 1970). The alfalfa whey (b) contains amino acids, sugars, minerals, and vitamins and thus has potential as an animal feed supplement. Fractions (c) and (e) could serve as poultry ration additives and (d) could be used for poultry, swine, or possibly as human food. Fraction (a), containing nearly 50% of the total protein, can meet standards for a good quality dehydrated alfalfa meal and would be suitable for ruminants. An evaluation of the use of pilot plant sugar cane rolls in alfalfa processing has been reported by Knuckles *et al.* (1970). Up to 33% of the total solids and almost 50% of the protein from fresh alfalfa can be extracted into the green juice. Recently, a pilot plant coagulation system capable of handling over 90 gal of juice per hr was evaluated (Spencer *et al.*, 1971).

Hartman, Akeson, Stahmann (1967), in an attempt to prevent the loss of water soluble nutrients and to solve the problems associated with drying the moist leaf protein cake, spray dried whole juice (alfalfa and

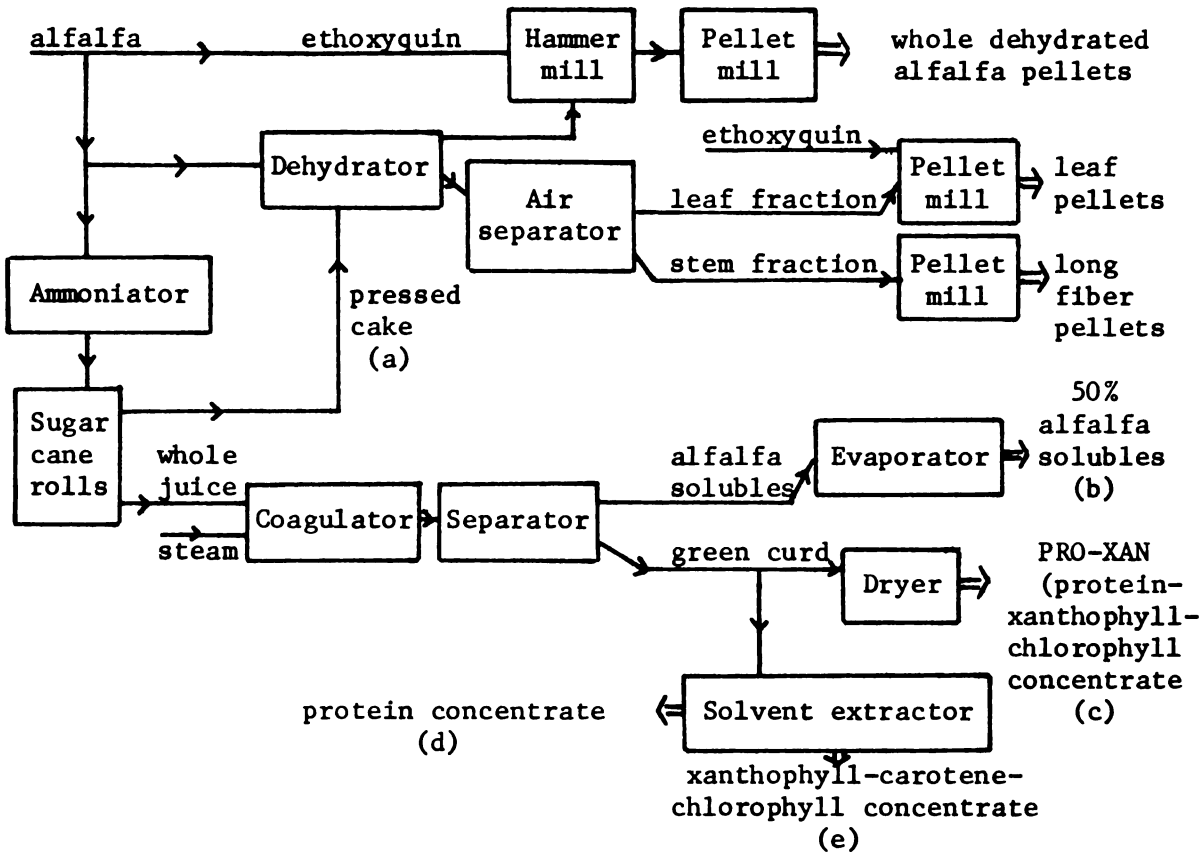

Figure 3. Flow sheet for wet fractionation of alfalfa.

pea vine) immediately after extraction. This yielded a green powder containing 18.5% and 35% protein for the pea vine and alfalfa, respectively. Oelshlegel *et al.* (1969a) noted that the spray dried juice will contain, in addition to soluble nutrients, toxic substances that are soluble in water or are bound to the protein.

In Portugal a protein isolate is obtained from grasses by a process based on reverse osmosis (Browning, 1970). Consequently heat is not required and the resultant protein denaturation is avoided (see Figure 4).

In Hungary, Tangl (1949) reported a method for extracting a protein concentrate from lucerne for pig and poultry rations; and in Russia, Parini (1953) developed machinery for extracting a protein paste from a wide range of green materials.

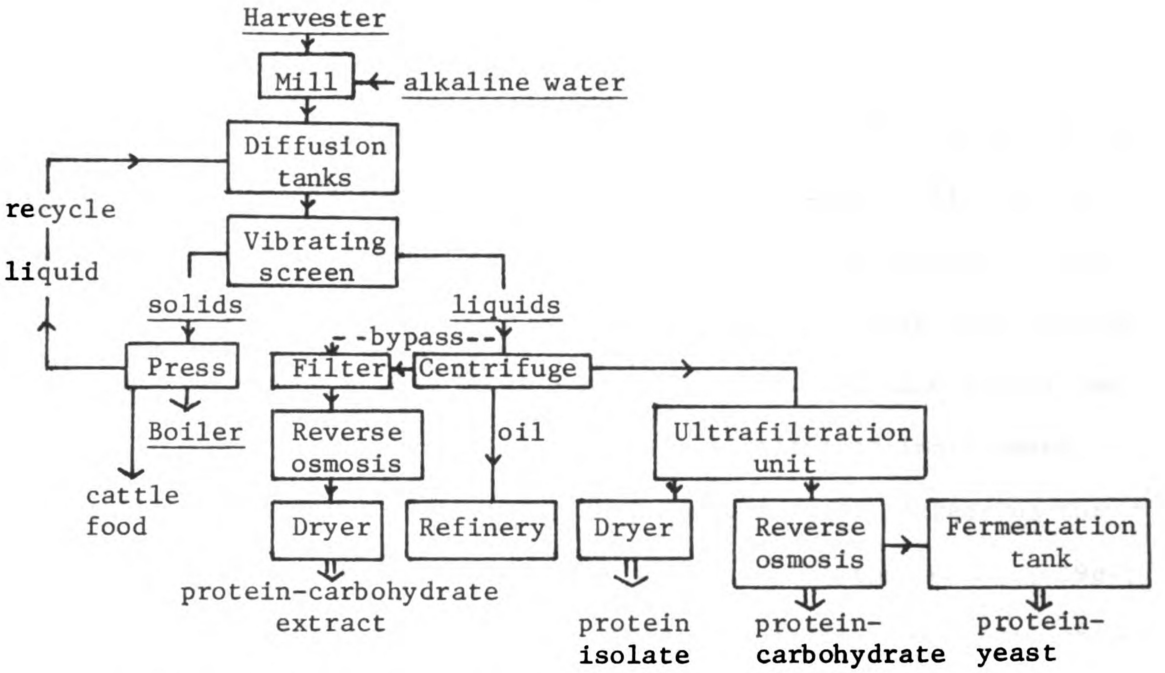

Figure 4. Flow sheet for extraction of protein from leaves by reverse osmosis.

Removal of the lipid solubles from the protein concentrate by solvent extraction results in a product lighter and more bland in flavor than the original cake. Slade *et al.* (1945) initiated decolorization by treatment with amyl alcohol. Pirie and associates found acetone convenient and efficient for the simultaneous removal of lipids and water (Byers *et al.*, 1956b; Pirie, 1957b, 1958, 1959a; Morrison and Pirie, 1961). The product keeps "indefinitely" in air. Acetone extraction was also used by Valli Devi *et al.* (1965) and Singh (1969). Chayen *et al.* (1961) extracted with acetone followed by ether and with a mixture of hexane and ethanol. Hartman *et al.* (1967) employed a 95% ethanol extraction for spray dried juice. Kohler *et al.* (1968) compared the efficiency of acetone, methanol, normal propanol, and isopropanol and concluded that the latter two were able to remove most of the pigments with two extractions. Poppe, Tobback, and Maes (1970) used successively water, ethanol, acetone and ether to yield a final product from lucerne which was a

colorless and tasteless powder. Simultaneously the protein content of the samples increased from 68% to 82-84%.

The merits of solvent extraction have been debated. Pirie (1969d) feels that solvent extraction should be avoided because: (1) lipids, particularly the unsaturated fatty acids, are valuable dietary components and (2) the additional cost and technology would make leaf protein production prohibitive on a local basis. However, Buchanan (1968) has shown that solvent extraction may produce a significant improvement in the nutritive value of leaf protein preparations by increasing the digestibility of the concentrate. The Americans (Hartman *et al.*, 1967; Kohler *et al.*, 1968; Spencer *et al.*, 1969) feel that solvent extraction is necessary to produce a product acceptable as human food.

Freeze-drying gives a stable and light colored powdery product which will keep well in air. The texture of the product is related to the method of freezing. A soft fluffy product will result only if the freezing process takes less than 2-3 min. Details of this process have been given by Morrison and Pirie (1963) and Pirie (1964a). The presence of various carriers gave a desirable product (Pirie, 1959a). However, this process is considered too expensive to be economically feasible in developing nations (Morrison and Pirie, 1960; Kohler *et al.*, 1968).

In summary, the basic extraction process is: (1) maceration of the leaves, (2) press extraction of soluble proteins, (3) precipitation of the protein by heat or acid, and (4) elimination of water (Kinsella, 1970). The large scale machinery which has been developed for the extraction of the juice can be divided into four classes according to the method used for maceration: (1) moving knives, (2) hammer mills, (3) rollers, and (4) screw expellers. The first two require a press for separation of the juice. All have been designed for continuous operation

(Tilley and Raymond, 1957). Under the International Biological Program (IBP), machinery for protein extraction has been sent to India, New Guinea, Nigeria, Uganda, United States and Canada (Smith, 1969). As a result of considerable engineering development, several processes are available for extracting the protein from a variety of leaves with reasonably good yield.

Factors Affecting Extraction

Extractability (i.e., the per cent of protein extractable into the juice or concentrate relative to the total protein content of the leaf) is affected by a variety of factors other than species. Apparently, it depends largely on the physical state of the leaf. Young, tender, lush leaves extract much more easily, giving higher protein yields, than mature, dry leaves of the same species (Crook and Holden, 1948; Byers *et al.*, 1956b; Byers, 1961; Festenstein, 1961; Morrison and Pirie, 1961; Byers and Sturrock, 1965; Pirie, 1942a, 1962, 1969b; Singh, 1964, 1967; Spencer *et al.*, 1969; Oelshlegel *et al.*, 1969a; Knuckles *et al.*, 1970). This can often be correlated with the greater initial water and nitrogen content of the former. Yield of extracted protein increases to a maximum when the dry matter is about 15% and diminishes as the dry matter increases and the plant matures (Pirie, 1964b). Byers and Sturrock (1965) stated that the protein nitrogen content decreased as the age of the plant increased. The suggestion was made that increased mechanical entrainment of the chloroplasts is associated with the decreased extractability (Pirie, 1960). Nazir and Shah (1966) agreed that more protein was extracted from young than from mature leaves although they found that neither total nitrogen nor dry matter were correlated with extractability. Byers and Sturrock (1965) also noted

that high extractability does not necessarily correlate with high nitrogen in the leaf. Young cereal leaves, even though they have a low nitrogen content, are easily extractable (Byers *et al.*, 1956b). Singh (1969) has reported that extractability varies with the bio-pH of the pulped mass, maturity of the chloroplasts, and nature of other nonprotein constituents present as well as the previously mentioned factors.

Unpublished results of Pirie indicated that the trichloroacetic acid (TCA)-induced precipitate of some wheat leaf extracts contained 10-20% more nitrogen than similar heat-induced precipitates (Singh, 1960). Singh (1960) compared protein precipitates prepared by four methods: heat, TCA, perchloric acid, and TCA with uranyl ions. The precipitate produced by TCA contained 10-15% more nitrogen than the precipitate produced by heat. He suggested that heat coagulation promotes either nuclease or protease activity. However, he has more recently stated that heat-induced coagulation yields a LPC with higher contents of dry matter and nitrogen than acid-induced precipitation (Singh, 1967, 1969). Sur (1967) could precipitate 96% of the juice protein by lowering the pH of the juice to 3.5 before heating.

Many researchers have noted that maceration in an alkaline solution or an increase in pH to about 8 prior to precipitation of the protein resulted in higher yields. Generally, they suggested that this was due to increased solubility of the protein (Lugg, 1939; Lugg and Weller, 1944; Crook, 1946; Smith and Agiza, 1951; Byers *et al.*, 1956b; Chayen *et al.*, 1961; Morrison and Pirie, 1961; Akinrele, 1963; Singh, 1964, 1969; Nazir and Shah, 1966; Pirie, 1966e, 1969b; Sentheshanmuganathan and Durand, 1969). Festenstein (1961) and Pirie (1959d) reported that the increased extraction under alkaline conditions was largely due to greater dispersion of the chloroplasts. It was noted by Spencer *et al.*

(1969), Browning (1970) and Knuckles *et al.* (1970) that the increase in pH in the PRO-XAN process also decreased the losses of xanthophyll, chlorophyll, and protein which result from enzymatic activity during rolling, and changed the consistency of the curd to one easier to process. However, Poppe *et al.* (1970) reported that although an increase in alkalinity increased yields, it decreased the purity (protein content) of the preparation.

Substances such as soaps and surface active alcohols influence the complexing of proteins with other leaf constituents (Pirie, 1942a; Chayen *et al.*, 1961), particularly chlorophyll and lipids (Festenstein, 1961). Smith (1941a,b) and Smith and Pickels (1941) studied the effects of various detergents in clarifying leaf extracts. Ke and Clendenning (1956) found that detergents may either free the pigment or leave it attached to lipoprotein prosthetic groups. Festenstein (1961; Pirie, 1959d, 1960), by using detergents to release nitrogen from the residual fiber, extracted 90-95% of the nitrogenous material in the leaf.

Various workers reported proteolytic activity in leaf juice (c.f., Byers, unpublished; Pirie, 1942a). However, few substantiated their statements with convincing experimental evidence. Tracey (1948) reported that both sap and fiber of green leaves showed protease activity. He noted that the difficulty in working on the protease of green leaves is due to its low concentration. Singh (1962) observed the autolysis of protein in leaf extracts and the proteolytic activity of these extracts against casein when incubated at 37 C for 2 hr. Autolysis was directly related to the temperature and the time between extraction and precipitation. Nazir and Shah (1966) investigated the autolytic breakdown of leaf protein at 25 C and concluded that juice can be kept for 1-2 hr without an appreciable loss in protein content. Davys *et al.* (1969)

concluded that the most critical period during processing is between pulping and pressing because protein is lost both through proteolytic activity and protein coagulation on the fiber (Pirie, 1967c).

A characteristic property of plant leaf extracts is the almost immediate development of a brown color, due presumably to the action of polyphenoloxidase. Cohen *et al.* (1956) have shown that browning can be prevented when all operations during homogenization and fractionation are conducted in a nitrogen atmosphere. Under such conditions an increase in solubility of the protein and a reduction in the brown color of the extract was noted.

Various workers have fractionated the leaf extract into (1) a chloroplastic protein and (2) a cytoplasmic or soluble protein. Festenstein (1961) obtained the two fractions, respectively, by centrifugation at 1500 g for 15 min and then at 12,000 g for 15 min. Pirie (1969e) also employed centrifugation to achieve a similar classification. Singh (1967), by differential heat treatment--heating at 50-55 C to obtain the chloroplastic proteins and further heating of the supernatant to about 80 C to precipitate the cytoplasmic proteins--found 40% of the total heat precipitable protein to be in the chloroplastic fraction (7% nitrogen) and 40-45% in the cytoplasmic fraction (11-12% nitrogen). He believed that this process may produce a high protein fraction of light color with a flavor suitable for human consumption.

According to Byers (1967b) and Pirie (1963, 1964b) the per cent chloroplastic to per cent cytoplasmic protein ratio varies with species, not maturity, although Henry and Ford (1965) claimed that there is a higher proportion of cytoplasmic protein present in extracts from older leaves.

Yields

In determining the desirability of a species, yield as well as ease of extractability must be considered. Stahmann, of the University of Wisconsin, has published valuable quantitative data concerning leaf protein yields (Akeson and Stahmann, 1966; Stahmann, 1968a,b). He and his associates have calculated the yield of essential amino acids and total protein from various U.S. forage and seed crops which could be processed into LPC. The forage crops, particularly the legumes, generally produced several times more protein and essential amino acids per acre than seed crops. Alfalfa gave the highest yields of essential amino acids (300 lb/acre) and total protein (2400 lb/acre) of all 25 crops considered. Therefore, Stahmann suggested that it would be the best crop for the highest production of LPC per acre (Akeson and Stahmann, 1966; Stahmann, 1968a,b).

The first reported yields (extracted protein/acre) from different crop leaves ranged from 26.2 lb for tares to 730 lb for sugar beet (Byers *et al.*, 1957; Pirie, 1958). Byers and Sturrock (1965) reported yields of leaf protein obtained from the large scale extraction of several temperate crops and suggested that the use of a suitable succession of crops should make it possible to obtain yields of 1000 kg protein/hectare/yr. Protein yield appeared to depend on species, variety, season, physiological age of the plant, ability to regrow after cutting, and time of harvest. Since then, Pirie (1967a,c, 1969b,d) has reported yields to be between 1200 and 1400 kg extracted protein/hectare/yr. Byers and Sturrock (1965) and Oke (1967) both found cereals to give the highest yield of nitrogen. Regrowth patterns and the effects of different forms of husbandry on the yield of extractable protein have been studied at Rothamsted (Pirie, 1966e). It was postulated

that the continual growth in the wet tropics would probably produce yields more than twice those achieved in Britain (Pirie, 1966c, 1967a, 1968; Oke, 1969).

Using estimates based on batch extraction yields of lucerne, Singh (1967) projected industrial yields to be 3-3.2 T extracted protein/hectare/yr, which is quite exceptional for Indian agriculture (Pirie, 1969a,b,d). Actual yields of lucerne and of by-product vegetation grown on non-agricultural land have been 1500 and 66-160 kg protein/hectare/yr, respectively (Singh, 1969).

Finally at Rothamsted, a yield of 2 T extracted protein/hectare/yr was realized with cereal crops which were followed by mustard or fodder radish (Pirie, 1969d,e; Arkcoll and Festenstein, 1971). This was a considerable improvement over Slade's studies in which he reported 200 lb protein/acre of grass (Slade *et al.*, 1945). In general, based on extractability and yield the legumes appear to be the best source of leaf protein (Buchanan, 1968).

Although the per cent extractability tends to diminish with crop maturity, the total amount of protein per acre increases. This results in an optimal harvest time which depends upon species and cultural conditions (Pirie, 1961c, 1962, 1969b). Chayen *et al.* (1961) noted that the yield of protein falls when grass reaches the flowering stage. Various studies on alfalfa have been conducted to determine the optimal time of harvest in relation to nutrient content and total yields (Van Riper and Smith, 1959; Smith *et al.*, 1966; Hanson *et al.*, 1965; Ogden and Kehr, 1968; Smith, 1970). Fiber content increased with maturity while protein content and digestibility tended to decrease. However, the yield of total dry matter increased with maturity. All workers agreed that harvesting at 1/10 bloom is the best compromise to give

optimal yield of high quality alfalfa protein from the vegetative plant.

Quality and Nutritive Value

Composition

Gross. The curd from the extraction method of Slade *et al.* (1945) was reported to have the following composition: protein, 17.5%; chlorophyll, 19.3%; carotene, 0.2%; xanthophyll, 0.3%; glycerides, 5.7%; wax, 3.4%; other lipid extractives, 18.0%; and ash, 5.6%.

The composition of fractions derived from the impulse rendering of alfalfa have been reported by Smith (1966). Some of his data are given in Table 1.

Table 1. Compositions of alfalfa fractions obtained by the impulse rendering process

Fraction	Total solids (%)	Crude protein (%)	Carbohydrate (%)	Lipid (%)
Alfalfa	20.0	22.0[1]	---	---
Lipid-protein isolate	---	61.0[1]	12.5[1]	17.0[1,3]
Protein effluent	---	0.01[2]	---	---

[1]Dry basis

[2]Wet basis

[3]Acetone-solubles

Hartman *et al.* (1967) compared the proximate compositions of fresh green plant, spray dried juice, ethanol extracted spray dried juice and the fibrous residues to other high protein foodstuffs. Some of their data are given in Table 2. Juice from the second cutting appeared to have a lower protein content.

Table 2. Compositions of alfalfa and alfalfa juice before and after extraction with ethanol

Fraction	Moisture	Protein	Fat	Ash
Alfalfa (first cut)	5.5	21.7	---	---
(second cut)	5.1	19.5	---	---
Spray dried juice (first cut)	4.9	34.9	6.6	12.7
(second cut)	4.2	31.3	5.8	12.1
Ethanol extracted spray dried juice (first cut)	2.6	42.8	0.6	14.4
(second cut)	2.3	37.0	0.6	14.1

The compositions of the products from the USDA wet fractionation process are shown in Table 3.

Table 3. Proximate composition of products from wet fractionation

Fraction	Moisture (%)	Protein[1] (%)	Fat[1] (%)	Ash[1] (%)
Alfalfa	79[2]	23.4(22.5)[3]	---	---
Juice	91	36.3(32.3)	---	---
Protein-xanthophyll-chlorophyll concentrate	80	55.7(50.5)[4]	6.7(8.1)	10.2(12.0)
Alfalfa solubles	94	28.9(18.3)	0.5(0.6)	19.7(21.1)

[1]Dry basis; [2]Figures not in parentheses reported by Spencer *et al.* (1969); [3]Figures in parentheses for alfalfa and whole juice reported later by Knuckles *et al.* (1970); [4]Figures in parentheses for PRO-XAN and solubles reported later by Spencer *et al.* (1971).

Subba Rau, Mahadeviah, and Singh (1969) reported the composition of fractions extracted from lucerne. Some of their data are given in Table 4. The cytoplasmic fraction was low in mineral matter and had the highest protein content. Most of the mineral matter, the ether extractives and carbohydrate, and somewhat less of the total coaguable leaf protein was in the chloroplastic fraction.

Table 4. Compositions of some fractions obtained from the extraction of lucerne

Fraction	Mineral matter[1] (%)	Ether extractives[1] (%)	Crude protein[1] (%)	Carbohydrate by difference[1] (%)
Batch I				
Lucerne	---	---	24.0	---
Juice	---	---	28.0	---
Batch II				
LPC	6.0	11.5	61.0	19.5
Chloroplastic	11.0	13.5	46.0	26.0
Cytoplasmic	1.5	5.0	77.0	16.0

[1]Dry basis

Oelshlegel *et al.* (1969a) reported the crude protein, crude fat, crude fiber, ash and nitrogen-free extract contents for fractions (starting material, residue, juice, protein precipitate) obtained during the extraction of LPC from green plant wastes.

The composition of Pirie's product (already given) varied with the age and species of the leaf (Morrison and Pirie, 1961). The nucleic acid content depended on the details of the extraction procedure but was only a minor component due to rapid hydrolysis by leaf ribonuclease (Morrison and Pirie, 1961; Pirie, 1961a).

The analyses of some protein concentrates extracted from fresh Indian flora by Valli Devi *et al.* (1965) showed wide variations in the protein (34-77%) and ash (1.3-9.0%) contents of the isolates. Other Indian workers, Sentheshanmuganathan and Durand (1969) observed a wide range in the protein content (27-75%) of the concentrate. Ash, calcium and iron were also determined. In the Philippines, Gonzales, Dimaunahan, and Banzon (1968) reported ranges in crude protein content of 9-39% and 9-63% for the leaf and protein isolate, respectively. And in West

Pakistan, Hussian, Ullah, and Ahmad (1968) found the crude protein contents of the leaf and the protein isolate to range from 17-30% and from 28-60%, respectively. The protein content of concentrates made from 58 tropical species in Ghana varied from 15-70%; the legumes exhibiting the highest protein contents and greatest extractability (Byers, 1961).

Eggum (1970) reported the composition--crude protein, ether extract, crude fiber, nitrogen-free extract, true protein, ash, Ca, P, Na, and Mg--of some leaves and LPC from eastern Nigeria. The crude protein contents were within a fairly narrow range of 31-37%. His results agree with those obtained by Oke (1966b) from leaf vegetables in Nigeria.

Amino acid. Numerous amino acid analyses of leaf protein have been published. In 1939 Chibnall considered the essential amino acid composition of leaf protein and concluded that it would have an excellent nutritive value. Lugg and his associates did a great deal of the early work in this area. In 1939, Lugg reported the contents of amide, tyrosine (Tyr), tryptophan (Trp), and the sulfur amino acids in the whole protein of leaves. He saw no evidence of compositional variation due to age, fertilization, climate or locality of growth; but a possible variance with species was observed. Lugg and Weller (1944) determined the amide, Tyr, Trp, cystine (Cys) and methionine (Met) nitrogen for the leaves of four pasture plants. Later, an estimation was made of the basic amino acids--arginine (Arg), lysine (Lys), and histidine (His) (Lugg and Weller, 1948a). It was determined that the composition of whole leaf protein from senescent tissue may vary markedly from that prevailing at an earlier stage of maturity (Lugg and Weller, 1948b). In 1949, Lugg reviewed the literature concerning plant proteins with regard to the amino acid composition and found little variation within species

due to conditions of growth and no outstanding differences between species.

Armstrong (1951) determined the Tyr, Trp, Met, Cys, Lys, and His contents of fifteen herbage species by microbiological assay. He concluded that there may be a wider variation in amino acids of herbage species than previously observed by Lugg and co-workers. Smith and Agiza (1951) colorimetrically determined 14 amino acids (separated by two dimensional paper chromatography) for several species. They reported that the proportions of certain acids, particularly Arg, Lys, and glutamic (Glu),were affected by such factors as age, stage of growth, season, nitrogenous fertilizers and species.

In 1953, Yemm and Folkes published data for the amino acids (determined chiefly by microbiological assay) for three fractions (total, cytoplasmic, and chloroplastic) of barley leaf protein. Their results showed a high degree of uniformity, with appreciable differences only for Lys. Reber and McVicar (1953) reported similar patterns of amino acid distribution in the bulk proteins of several cereal grasses with increases in glutamine (Gln) and isoleucine (Ile) and decreases in Lys in older plants. In contrast, others reported that the protein extracted from four species of grass at varying stages of growth showed considerable uniformity in Lys contents (Waite, Fensom, and Lovett, 1953). By microbiological assay, Kelley and Baum (1953) concluded that leaf meals from several vegetables contained a well balanced mixture of the eight essential amino acids plus His and Arg.

The amino acid composition of amaranth leaves was determined by Deshpande and Rao (1954). Also that year, Kemble and MacPherson reported analyses of protein fractions extracted by several methods from a variety of herbages. Although the protein varied greatly in purity, there were

no significant differences in the monoamine and monocarboxylic acid contents. Except for Ile and alanine (Ala), their results are in good agreement with those obtained by Yemm and Folkes (1953). Kuppuswamy, Srinivasan, and Subramaniam (1958) noted changes in amino acid composition as the plants aged.

The results of Pleshkov and Fowden (1959) confirmed the well known fact that total nitrogen and protein nitrogen decrease with age of the plant and vary with soil mineral treatments. The amino acid composition of barley leaf protein showed a trend toward decreased amide and Arg with slightly increased Lys contents in the older plants.

The amino acid compositions of protein extracted from various leaves (lucerne, spinach, cabbage, and burr medic) exhibited little compositional variation and the concentrations for lucerne proteins are presented in Table 5 (Chibnall, Rees, and Lugg, 1963).

Wilson and Tilley (1965) determined, by ion exchange chromatography, the amino acid compositions of protein isolated by alcohol precipitation from lucerne and grasses. They were similar and had no appreciable differences due to stage of growth (Table 5).

The classical and most comprehensive set of amino acid analyses on leaf protein concentrates was published by Gerloff, Lima, and Stahmann (1965). Protein concentrates extracted from green leaves of ten species harvested under different conditions of fertilization and maturity by the mechanical methods of Morrison and Pirie (1961) and Chayen *et al.* (1961) were analyzed by ion exchange chromatography. Although the protein content ranged from 31.5-83.8% and was subject to crop condition, extraction procedure, and subsequent processing, the samples did not, with the exception of Lys, show large variation in amino acid content. The range of amino acid contents are given in Table 5 along with the

Table 5. Amino acid composition of several alfalfa leaf protein preparations

Amino acid[1]	Chibnall		Wilson and Tilley		Gerloff		Hartman			Smith	
	1[2]	2[3]	Total	Cyto-plasmic	Range[4]	Alfalfa[5]	First	Second[6]	Total	Chloro-plastic	Cyto-plasmic
Lys	8.6	8.7	9.4	9.4	4.5-7.3	6.3	5.5	6.4	6.3	6.2	6.5
Thr	4.4	4.2	4.2	4.6	4.3-5.8	5.2	5.6	5.3	5.0	4.9	5.2
Val	4.9	4.9	5.9	5.1	5.9-6.9	6.1	5.6	5.9	5.8	5.7	5.6
Met	1.4	1.4	1.2	1.3	0.9-2.8	1.4	1.7	1.4	1.8	1.6	1.8
Cys	1.2	1.3	0.8[7]	1.1[7]	0.3-1.1[7]	0.8[7]	1.0[7]	1.3[7]	0.8	1.1	0.9
Ile	3.5	3.2	4.4	5.1	4.9-6.6	6.0	4.6	4.9	5.3	5.0	5.0
Leu	6.7	6.5	7.5	7.5	9.1-10.7	9.8	8.0	8.6	9.6	7.0	10.3
Tyr	2.3	2.8	2.8	3.1	3.1-6.1	4.3	4.7	4.7	5.0	4.8	4.9
Phe	2.3	3.0	3.9	4.4	4.9-6.8	6.6	6.4	6.9	6.1	6.5	6.5
Trp	1.9	1.9	1.9[7]	2.2[7]	1.2-2.2[7]	1.8[7]	1.7[7]	1.8[7]	2.0	1.6	1.2
His	4.1	5.2	5.1	5.0	1.3-2.9	2.3	2.5	2.4	2.0	3.0	1.9
Arg	13.0	16.5	16.6	15.7	5.8-7.3	6.2	5.9	6.7	7.3	8.1	6.6
Asp	6.5	6.5	7.8	8.0	9.0-10.8	10.2	13.8	11.3	9.7	9.4	9.1
Ser	4.5	4.1	4.2	3.9	3.8-5.4	4.8	5.2	4.9	4.5	4.8	4.2
Glu	7.3	7.0	7.7	8.6	11.0-13.2	11.3	12.0	11.4	10.6	10.0	10.4
Pro	3.8	3.2	4.5	4.0	3.5-6.7	5.1	4.9	4.7	4.5	4.4	4.9
Gly	6.6	6.3	7.5	6.7	5.3-6.1	5.7	5.1	5.3	4.7	4.8	5.6
Ala	6.2	6.1	7.1	7.5	5.8-7.4	6.5	5.7	6.0	5.8	5.7	6.0

[1]Expressed as per cent; [2]Extracted by maceration method of Lugg and Weller (1944); [3]Extracted by ether method of Chibnall and Nolan (1924); [4]Range of ten species; [5]Average of two samples prepared by impulse rendering; [6]Refers to cutting; [7]Determined separately.

values determined for alfalfa. No significant effect could be identified with species, variety or maturity. A comparison of the average essential amino acid values of LPC to those of animal proteins showed that only Lys and Met consistently were lower in leaf protein. When compared with other higher protein foodstuffs LPC is high in Met and Lys (Oke, 1969). With Met supplementation leaf protein was suggested to be a well balanced source of dietary protein (Gerloff *et al.*, 1965). The amino acid composition of lucerne LPC reported by Singh (1967) is similar to that obtained at Wisconsin except for differences in leucine (Leu), valine (Val), and Arg.

Hartman *et al.* (1967), in their analysis of spray dried alfalfa and pea vine juice, obtained amino acid compositions similar to those reported by Gerloff *et al.* (1965); see Table 5. With only two exceptions the Met contents were as high or higher than those of the other protein supplements to which they were compared. On the basis of amino acid analyses, the authors concluded that leaf protein could have a nutritive value equal to or greater than the protein supplements currently available except possibly fish meal. Unpublished results of Oelshlegel *et al.* (Stahmann, 1968b) indicated that the distribution of the eight essential amino acids in alfalfa juice was similar to that recommended by FAO.

The amino acid compositions for alfalfa leaf protein, alfalfa leaf cytoplasmic fraction, and alfalfa leaf chloroplastic fraction obtained by the impulse rendering process have been reported by Smith (1966). All three fractions showed similar amino acid distribution. The contents of the essential amino acids are given in Table 5. The sulfur

amino acids are limiting.* However, small differences in their content do not seem to warrant the rather large variation given for the chemical scores--57, 65, and 54 for LPC, cytoplasmic and chloroplastic fractions, respectively.

Valli Devi *et al.* (1965) determined, by microbiological assay, the Met and Lys contents of LPC prepared from several Indian leaves to be 1.3-2.2% and 3-4%, respectively. In contrast to such low values (rarely more than 2 g/16 g N) reported for Met, Surinder Kaur and Vijayaraghavan (1961) found, in a survey of the Met contents of a number of protein concentrates prepared from various local sources, that in seven out of eight species a range of 2.2-2.7 g Met/16 g N existed.

Amino acid analyses of various tropical leaf proteins by Sentheshanmuganathan and Durand (1969) indicated that, except for the sulfur containing amino acids, the essential amino acids were in amounts comparable with those in milk and soya flour and greater than those in the 1957 FAO provisional pattern. They calculated that the chemical scores (CS) (Mitchell and Block, 1964) of the various leaf protein isolates ranged from 50 to 73 against the 1957 FAO provisional pattern. Pirie (1962, 1969e) reported that there were small but definite differences between the amino acid compositions of the whole protein, the chloroplastic and the cytoplasmic fractions extracted at Mysore.

The amino acid composition of some leaves and LPC, principally cassava, from Biafra have been reported by Eggum (1970). There was little variation between species and variety and between whole leaves and LPC. And once again the essential amino acid pattern was adequate except for Met. Shanley and Lewis (1969) determined the Lys, Trp, Cys,

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*Limiting amino acid is that essential amino acid available in least amount in relation to its requirement (Bergen, 1971).

and Met contents of the leaves of a number of plants to estimate their value as a supplement to the maize meal staple diets of South Africa. In all cases the CS of the maize meal-leaf mixture (86) was much greater than that of maize meal alone (65).

Table 6 (Stahmann, 1968a; Oke, 1969) lists the essential amino acid compositions of protein from green plants, maize, soya beans, meats, and milk. LPC had a higher content of Lys than maize, more Met than soya bean and compared favorably with the animal proteins.

Leaf protein is a mixture of many different proteins from a metabolically active organ of the green plant. Therefore, large differences

Table 6. The essential amino acid composition of protein from leaves, corn, soybeans, meats, and milk

Protein source	Lys	Phe	Met	Thr	Ile	Leu	Val	Trp
LPC	6.3*	6.0	2.1	5.2	9.8	5.3	6.3	1.6
Opaque 2 maize endosperm	3.6	4.5	2.1	3.7	10.5	3.8	5.7	---
Soya bean meal	6.4	4.8	0.6	3.7	3.5	6.1	5.0	1.2
Meat, poultry, fish	8.1	4.9	3.3	4.6	7.7	6.3	5.8	1.3
Milk	8.2	5.7	3.4	4.5	8.5	11.3	8.5	1.6

*Expressed as g amino acid per 100 g protein

in the amino acid composition of protein from different species, or the same species in different physiological states, are not expected because it is unlikely that the same excess or deficiency would characterize most of the different component proteins (Singh, 1967; Oke, 1969; Pirie, 1957b, 1959c, 1966a,c, 1969b; Morrison and Pirie, 1961). In agreement with this speculation, the observed differences in amino acid composition with the possible exceptions of Lys and Met according to the studies

reviewed herein are generally small. Differences between species seem no more significant than those within a species. Methionine appears to be the amino acid which varies most. Jennings and Lewis (1969) demonstrated a high degree of Met loss during the hydrolysis of protein in leaf material which had not been previously oxidized. The range of Met loss was from 30-50%. The actual degree of destruction seemed to be unpredictable, as evidenced by poor correlation between some unoxidized duplicates. In summary, the various amino acid analyses suggest that leaf protein should be a better supplement than most of the cereal and seed proteins, but not as good as casein or egg (Pirie, 1959c, 1969b,d).

Biological Assays

While amino acid analyses and resulting chemical scores provide an indication of protein quality, they give no evaluation of availability or digestibility (Carpenter, 1958). That amino acid composition is not a reliable indicator of protein quality as determined by biological assays may be due to: (1) low digestibility of the protein because of improper processing, (2) reduction in the amount of metabolic activity of one or more critical amino acids, (3) differential rates of hydrolysis and absorption in the gut yielding an amino acid composition unlike that of the native protein, (4) failure to destroy, inactivate, or remove physiologically deleterious substances naturally present in the raw material, (5) contamination with foreign toxic substances during processing or (6) formation of physiologically harmful substances during storage (Duckworth and Woodham, 1961; Bergen, 1971). The nutritive value is also affected by such factors as heat, interactions of components in the food system, other non-protein components in the diet, and individual physiological variability (Liener, 1958; Kreyl and Barboriak, 1960). Hence, dietary studies are always necessary for a

valid evaluation of the nutritive value of a protein source. Correlations between the chemical score and various biological assays have been discussed in a FAO publication (1957) and by Cresta *et al.* (1969). The traditional method for determining the biological quality of a protein is to compare the performance of animals (classically rats) on a diet with a recognized good protein such as casein or egg, usually at a level of 10%, with the performance on a diet in which part or all of the "good protein" is replaced by the novel protein (Carpenter, 1951; Pirie, 1966e).

One of the first studies was that of Davies, Evans, and Parr (1952), who determined that the biological value (BV) and digestibility of whole leaf protein were low. They also noted that the cytoplasmic fraction had a higher BV and digestibility than the chloroplastic fraction and that there appeared to be little difference between plant species. An increase in the age of the plant was accompanied by a decrease in BV and digestibility.

That same year Carpenter, Duckworth, and Ellinger (1952) determined, using the gross protein value (GPV) for chicks--i.e., a comparison of the capacity of the protein under test to that of casein to supplement the cereal protein in rations for growing chicks--that leaf protein fed at low levels (3%) was superior to groundnut meal, but was inferior at higher concentrations (11%). Protein efficiency ratios (PER), true digestibility (TD) and BV determined with rats revealed that leaf protein was inferior to white fish meal but similar to groundnut meal. Hughes and Eyles (1953a,b) concluded that leaf protein from lucerne was as valuable as fish meal when incorporated as the main supplementary protein in chick rations.

However, Cowlishaw *et al.* (1954) found lucerne protein concentrate (roller dried at 120 C) to be inferior to fish meal but better than

groundnut meal when added as the only supplementary protein to cereal rations for chicks. In a continuation of this study, using the GPV technique of Carpenter, Ellinger, and Shrimpton (1955), in which LPC provides 27% of the total dietary protein, Cowlishaw *et al.* (1956a) determined that different crops had widely differing nutritive values that in all cases were inferior to casein. Lucerne contained a growth depressing factor (suggested to be saponin) which was counteracted by adding cholesterol. The addition of Lys increased the value of the leaf protein diets indicating low availability of Lys in LPC. A complementary study by Cowlishaw *et al.* (1956b), also using the GPV technique, showed that hot water washing improved the value of lucerne concentrates by removing the water soluble saponins. No appreciable improvements were obtained by varying the pH of precipitation or by solvent extraction. Their results are in agreement with Carpenter, Duckworth, and Ellinger (1954); i.e., in that the GPV of LPC is similar to that of groundnut and less than that of soya bean. The GPV of freeze-dried concentrates were significantly greater than those of roller dried preparations, indicating that the proteins may be damageable by heat. Carpenter and Ellinger (1955) found a highly significant correlation between GPV and estimates of available Lys of dietary proteins, thus providing confirming evidence for the postulate that the ε-amino group of Lys reacts to form a nutritionally unavailable complex when intact proteins are damaged. Widely varying estimates and low value of the nutritive quality of LPC up to 1957 were attributed to improper processing, especially poor washing and over heating during drying (Buchanan, 1969b).

Barber, Braude, and Mitchell (1959) concluded from preliminary tests that wet LPC, when fed as a protein supplement to practical rations

(7% LPC) or diets suboptimal in protein (3.5% LPC), was equal to white fish meal for growing pigs. Leaf protein concentrate was tested as a source of supplementary protein in the diets of chicks and growing rats by Duckworth and Woodham (1961). Provided that the method of drying did not involve high temperatures (greater than 81 C), all preparations had uniformly high GPV values (mean of 79) similar to values for soya bean, i.e., 74. In another study Duckworth, Hepburn, and Woodham (1961) found that wheat LPC, when used at a 7% concentration as a source of supplementary protein in the practical diets for newly weaned pigs, promoted the same rate of growth and efficiency of feed utilization as standard diets with 7 or 8% fish meal.

The first study utilizing humans as test species was that of Waterlow (1962). Twenty-one Jamaican infants who were recovering from severe malnutrition were put on diets supplemented with LPC. Since leaf protein might never be used as the sole source of nitrogen in the diet, this study proposed to determine if leaf protein could be used as a supplement to marginal quantities of milk. Therefore, mixtures in which one-half to two-thirds of the nitrogen was derived from leaf protein and the remainder from milk were given with two controls--milk at an equal nitrogen level and milk at a low level of nitrogen. Balance studies (details given by Waterlow and Wills, 1960, and Waterlow, 1961), which measure the intake and excrement of nutrients, were made. Leaf protein concentrate was tolerated well by the infants and neither weight gains nor nitrogen retention differed significantly between the leaf protein milk (LPM) mixture and milk alone. With low protein milk diets nitrogen retention was nil, but highly significant positive balances were achieved with the addition of leaf protein. Therefore, it was concluded that leaf protein could act as a valuable supplement to a marginal

intake of milk. The mean values for nitrogen retention are given in Table 7.

Table 7. Mean values for the retention of nitrogen by malnourished infants fed milk (M) or leaf protein and milk (LPM)

Group	Diet	N intake*	N retained*	% N retained
A_1	LPM	727	156 ± 21.4	21.4 ± 1.8
B_1	M	743	175 ± 32.9	23.9 ± 3.6
A_2	LPM	471	105 ± 16.5	21.2 ± 2.5
B_2	M	491	108 ± 19.0	22.4 ± 4.3
C_1	M	246	14 ± 20.7	5.4 ± 9.3
C_2	M	159	13 ± 17.1	6.2 ±11.9

*Expressed as mg N/kg body weight/day

In a later study Fox and Waterlow (1967) investigated the value of leaf protein as a partial replacement for dry skim milk (DSM) and as a supplement to the poor Jamaican diet. In animal trials the BV, TD, and net protein utilization (NPU) of the LPC ranged as follows: 41-54, 60-80, and 30-40, respectively; except for banana leaf protein. The suggestion was made that this latter preparation might contain some toxic constituent. Since cow pea LPC appeared to be the most economical to produce locally it was assessed as a supplement. With rats the addition of cow pea LPC to the poor Jamaican diet caused a doubling in per cent weight gain. In long term rat trials no gross post mortem abnormalities were found which could be attributed to the cow pea LPC. Balance studies were conducted with normal young children who had recovered from protein malnutrition. The three diets fed were: (1) DSM, (2) 1/2 DSM protein and 1/2 solvent extracted LPC protein (LPI), and (3) 1/2 DSM protein and 1/2 LPC protein (LPII). The results are given in Table 8. With the leaf protein diets, in spite of positive

Table 8. Mean values for nitrogen absorption and retention by malnourished infants fed a milk diet (DSM) and a leaf protein and milk diet (LP)

Evaluation criteria	DSM	LP I	LP II
Weight change (g/kg/day)	+1.7	-3.0	-0.34
N intake (mg/kg/day)	195	201	196
N absorbed (as % of intake)	85 ± 4.8	87 ± 2.1	79 ± 5.1
N retained (as % of intake)	32 ± 3.5	33 ± 6.9	26 ± 4.4
True N digestibility	86.5	86.8	78.8
Net protein utilization	83	83	78

nitrogen retention there was a weight loss. With the exception of weight change there was little difference between DSM and DSM partially substituted with solvent extracted LPC. However, the non-solvent extracted LPC gave the poorest responses. The investigators concluded that it was difficult to make a realistic assessment of the value of cow pea LPC, but it appeared promising if it replaces less than 50% of the protein of DSM.

Henry and Ford (1965) determined BV and TD values for a number of LPC preparations by the method of Mitchell (1924) at an 8% level of protein intake on young growing rats. In general, BVs of the LPC (over half greater than 70) were of the order found for legumes, cereal seeds and yeast, but the TDs were lower (range of 71-91). Both values varied with species. Freeze-drying, acetone extraction and drying on starch were satisfactory but oven drying at 100 C caused marked decreases in BV and TD. BV and TD increased with maturity of the leaf due to increased availability of Met; when LPC from tares was supplemented with sufficient Met to bring the level equal to that of whole egg, the BV was increased to that of egg. The availability of some essential amino

acids was determined microbiologically by the methods of Ford (1960, 1962, 1964) and the results were consistent with the previously stated biological findings (i.e., BV and TD). In agreement with earlier work (Davies *et al.*, 1952), both the BV and TD were greater for the cytoplasmic fractions than for the chloroplastic fractions.

The results of GPV (modified method of Duckworth, Woodham, and McDonald, 1961) and PER studies by Woodham (1965) confirmed the potentially high value of leaf protein. Oke and Tella (1967) showed from feeding experiments with rats that leaf protein was as good as milk protein.

Smith (1966) reported that alfalfa leaf protein had a BV, digestibility, and NPU of 59, 75, and 44, respectively, for the rat. However, Met supplementation increased these parameters to 72, 77, and 56, respectively. He stated the relatively low digestibility was not associated with processing, drying conditions, or a saponin factor.

Nutritive values of leaves and LPC, mostly cassava, prepared in Nigeria were determined by Eggum (1970) using rats. The following ranges were noted: BV, 44-57; TD, 70-78; and NPU, 34-41. The preparation with the highest Met content gave the highest BV. Methionine supplementation of cassava leaves raised its BV from 49 to 80. Cassava leaves combined with Norwegian dried cod, an important protein source in Nigeria, showed a mutual supplementation effect (i.e., the BV of cassava leaves was raised from 49 to 73).

Nutritional studies conducted in India generally indicated at least some supplementary value for most leaf protein preparations. Subrahmanyan and Sur (1949) showed that lucerne powder is an excellent supplement to the poor South Indian rice diet when fed at levels of 10%

to rats. In 1954 Sur and Subrahmanyan reported that lucerne has a supplementary value even at low levels. Studies by Shurpalekar, Singh, and Sundaravalli (1967, 1969) revealed a PER (with rats) for freeze-dried lucerne LPC of only 1.54 (skim milk powder 3.3), but supplementation with Met increased this to 2.77. No change in the PER was noted with Lys supplementation. The greatest growth was found at a 15% protein level when leaf protein was the sole protein source. However, Met supplementation gave the best growth at a 10% protein level. They noted that the addition of cholesterol was unnecessary to counteract the possible presence of growth depressing saponins due to the satisfactory removal of soluble constituents by washing of the curd. Also the quality of leaf protein precipitated by acid was found to be slightly inferior to that coagulated by steam. Leaf protein concentrate--at a 5% protein level--plus vitamins and minerals was equal to skim milk powder in its ability to supplement a poor rice diet.

Sur (1967) in rat feeding studies with water hyacinth leaf protein found that Met supplementation increased both BV and digestibility. Leaf protein and rice protein had complementary amino acid patterns. Ghosh (1967) also improved the nutritive value of water hyacinth leaf protein by Met supplementation.

At CFTRI supplementation with leaf protein led to significant nutritive improvement of rice diets (Singh, 1969). Singh also reported that there were differences in nutritive value between species and between different preparations of lucerne LPC. This latter difference was attributed to differences in age of the plants. The following PER values were given: carrot, 0; Dhaincha, 0.8; lucerne, 1.3-1.8; and crucifers, 1.8-2.2. Histological examination of the liver and intestine of those animals which did not grow did not show any signs of toxicity.

Subba Rau *et al.* (1969), using rats, compared the nutritive values of spray dried juice, LPC, as well as the chloroplastic and cytoplasmic fractions of leaf protein, prepared from lucerne. Their results are given in Table 9. The values obtained for the whole juice were extremely low. The chloroplastic fraction was inferior to the unfractionated

Table 9. Nutritive value of lucerne leaf juice, LPC, and the chloroplastic and cytoplasmic fractions of leaf protein

Protein source	PER	Apparent digestibility
Batch I		
Spray dried juice	0.34	---
LPC	1.38 ± 0.13	---
Batch II		
LPC	1.36 ± 0.13	73
Chloroplastic fraction	0.78 ± 0.13	65
Cytoplasmic fraction	2.02 ± 0.13	80

leaf protein which was inferior to the cytoplasmic fraction. The digestibility values are in agreement with those reported earlier by Smith (1966) for similar lucerne preparations. The small differences in Lys and Met values between the preparations cannot account for the nutritional variations. The authors suggested that nonprotein constituents act as low digestibility diluents and probably lower the nutritive value through various interactions. Speculation was made that the toxicity of the spray dried juice might be due to high contents of soluble mineral matter and nitrogen constituents, some antigrowth factor, or nutritionally unavailable protein damaged by interactions with other substances. This study illustrated the necessity for bioassays; for an *in vitro* digestion (Hartman *et al.*, 1967) failed to indicate the unsuitability of whole juice as a dietary additive.

Singh (1967) reported that in a preliminary metabolic study of eight children on LPC diets the TD and BV were 85 and 65, respectively. In a six month trial with children, diets based on ragi--a millet cultivated widely in South India, contributing a major ingredient in the diet and reported to be deficient in lysine--were supplemented with Lys, lucerne LPC, or sesame flour. Nitrogen retention, apparent digestibility, height, weight, and hemoglobin were measured. All three supplements resulted in improved nutritional response, but leaf protein gave the greatest growth response because it improved both the quality and content of protein in the diet (Doraiswamy, Singh, and Daniel, 1969; Singh, 1967). However, extensive tests indicating the long term nutritional effects of leaf protein supplementation have not been made (Smith, 1969).

In vitro Digestion Methods

Various investigators have used *in vitro* methods for assessing the nutritive value of extracted leaf protein. Akeson and Stahmann (1965) estimated the biological value of freeze-dried LPC with an *in vitro* enzymatic digestion--based on the amount of eight essential amino acids released by pepsin followed by pancreatin hydrolysis and compared to that released by whole egg. Excellent correlation, better than the essential amino acid index or chemical score, has been obtained between this pepsin pancreatin digest index (PPDI) and published BVs for eight reference proteins (Akeson and Stahmann, 1964). The estimated BVs of LPC (78-89) exhibited little difference between species and in general were less than the values for egg (97), about equal to whole milk (83), and greater than beef (75), casein (76), yeast (71), soybean (65), cottonseed (64) and wheat (50) flours, gluten (45), and zein (26).

This indicated that LPC would be a high quality protein source for human consumption (Stahmann, 1968a,b). Hartman *et al.* (1967) reported that BV, estimated as PPDI, of spray-dried alfalfa juice samples was similar to those reported for freeze-dried LPC by Akeson and Stahmann (1965).

Smith (1966) reported the *in vitro* digestibility of alfalfa leaf cytoplasmic and chloroplastic fractions to be 95-100 and 60-70, respectively. Solvent extraction of the chloroplastic fraction did not increase its digestibility.

Byers (1967a) found that maize leaf protein was not digested by papain under conditions in which casein is hydrolyzed (Pirie, 1963). Maximum digestion was achieved at pH 6.6 and 70 C with KCN-activated papain. Evidence was presented indicating that lipids do not protect the protein against proteolytic digestion. Although *in vivo* experiments have shown higher nutritive value with protein from mature wheat leaves, there was no corresponding increase in the *in vitro* digestibility. In the following paper Byers (1967b) described the *in vitro* digestion by papain of LPC extracted from 14 species of plants. The extent of hydrolysis, which was never complete, varied with species. The effect of leaf age was uncertain. For all species the chloroplastic fraction (precipitated at 53 C) was digested less and the cytoplasmic fraction (precipitated at 80 C) more than the corresponding whole protein. She suggested that the protein digestibility of processed LPC preparations could be assessed rapidly by papain hydrolysis as a preliminary evaluation to *in vivo* tests. Pirie (1969e) reported the analyses of seven whole leaf preparations from Mysore by this method.

Buchanan (1969b; Pirie, 1967c) compared several *in vitro* methods with *in vivo* evaluations for estimating the nutritive value of LPC. The thioglycollic acid-activated papain solubilization procedure of

Buchanan and Byers (1969) gave the best correlation with *in vivo* results for rats, but the PPDI seemed promising. Heating moist LPC decreased the TD due to protein-unsaturated lipid complexing, and therefore also the PER and NPU values. The latter two were almost restored to normal values by lipid extraction, since this caused the splitting of these lipid-protein complexes (Buchanan, 1968).

Although the sulfur amino acids are usually limiting, Buchanan (1969b) stated that Lys may be limiting if LPC is heated. Shah, Riaz-ud-Din, and Salam (1967) studied the effect of heat on the *in vitro* digestibility of LPC. Treatment of LPC at 100 C decreased its digestibility with human proteolytic enzymes. Digestibility increased when the LPC was defatted with chloroform-methanol. The oxidation products of the lipids and their polymers with proteins were determined to be toxic to trypsin, pepsin, and pancreatic enzymes.

Effects of Storage

Pirie (1961c, 1962) reported decreased nutritive value and TD with drying or storage at high temperatures, and suggested that products of lipid oxidation combine with protein breakdown products to form insoluble compounds. Shah (1963) noted that after storage at 80-100 C the extractability of LPC lipids decreased. Pirie (1966e) reported that the digestibility of barley protein was increased by partially extracting the lipid, indicating that the resistance of leaf protein to proteolysis depends in part on the formation of lipid complexes. This concept was in contrast to the opinion of Byers (1967a). In a study by Buchanan (1969c) at 4 and 28 C, extensive oxidation of LPC occurred, but with little concomitant change in its papain digestibility. Losses in digestibility after heating at 100 C were attributed to two different reactions: (1)

a lipid-protein complexing reaction which is reversible by solvent extraction and (2) modification of the protein which was not influenced by the presence of leaf lipid. It was not possible to conclude how LPC should be stored, only that the interactions occurring during storage are complex. Therefore, Pirie (1966a,c, 1969b,d) suggested that variations in nutritive value, despite uniform amino acid composition, may be the result of changes taking place during preparation or storage due to reaction of carbohydrate, phenolic compounds or fatty acids with amino acid residues.

In summary, the several studies showed that leaf protein extracted from many green plants, if not damaged by inappropriate processing, has a food value as great as that of fish meal or the best seed protein but less than that of casein and egg (Pirie, 1966c, 1967a; Stahmann, 1968a; Kinsella, 1970). Total protein in the diet is rarely from one source, therefore the manner in which the various sources complement each other is as important as the composition of each source separately; e.g., leaf protein is limiting in Met, fish and maize are not (Pirie, 1957b; Morrison and Pirie, 1960; Oke, 1966a). Since leaf protein is intended as a supplement to a diet which contains some but insufficient amounts of protein (Pirie, 1959a, 1968), more feeding trials are needed in which leaf protein is compared not only with a reference protein, but combined with other protein that constitute the major portions of local diets. That is, leaf protein should not be considered in isolation, but rather in relation to other dietary components (Pirie, 1968). There is little doubt of the potential value of LPC as a supplement for improving the quality of conventional dietaries based on cereals (Anon., 1970).

Indigenous Toxic Substances

Many plants have the capacity to synthesize a wide variety of chemical substances which are known to exert a deleterious effect when ingested by man or animals (Liener, 1969). While most plant species undoubtedly contain potentially toxic* compounds, low levels of exposure generally preclude intoxication; for toxicity is determined not only by the intrinsic properties of the toxicant and the exposed individual, but also by the level and duration of exposure (Crosby, 1969). The subject of naturally occurring toxicants in foods has been reviewed by Liener (1962, 1966), Mickelsen and Yang (1966), Strong (1966) and NAS-NRC (1966). This review will concern those which might have possible consequence in the production of protein from green leaves, particularly from alfalfa.

Substances which have the ability to inhibit the proteolytic activity of certain enzymes are found throughout the plant kingdom, particularly among the legumes (Liener and Kakade, 1969). Ramirez and Mitchell (1960) described the partial purification of a trypsin inhibitor from alfalfa which they believed to be a non-dialyzable polypeptide or a noncoaguable protein. Mooijman (1964) was of the opinion that the alfalfa trypsin inhibitor is a saponin-peptide or saponin-amino acid complex. Liener and Kakada (1969) discussed the following aspects of protease inhibitors: distribution in plants; physical and chemical properties; specificity, stoichiometry, kinetics, and mechanisms of inhibitor-protease reactions; nutritional and physiological significance; and effects of processing.

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*Toxic is defined as producing an adverse physiological response in man or animal by a particular food (Liener, 1969).

Certain grasses, pulses, root crops, and fruit kernels contain relatively high concentrations of cyanogenetic glucosides (Montgomery, 1969). These plant tissues may be rendered less toxic by extraction or by maceration and dehydration (Conn, 1969). The cyanogens are important in the production of leaf protein from cassava. Their chemistry and biosynthesis have been reviewed by Conn (1969).

The family *Cycadaceae* which is indigenous to the tropics and possesses large leaves is also toxic when not properly prepared (Mickelsen and Yang, 1968; Yang and Mickelsen, 1969).

Natural thioglucosides from the *Cruciferae* and related plant families are a source of goitrogens or antithyroid compounds (VanEtten, 1969). Included are some common crucifer plants--kale, rape, radish, mustard, turnip--which have been considered for leaf protein production. Fortunately the thioglucosides occur in highest concentration in the mature seed (VanEtten, 1969). The chemistry and biological effects of thioglucosides (or glucosinolates) have been reviewed by VanEtten (1969) and VanEtten, Daxenbichler, and Wolff (1969).

Alkaloids represent a variety of unrelated structural types, common only in the classical sense that they contain nitrogen, have a bitter taste, and are very active biologically, but without showing post-mortem lesions (Sollman, 1957; Keeler, 1969). Alkaloids appear to be the active principals in many range plants which produce acute toxicosis or teratogenic effects (Keeler, 1969). Metabolites detrimental to the growth of meadow vole weanlings were extracted from individual alfalfa clones and were tentatively determined to include the following alkaloids: trigonelline, stachydrine, and homostachydrine (Elliott, 1963b).

The toxicity of non-nitrogen containing plant phenols has been reviewed by Singleton and Kratzer (1969). These are toxic only if the

natural barriers or detoxification mechanisms are overloaded or evaded. Generally the toxicity limits are not even approached in plant foods, but the potential toxicity should not be neglected (Singleton and Kratzer, 1969).

Tannins--polyphenolic substances which have the ability to form a precipitate with gelatin under certain conditions (Kirby, 1960)--are present in a number of plant materials at very high levels (Singleton and Kratzer, 1969) and are present in toxic concentrations in certain foods (Glick and Joslyn, 1970). Although Kirby (1960) found tannins injected subcutaneously induced tumors in rats, oral administration to mice caused no adverse effects.

The chemical and physiological properties of some toxic amino acids and peptides occurring naturally in foods have, along with the mechanisms of toxicity, been reviewed by Hylin (1969) and Fowden, Lewis and Tristam (1967). They point out that most food consumed by man does not contain toxic amino acids and/or peptides. Those which do are usually unique to certain limited climatic areas of the world and are consumed by relatively small populations (Hylin, 1969).

Pudelkiewicz and Matterson (1960) showed that alfalfa contains an ethanol soluble fraction which reduces the availability of vitamin E. Elliott (1963a) found critical levels of a competitive antimetabolite to niacin in certain individual alfalfa plants. Later, a source of diploid alfalfa was found to contain plants with antimetabolic activity as evidenced by bioassays. This activity could be counteracted by a combination of aspartic acid, glutamine, and glycine. Differences in nutritive value between the plants could not be associated with amino acid deficiencies (Schillinger and Elliott, 1966b).

Saponins are glycosides that occur in a wide variety of plants. They are characterized by a bitter taste, formation of a stable foam in aqueous solution, hemolysis of red blood cells, formation of molecular compounds with cholesterol and other hydroxy steroids, and an irritating effect on mucous membranes (Sollman, 1957; Hanson *et al.*, 1963; Scardavi and Elliott, 1967; Birk, 1969). They exhibit a wide variety of physiological effects, mostly adverse, on animals. Most of these activities arise from the strong surface activity of saponins and from their ability to form complexes with sterols and proteins (Birk, 1969).

There are two main classes of saponins: steroids (C_{27}) and triterpenoids (C_{30}) (Scardavi and Elliott, 1967; Birk, 1969). They differ markedly in chemical composition with respect to both alglycones and carbohydrate and on hydrolysis they yield sapogenins (the alglycone moiety) plus sugars (Birk, 1969). The chemistry and biosynthesis of the steroidal saponins (and glycoalkaloids) has been discussed by Heftmann (1967).

The exact nature of saponins and their potency in foods have been studied relatively little; and some of the properties attributed to them have not always been conclusively demonstrated (Birk, 1969; Gestetner *et al.*, 1970). Among the biological phenomena which may involve alfalfa saponins are growth depressing effects, depressed egg production in laying hens, ruminant bloat, hemolytic effects, respiratory inhibition, nonspecific inhibition of alpha-chymotrypsin, proteases, cholinesterase, and retardation of seed germination (Hanson *et al.*, 1963; Scardavi and Elliott, 1967; Birk, 1969; Shany *et al.*, 1970a).

Saponins appear to be partly responsible for the depressing effect of alfalfa on growth, food consumption and diet utilization of chicks. Initial studies included those of Peterson (1950) and Lepkovsky *et al.*

(1950) noted that fractions obtained from an aqueous extract of alfalfa meal produced depression of growth in young chicks proportional to the level of alfalfa meal in the ration. The strong foaming properties of these fractions suggested saponins as the growth depressing agent. All samples having growth depressing action also had hemolytic activity. Most of the inhibitory effect on growth could be removed by exhaustive extraction of the alfalfa meal with hot water (Lepkovsky *et al.*, 1950).

Walter *et al.* (1954) investigated the water soluble fraction of alfalfa to test the hypothesis that it contains saponin capable of inhibiting growth of chicks and of contributing to ruminant bloat. Coulson and Davies (1962) reviewed the implication of alfalfa saponins in the etiology of bloat and respiratory inhibition. The studies of Coulson and Davies (1962) and Lendahl *et al.* (1957) suggested that alfalfa saponins are involved in the formation of bloat in ruminants by altering the surface tension of the ruminant contents. In 1959 Jackson and Shaw compared alfalfa saponins, purified by different methods, on the basis of respiratory inhibiting activity, specific rotation, IR spectra, and monosaccharides released on hydrolysis. Pederson *et al.* (1966) conducted a comparative study with saponins from different alfalfa varieties of their biological activities.

Most saponins are so poorly absorbed in the intestine that acute oral doses produce only local effects on the mucous membranes. Consequently, death is due to inflammation of the alimentary canal rather than to absorption and systemic action of the saponin (Sollmann, 1957).

Varieties differ in saponin quantity as well as biological value. Hanson *et al.* (1963) studied the magnitude and variation of the saponin content in alfalfa as a function of location, cutting, variety, and

other variables. The first cutting was lower in saponin content than the last two, which were approximately equal. Saponin content, which varied from 2% to over 3%, was positively correlated with contents of protein, ash, fat, and nitrogen-free extract; and negatively correlated with crude fiber and hay yield.

The chemical composition of alfalfa saponins, which belong to the class of triterpenoids, has been reviewed by Hanson *et al.* (1963) and Birk (1969). Actually, little work has been done in this area due to the difficulties in purification and characterization and to the heterogeneity of saponins. By improved methods of paper chromatography, Coulson and Davies (1962) revealed 10 constituents of lucerne bulk saponins. Pederson *et al.* (1966) found alfalfa to contain a mixture of at least 5 saponins. Methods for their detection, extraction, purification, determination, and characterization have been reviewed by Scardavi and Elliott (1967) and Birk (1969). But a simple quantitative method for the determination of saponins is not yet available (Scardavi and Elliott, 1967).

Recently, Shany and associates (Shany *et al.*, 1970a,b; Gestetner *et al.*, 1970) compared the chemical composition and biological activity of saponin preparations from lucerne tops and roots. The root extracts possessed stronger hemolytic activity and surface activity than the top extracts. They found the toxicity of lucerne saponins due mainly to those saponins which contain medicagenic acid or an unidentified sapogenin as their aglycone moiety. It was also reported that the amount of cholesterol needed to fully counteract the saponin-induced growth impairment depends on the toxicity of the extract preparation and on its concentration in the diet.

Toxins may also be introduced in a food during processing. Browning, which can be enzymatic or nonenzymatic, may occur due to the formation of unstable polymers of varying composition. It may result in the following adverse effects: (1) development of brown discoloration; (2) production of stale, carmelized, or bitter odors or flavors; (3) loss of solubility of the protein; (4) increased tendency to foam; (5) loss of nutritive value of the protein; and (6) possible toxic effects (Friedman and Shibko, 1969). A complete study of the circling syndrome produced in mice by dimethylaminohexose reductone--a product of the browning reaction--administered orally or by injection, has been reported by Cutting *et al.* (1960). The syndrome was characterized by head tossing and running in circles. At higher dosages early deaths occurred and at lower dosages the syndrome was absent or incompletely developed. Ambrose, Robbins, and DeEds (1961) also studied the toxicity of amino-hexose-reductones, administered orally. They noted typical symptoms of hyperexcitability, whirling, and elevations and nodding of the head.

Although there is evidence that proper processing can eliminate toxic substances from LPC, new leaf protein isolates should be examined for toxicants (Buchanan, 1968).

Acceptability

Evidence of good nutritional value is of little practical significance if the physical characteristics--color, flavor, and odor--are objectionable to humans. Leaf protein concentrate which has not been solvent extracted has a dark green color and a slight spinach, tea-like or leafy flavor and consequently does not elicit immediate appeal (Byers *et al.*, 1957; Morrison and Pirie, 1960; Pirie, 1957b, 1959a,

1966a; Buchanan, 1968, 1969a; Singh, 1969; Oke, 1969; Kamalanathan, Usha, and Devadas, 1969; Smith, 1969; Knuckles *et al.*, 1970). However, no novel food, regardless of intrinsic merits, is likely to be accepted by adults (Pirie, 1969b); for acceptable foods are determined largely by convention. This bias is less pronounced in children (Morrison and Pirie, 1960). Pirie, who for many years has been eating 5-10 g/day quantities of leaf protein (Pirie, 1966a,b), maintains that the problem is mainly an aesthetic one, and that it is only a myth that food habits are unchangeable for people have few food instincts (Pirie, 1959a, 1966d; Byers *et al.*, 1965; Buchanan, 1968). He does admit that people are reluctant to change and that popularization of novel foods may be difficult (Pirie, 1968, 1969a,d; Buchanan, 1968).

Wilson (1964) and Onrust (1969) have discussed general factors which must be considered in attempting a nutritional change with non-conventional proteins. Pirie (1966a,d, 1967b, 1968, 1970) outlined steps for introducing a novel food source: (1) be sure that the novelty can be made in sufficient quantity in the region where it is to be consumed, (2) be sure that it has the feeding value for people that animal experiments suggest, (3) be patient and eat it manifestly, (4) resist attempts to use it first on underprivileged groups, i.e., establish a status associated with the upper class, (5) devise dishes acceptable to those doing and financing the research. After accomplishing these steps, acceptance is simply a matter of studying the local food patterns to see which dishes are amenable to modification.

Rate and degree of acceptance will depend on dishes normally eaten. Fortunately, tastes in the wet tropics, where the need is the greatest, differ from our own (Buchanan, 1968). Dark colored and greenish foods, as well as powdered dried leaves, are part of the normal diets in

India, West Africa, Southeast Asia, and New Guinea--regions where the people are primarily vegetarian (Pirie, 1966a, 1969b; Buchanan, 1969a). Guha (1960) stated that color and flavor do not matter when fresh LPC is incorporated into common Indian dishes like curries, pakora, and purries; for they have been well accepted and were, in fact, consumed during the famine in Bengal in 1943.

Akinrele (1963) removed the leafy flavor by a thorough water wash and by boiling for a short time or by masking with spices. He and Oke (1966a, 1969) agreed that color should not be a problem in Nigeria where foods of widely varying colors, particularly green vegetable stews and soups, are commonly eaten. However, Akinrele noted that a white LPC, which could be mixed with cassava or yam flour, would be desirable. Oke (1966a, 1968a) included lists of some Nigerian dishes into which freeze-dried LPC was successfully introduced.

Singh (1967, 1969; CFTRI, 1968) reported that preliminary trials with several common Indian dishes and some processed foods showed that LPC was acceptable as a base material for incorporation into certain foods, including chutneys, vegetable curry bases, soup powders, and weaning foods. Mahadeviah and Singh (1968) removed the bitterness of chicory LPC by boiling with water.

Kamalanathan *et al.* (1969) published their results on an investigation of the incorporation of LPC into foods, and gave an evaluation of the acceptability of LPC in foods. Leaf protein concentrate prepared from lucerne was incorporated into six preparations at levels of 5 g, 10 g, and 15 g per serving. Appearance and color, texture, flavor, taste, and overall palatability were evaluated. Ratings for the various preparations are given in Table 10. Color was not as great a problem as the leafy flavor. The workers concluded that the flavor of LPC

Table 10. Mean hedonic ratings of non-LPC and LPC incorporated products

Preparations	Scores for variations*			
	Non LPC	5 g level	10 g level	15 g level
Dhal balls	6.56	5.04	3.80	2.56
'Chutney powder'	6.00	4.88	4.40	4.04
Sweet potato 'curry'	5.76	3.80	3.36	2.68
Ragi 'adai'	6.16	5.48	4.44	4.20
Potato 'bath'	6.28	4.24	3.24	2.64
Greens 'chutney'	6.84	6.00	5.56	4.68

*Maximum score = 7

influenced the preparations adversely and for better acceptability LPC should be incorporated into highly spiced or flavored preparations.

In an acceptability trial of 55 Jamaican recipes, Fox and Waterlow (1967) found the objection dependent on the recipe in which LPC was used. Color was generally a deterrent. According to Shorland (1969), replacement of flour in sponge cake with alcohol extracted grass leaf protein gave a product which was acceptable except for the detectable residual chlorophyll. In New Guinea, Pirie (1969b) reported that both children and adults ate leaf protein readily. It has also been reported that in New Guinea leaf protein is used for the manufacture of a bread which has been acceptable to the natives (Anon., 1967).

According to Parpia (1967) a fair amount of progress has been made in developing acceptable recipes of traditional food preparations incorporating LPC. Others feel that a better understanding of food habits and traditional methods of food preparation is required for the regions where leaf protein might be used (Pirie, 1966c; Buchanan, 1968; Singh, 1969).

An investigation of methods for presentation of green LPC--prepared by the method of Morrison and Pirie (1961)--as a food is proceeding at Rothamsted. The first studies were based on the assumption that leaf protein should be used to satisfy 10% of the protein need, about 4-8 g/day (Pirie, 1959c; Morrison and Pirie, 1960). Several recipes--e.g., peanut butter spread, stuffed tomatoes, ravioli, and doughnuts--have been formulated and published (Morrison and Pirie, 1960; Byers *et al.*, 1965). These are categorized into three basic types: (1) dry, stable food, (2) mixtures enclosed in a batter or pastry, (3) soups and stews prepared in the conventional way but with leaf protein added (Byers *et al.*, 1965). Those who see leaf protein regularly accepted it (Pirie, 1959a) and therefore, according to Byers *et al.* (1965), the problem is to devise a food sufficiently attractive to appeal to those responsible for the agricultural and food policy of a developing country.

There is little information concerning flavors of LPC (Kinsella, 1970). The bitter flavor of forage extracts has been attributed to inorganic ions (especially Zn) which are claimed to be removable by electrodialysis and ion-exchange (Jennings, 1949). Buchanan (1968) stated that the flavor differs little between species and may become rancid in unextracted preparations. According to Pirie and associates (Pirie, 1959c; Morrison and Pirie, 1960) flavor is no problem when LPC is encased in pastry. Thorough washing with hot acidified water can even make leaves which have a strong flavor yield a bland or mildly flavored product (Singh, 1969). In general, flavors such as fish, cheese or egg blend better with leaf protein than sweet flavors (Byers *et al.*, 1965; Pirie, 1957b). Texture is also important. Fresh leaf protein and properly processed freeze-dried preparations can be converted to a smooth paste (Byers *et al.*, 1965).

There appears to be slight variation in the color of LPC among species of plants (Buchanan, 1968). The dry, gray-brown powder made by solvent extraction is easier to incorporate into foods than the green concentrate (Byers *et al.*, 1965). However, the following reasons were given for not using it: (1) it involves sophisticated technology, whereas the objective is to devise methods of producing and utilizing a protein supplement in technically unadvanced countries; and (2) the color has not been found to be a serious obstacle. Singh (1967) stated that the predominant feeling among the experts that only white bland products would be acceptable as protein supplements must be overcome. However, Fox and Waterlow (1967) in Jamaica and Smith (1966) in England agreed that although it may be possible to conceal small amounts of the green LPC in certain highly flavored dishes, for most human feeding purposes the strongly flavored components must first be removed. Opinions in the United States also generally support the development of a bland, light colored concentrate which can be incorporated into common foods without vastly changing their physical properties (Kinsella, 1970).

Potential

The potential of leaf protein as a human food has been discussed by many writers. Slade (1937) stated that if used as "grass cheese" four to five times more food per acre could be produced than produced by raising wheat. The premise that it would be more efficient to convert the protein in leaf into a human food by industrial, biochemical methods rather than by the ruminant has been expounded many times (Slade *et al.*, 1945; Pirie, 1942a, 1953, 1956b, 1957a, 1959a, 1967a, 1968; Akinrele, 1963; Tilley *et al.*, 1954; Raymond and Tilley, 1956; West,

1968; Stahmann, 1968a,b; Kinsella, 1970). Biological conversion is very wasteful. The conversion efficiency of the ruminant is never greater than 30% and is usually 5-15%.

In contrast to Pirie's (1942a,b, 1953) early optimistic advocation of leaf protein as a human food, Raymond and Tilley (1956) stated that few papers contained sufficient data to substantiate their claims to the promising future of leaf protein; and that there was no reason to recommend it over legumes or oilseeds. However, under conditions of inadequate food supplies, any method capable of making more food should be of interest. They (Tilley and Raymond, 1957) concluded that the utilization of LPC as a major agricultural product is dependent on the demonstration that the protein could be produced cheaper and in greater yield than alternative sources of protein of equivalent nutritive value, and that it could be converted into a satisfactory food product.

Akinrele (1963) felt that its prohibitive cost and restricted availability relative to other protein sources will keep the production of leaf protein a problem of academic rather than practical interest. However, he added that it is a potentially important protein reserve which should not be forgotten. An opposing view is held by another Nigerian investigator, Oke (1968a,b), who feels the main hope for needed protein supplementation lies in the protein of green leaves. He suggested that if the three main crops of Nigeria--cassava, maize, and cow pea--are planted for seeds, the yield of crude protein per acre would be about 8, 40, and 100 kg, respectively. Whereas, if they are harvested for their leaves the corresponding yields will be about 70, 200, and 400 kg, respectively.

In 1964 it was the opinion of the FAO that although there were a number of problems--unattractive color, strong hay taste, and financial

support--to be solved before a useable, low cost product could be produced, the potential food value of LPC is unquestionable.

Pirie (1966a) stated that the concept of leaf protein significantly alleviating the world protein shortage has not yet gained sufficient acceptance to initiate large scale production. He feels that emphasis should be placed on the fact that palatable leaf protein, with sufficient nutritive value, can be made in bulk from a number of plants. Inertia and a plentiful supply of protein in those nations capable of carrying on the research have thus far reduced investigative efforts. Akeson and Stahmann (1966) concluded that further study of leaf protein production should be undertaken in order to increase the protein supply in those areas in which it is inadequate. However, Parpia (1967) felt that the utilization of LPC on a large scale is beset with several problems such as keeping quality, taste, and acceptability.

According to Singh (1967), the results of the studies on LPC completed thus far have given substantial support to the expected potentialities inherent in the direct exploitation of green vegetation. He concluded that a program utilizing some legume fodder plants and the by-product vegetation of several green vegetable crops would be workable for leaf protein production without changing current Indian agricultural practices. Gonzales *et al.* (1968) in the Philippines stated that leaf protein can be considered a valuable supplementary food in their country.

The International Symposium of Protein Foods and Concentrates (1967), feeling that LPC has potential that merited further investigation and study, went on record to support research on leaf protein. The President's Science Advisory Council (1967) reported that LPC should be given consideration primarily because the raw materials are frequently wasted or are at best fed to animals with concomitant inefficient utilization of the protein.

Stahmann (1968b) speculated that enough alfalfa leaf protein to meet world needs could be produced on only 302,000 square miles. An equal amount of protein would remain unextracted in the residue. This could be fed to cattle to produce additional meat and milk. Although only speculation, it does demonstrate the vast potential of forage protein if utilized as a primary food source (Kinsella, 1970). Browning (1970) declared alfalfa and grasses to be the most promising sources for economical production of edible LPC. Stahmann (1968b) concluded that alfalfa has the greatest potential of the common crops for producing a maximum quantity of high quality protein. He is convinced that, in the future, mechanical leaf protein production will be utilized and will assume a significant role in meeting the increasing protein requirement. It is the opinion of Smith (1969) that mechanical extraction of protein from leaves promises to provide a cheap source of concentrated protein that will rival traditional animal sources. He felt that North Americans must be persuaded to adopt leaf protein additives, thus setting an example for developing countries. Shorland (1969) and Kinsella (1970) also concluded that plant leaves can supply an important quantity of protein for future requirements.

Although LPC has unquestionably been shown to have potential as a protein supplement, whether or not it will realize this potential will depend to a large extent on whether or not it can meet the criteria by which a food protein is judged--nutritional value, functionality as an ingredient, compatibility with other foods and food ingredients, keeping quality, sensory properties, and social acceptability (Kinsella, 1970). Considering the potential of leaf protein, especially in tropical areas, research efforts are still extremely small (Pirie, 1969a; Floyd, 1970). This contrasts to the much larger efforts being given to other potential

protein sources: oilseeds, etc. (Singh, 1967; Pirie, 1967a, 1969d; Buchanan, 1968). This disparity should be recognized when quality comparisons are made of different end products. Pleas have been made for international, cooperative research and training of scientists in the methods developed for leaf protein production (Pirie, 1967a; Kinsella, 1970). But research is still needed, particularly in crop husbandry, preservation, biological assessments of nutritive value, economics, and acceptability and presentation, as well as on many unexamined species of plants (Buchanan, 1968; Stahmann, 1968b; Singh, 1969; Kinsella, 1970). However, the nations most in need of the results of this research lack the resources to conduct it (Stahmann, 1968a).

EXPERIMENTAL

Chemicals and Materials

Chemicals

The principal chemicals used in this study and their sources are listed in the Appendix, Table A1. All were reagent grade unless otherwise stated. Water was distilled and deionized.

For the enzyme extractions the following enzyme preparations were specially procured: (1) Wallerstein Cellzyme (R) from Wallerstein Co., Morton Grove, Ill.; (2) cellulase, purified (15,000 units/mg), *T. viride*, from Seikagaku Fine Biochemicals, Seikagaku Kogyo Co., Tokoyo, Japan; (3) alpha-amylase (liquifying), 3x crystallized (643 units/mg), *B. subtilis* from Seikagaku Fine Biochemicals; (4) pectinase, purified (1.1 unit/mg), fungal origin from Sigma Chemical Co., St. Louis, Mo. The cellulase and alpha-amylase were purchased from Miles Laboratory, Elkhart, Indiana.

Alfalfa

There were three major sources of the fresh green alfalfa. The first was from a green house during winter and spring, 1970. The second and third were Fields A and B available during the summer season, 1970. All three areas were under the care of the Department of Crop and Soil Sciences at Michigan State University. This alfalfa included *Medicago glutinosa* and primarily *Medicago sativa*, varieties Vernal, Culver/Vernal, MSB, MSA, and Hardy moapa. All had been genetically selected to be low or medium low in saponin level.

"Greenhouse" alfalfa was used for initial investigations of: the standard extraction procedure, yields, and enzyme extractions (Modifications A, B, C, D). Field A provided the raw material for further yield studies and for extraction of the two fractions which were used for chemical analyses and biological assay. The first-cutting alfalfa from Field B was the source for further enzyme extractions (Modifications E, F, G). The fractions for organoleptic evaluation were obtained from the second cutting. The last cutting of this field furnished alfalfa for extractions by the method of Pirie and Modification I.

Preparative Procedures

Standard Extraction Method, Modifications A, B, C, D

The standard extraction method, Modifications A, B, C, and D, are illustrated in Figures 5, 6, 7, 8, and 9, respectively. The figure descriptions are intended to be a part of and to be read in sequence with the remainder of the text.

Figure 5. Flow diagram for standard extraction method.

Freshly cut alfalfa was blended with water in a Waring, commercial-sized blender, model CB-3 for 15 sec on low speed, 15 sec on medium speed, and 20 sec on high speed. Blending was most satisfactorily achieved with units of 200 g of alfalfa plus 800 ml of water. Two "units" of leaf pulp were poured into a press cylinder which was placed in a plastic pail with an adjustable outlet at the bottom to aid in the collection of the juice. Pressure was applied to the constrained pulp with a Carver laboratory press, model B.

The juice was heated with a magnetic stirrer-hot plate. The time required to induce chloroplast coagulation varied with the quantity of the juice, but usually was about 10-15 min for 3500 ml of juice. Centrifugation was principally performed in an International model V, size 2, with rotor #226, and occasionally performed in a Sorvall RC2-B refrigerated centrifuge equipped with a type GSA rotor. Adjustments in pH of supernatants 1 and 2 were monitored with either a Beckman Zeromatic pH meter or a Corning Model 12 pH meter.

The two final fractions of interest were precipitate 3 (P3 or the acid precipitate) and supernatant 3 (S3 or the acid supernatant). The latter fraction was condensed in a laboratory constructed vacuum condensing unit operated at a vapor temperature of about 23 C. The rate of evaporation was 0.7 to 1.0 liter/hr. The fraction was concentrated about 10:1 and dialyzed, with dialysis water changed about every 6 hr.

Three freeze-driers were used for lyophilization of the alfalfa fractions: (1) a laboratory constructed lyophilizer which was connected to a Cenco Hyvac 28 vacuum pump; (2) a Virtis Unitrap with combination bulk and manifold freeze-drying chamber connected to a DuoSeal vacuum pump, model 1397 by Welch Scientific Co.; (3) a Virtis RePP #FFD 42 WS freeze-dryer, with a capacity of 50 lb of water removal per drying run and equipped with instrumentation for control of freeze-drying variables.

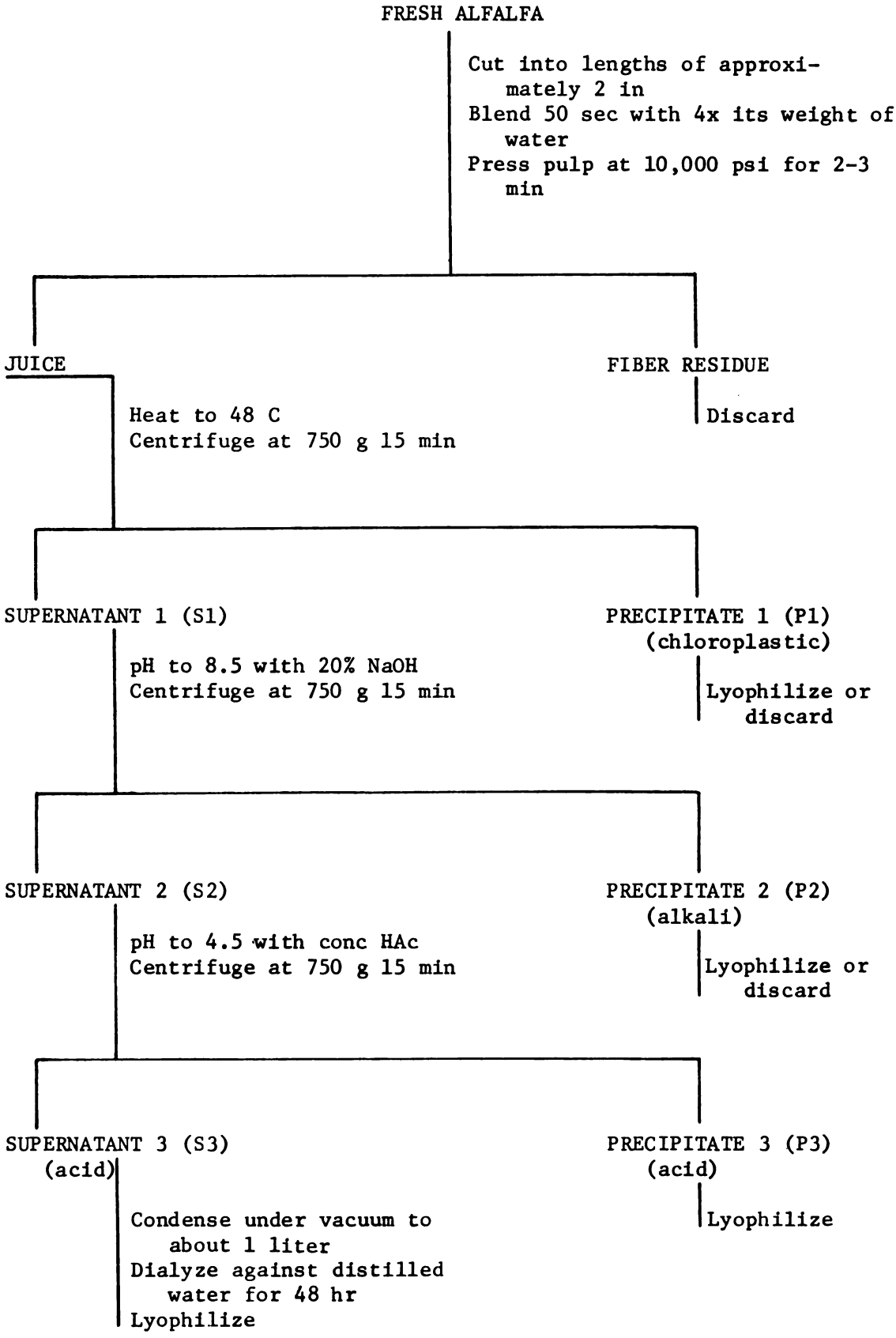
FRESH ALFALFA
Cut into lengths of approximately 2 in
Blend 50 sec with 4x its weight of water
Press pulp at 10,000 psi for 2-3 min
JUICE
FIBER RESIDUE
Discard
Heat to 48 C
Centrifuge at 750 g 15 min
SUPERNATANT 1 (S1)
PRECIPITATE 1 (P1) (chloroplastic)
pH to 8.5 with 20% NaOH
Centrifuge at 750 g 15 min
Lyophilize or discard
SUPERNATANT 2 (S2)
PRECIPITATE 2 (P2) (alkali)
pH to 4.5 with conc HAc
Centrifuge at 750 g 15 min
Lyophilize or discard
SUPERNATANT 3 (S3) (acid)
PRECIPITATE 3 (P3) (acid)
Condense under vacuum to about 1 liter
Dialyze against distilled water for 48 hr
Lyophilize
Lyophilize

Figure 5

Figure 6. Flow diagram for Modification A of the standard extraction method.

Fresh alfalfa was cut, blended, and pressed as in the standard extraction method. Fifty milliliters of unheated juice were allowed to remain at room temperature for nonprotein nitrogen (NPN) determinations after 0, 1, 3, and 8 hr. The bulk of the juice was heated as previously described. Fifty milliliters were held at room temperature for NPN determinations as above. Precipitates 1 and 2 were separated from the remaining juice by the standard extraction method. Supernatant 2 was split in half (S2 and S2'). Fraction S2 was acidified and centrifuged, yielding precipitate 3A and supernatant 3A (S3A). Fraction S3A was heated in a water bath and centrifuged, giving precipitate 3B and supernatant 3B (S3B). The precipitation process was then reversed with fraction S2', which was first heated. The resulting supernatant 3A' was acidified. The final supernatants (i.e., S3B and S3B') were passed over a mixed bed ion exchange resin prior to concentration and lyophilization. A yield study was conducted simultaneously (see Figure 10 for flow diagram and for total solids (TS) and nitrogen (N) sample sizes).

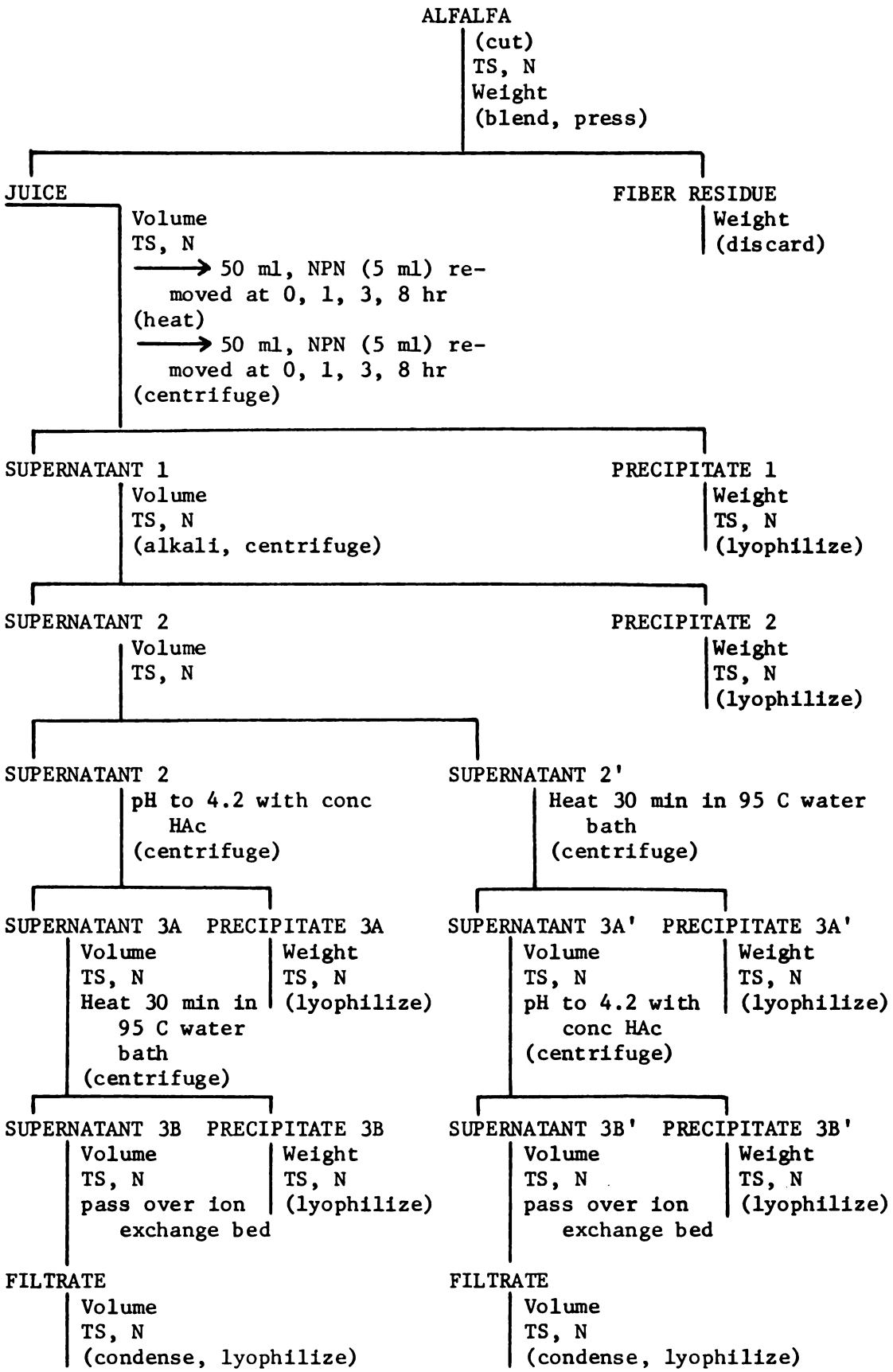
ALFALFA
(cut)
TS, N
Weight
(blend, press)
JUICE
Volume
TS, N
→ 50 ml, NPN (5 ml) removed at 0, 1, 3, 8 hr
(heat)
→ 50 ml, NPN (5 ml) removed at 0, 1, 3, 8 hr
(centrifuge)
FIBER RESIDUE
Weight
(discard)
SUPERNATANT 1
Volume
TS, N
(alkali, centrifuge)
PRECIPITATE 1
Weight
TS, N
(lyophilize)
SUPERNATANT 2
Volume
TS, N
PRECIPITATE 2
Weight
TS, N
(lyophilize)
SUPERNATANT 2
pH to 4.2 with conc HAc
(centrifuge)
SUPERNATANT 2'
Heat 30 min in 95 C water bath
(centrifuge)
SUPERNATANT 3A
Volume
TS, N
Heat 30 min in 95 C water bath
(centrifuge)
PRECIPITATE 3A
Weight
TS, N
(lyophilize)
SUPERNATANT 3A'
Volume
TS, N
pH to 4.2 with conc HAc
(centrifuge)
PRECIPITATE 3A'
Weight
TS, N
(lyophilize)
SUPERNATANT 3B
Volume
TS, N
pass over ion exchange bed
PRECIPITATE 3B
Weight
TS, N
(lyophilize)
SUPERNATANT 3B'
Volume
TS, N
pass over ion exchange bed
PRECIPITATE 3B'
Weight
TS, N
(lyophilize)
FILTRATE
Volume
TS, N
(condense, lyophilize)
FILTRATE
Volume
TS, N
(condense, lyophilize)

Figure 6

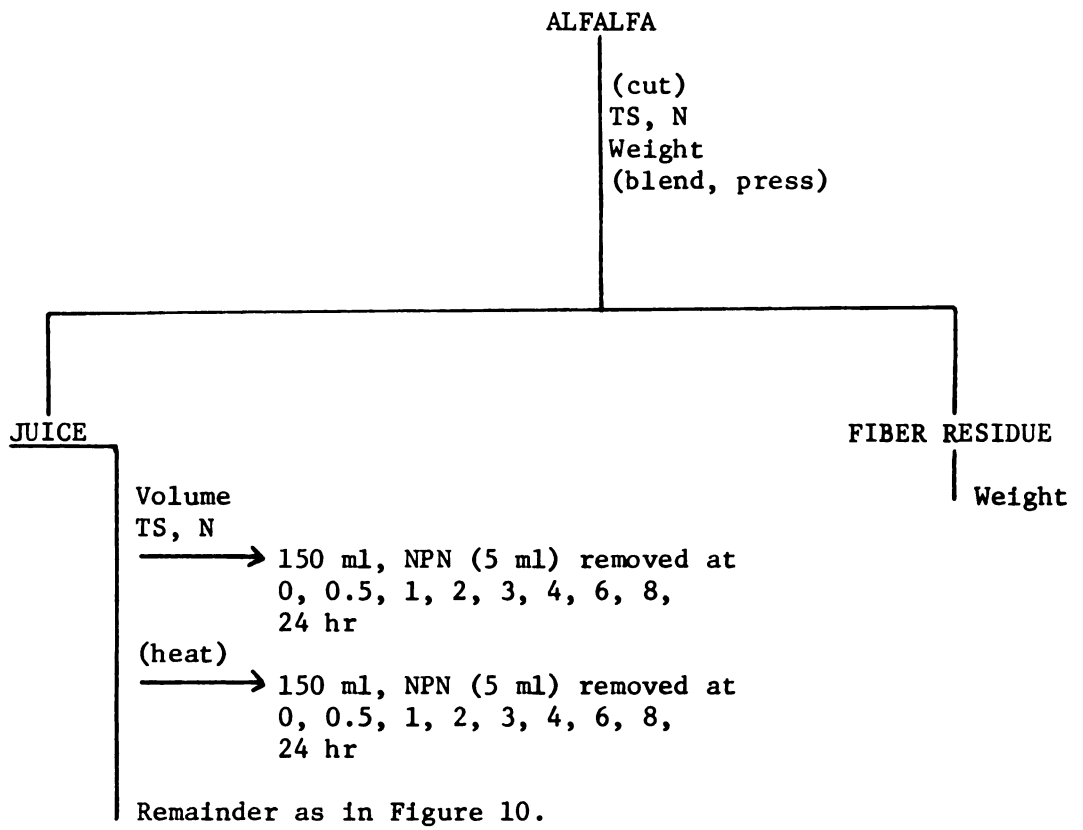

Figure 7. Flow diagram for Modification B of the standard extraction method.

A simultaneous yield study (see Figure 10) was conducted with this modification as with Modification A. Fresh cut alfalfa was blended and pressed by the standard extraction method. One hundred fifty milliliters of the unheated juice were set aside at room temperature from which samples were removed for NPN at the following times: 0, 0.5, 1, 2, 3, 4, 6, 8, and 24 hr. The remaining juice was heated as in the standard procedure and a 150 ml portion set aside for determinations of NPN at the same time intervals as the unheated juice. The remainder of the extraction was as in a standard yield study.

Figure 8. Flow diagram for Modification C of the standard extraction method.

Fresh cut alfalfa was sampled for nitrogen and TS and weighed into two 300 g units. To each unit was added 1200 ml water and 7.5 ml Cellzyme (R). An additional unit of 165 g alfalfa plus 660 ml water was processed without added enzyme and served as a control. Each was blended and pressed as in the standard extraction procedure. Juice from the two enzyme units was combined. Samples for TS and N analyses were taken from each experimental batch (i.e., enzyme and control). The enzyme-treated juice was divided into four approximately equal volumes which were held at room temperature for 0, 4, 6, or 8 hr. The control was split in halves which were held for 0 or 8 hr under the same conditions as the enzyme-treated juice. At the conclusion of the holding periods, each juice sample was heated and centrifuged as in the standard extraction method. Samples were taken from supernatant 1 for TS, N, and NPN determinations and from precipitate 1 for TS and N analyses. Then the standard extraction procedure was followed, yielding supernatant 3 and precipitate 3. Samples from these were taken for both TS and N, as well as a sample from the supernatant for NPN.

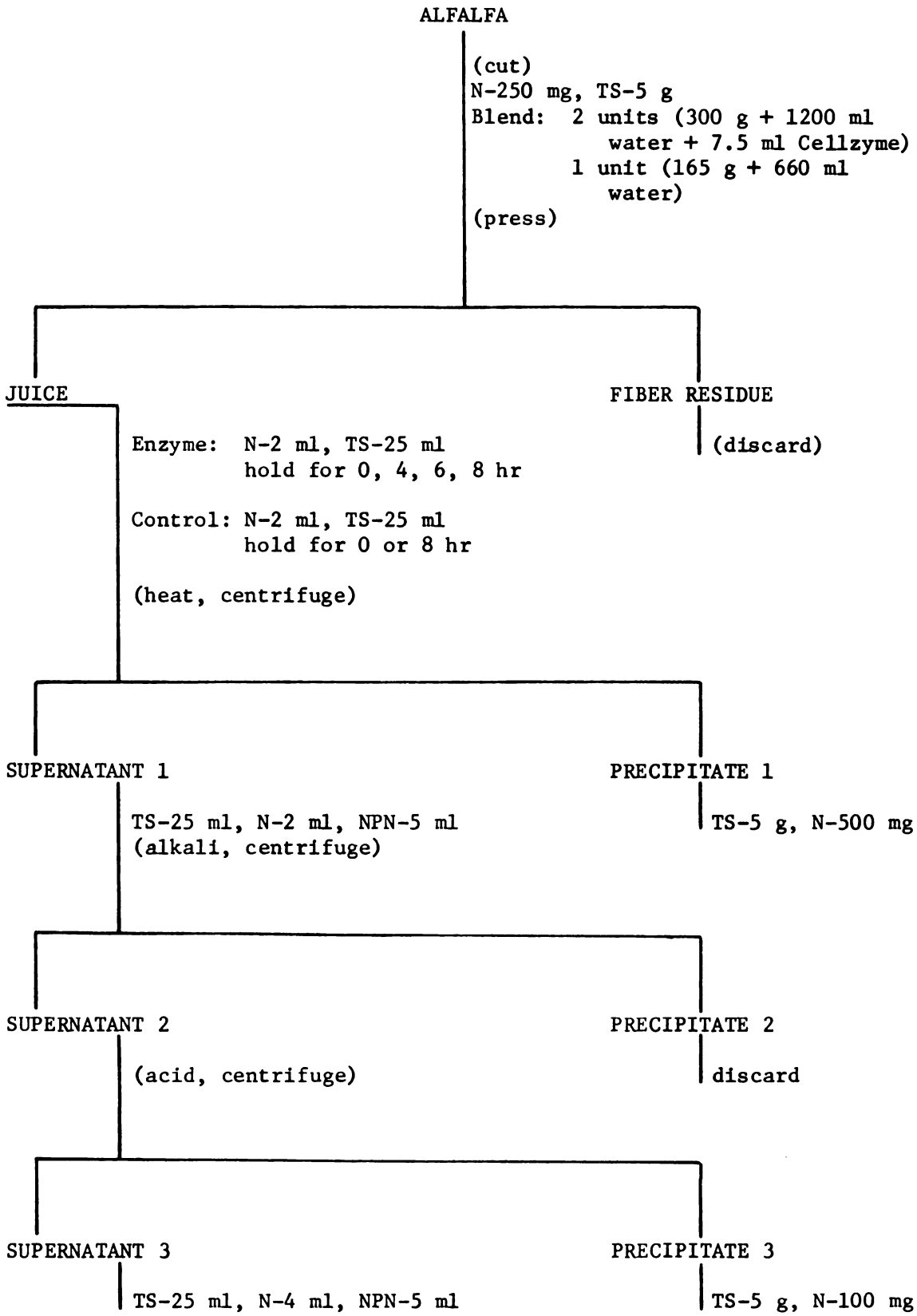
ALFALFA
(cut)
N-250 mg, TS-5 g
Blend: 2 units (300 g + 1200 ml water + 7.5 ml Cellzyme)
1 unit (165 g + 660 ml water)
(press)
JUICE
FIBER RESIDUE
(discard)
Enzyme: N-2 ml, TS-25 ml
hold for 0, 4, 6, 8 hr
Control: N-2 ml, TS-25 ml
hold for 0 or 8 hr
(heat, centrifuge)
SUPERNATANT 1
PRECIPITATE 1
TS-25 ml, N-2 ml, NPN-5 ml
(alkali, centrifuge)
TS-5 g, N-500 mg
SUPERNATANT 2
PRECIPITATE 2
(acid, centrifuge)
discard
SUPERNATANT 3
PRECIPITATE 3
TS-25 ml, N-4 ml, NPN-5 ml
TS-5 g, N-100 mg

Figure 8

Figure 9. Flow diagram for Modification D of the standard extraction method.

Fresh alfalfa, prepared for extraction and sampled for TS and N, was blended and pressed as in the standard extraction procedure. Samples from the juice were taken for TS and N analyses and six 450 ml volumes of juice were measured. Two of these served as controls and were held at room temperature for 0 or 8 hr. Two and one tenth milligrams of cellulase (0.465 mg/100 ml juice) were added to each of the remaining four portions which were held at room temperature for 0, 4, 6, or 8 hr. At the conclusion of each holding period, the juice was heated and centrifuged as for the standard extraction method. Samples for TS and N determinations were taken from supernatants 1 and precipitates 1 obtained from the juices held the times indicated in Figure 9. The standard extraction method was followed to produce supernatants 3 and precipitates 3. Samples for TS and N determinations were also removed from the latter two fractions obtained from juices held the times indicated.

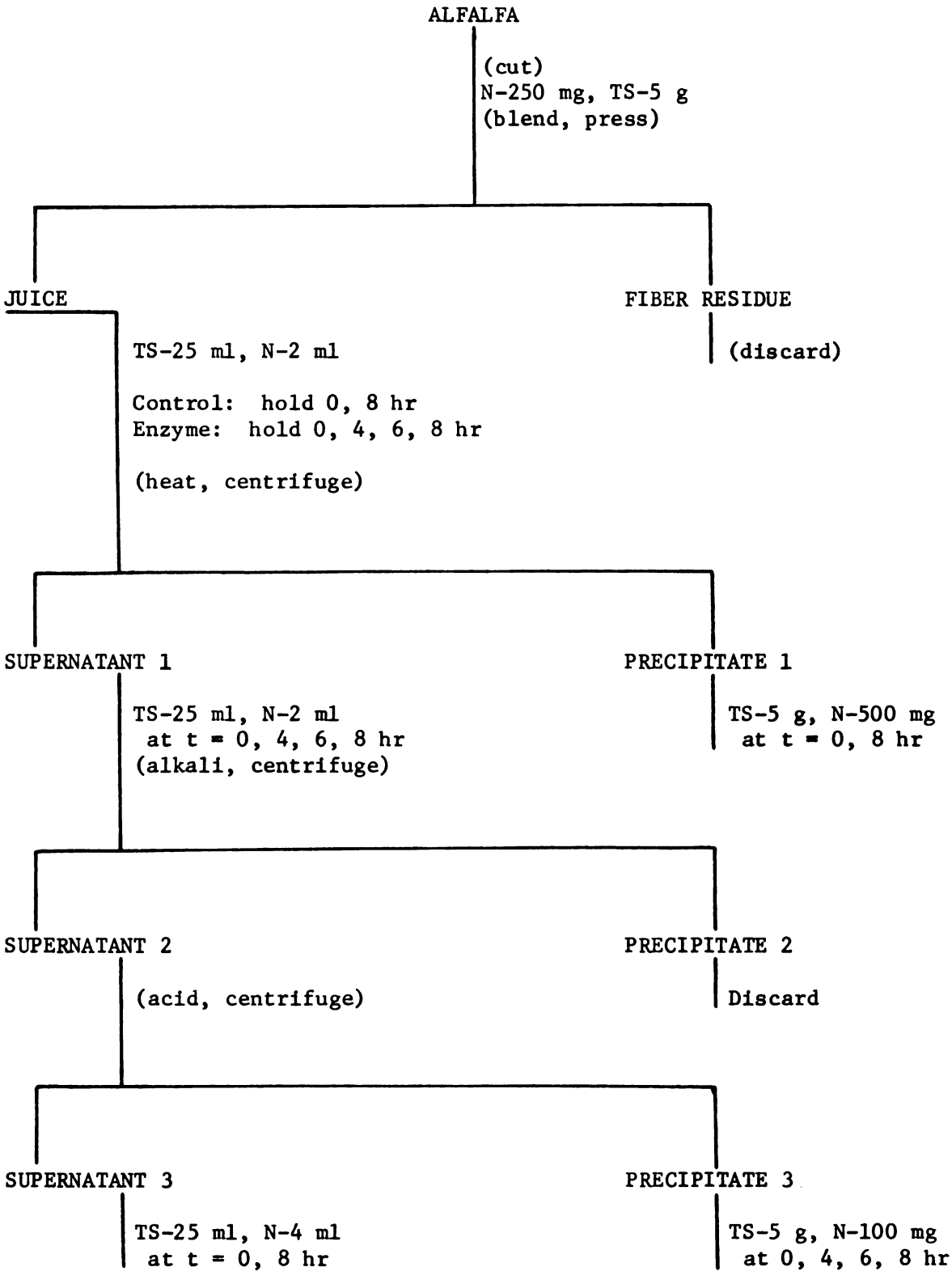
ALFALFA
(cut)
N-250 mg, TS-5 g
(blend, press)
JUICE
FIBER RESIDUE
TS-25 ml, N-2 ml
Control: hold 0, 8 hr
Enzyme: hold 0, 4, 6, 8 hr
(heat, centrifuge)
(discard)
SUPERNATANT 1
PRECIPITATE 1
TS-25 ml, N-2 ml
at t = 0, 4, 6, 8 hr
(alkali, centrifuge)
TS-5 g, N-500 mg
at t = 0, 8 hr
SUPERNATANT 2
PRECIPITATE 2
(acid, centrifuge)
Discard
SUPERNATANT 3
PRECIPITATE 3
TS-25 ml, N-4 ml
at t = 0, 8 hr
TS-5 g, N-100 mg
at 0, 4, 6, 8 hr

Figure 9

Modification E

Modification D was followed with one exception--1 mg cellulase was added per 100 ml juice.

Modification F

Modification D was followed with 10 mg alpha-amylase instead of cellulase added per 100 ml juice.

Modification G

Modification D was followed with 10 mg pectinase in place of cellulase added per 100 ml juice.

Modification H

Two freeze-dried samples (from the standard extraction procedure) of acid precipitate (0.2 g) and acid supernatant (0.1 g) were weighed. To one of each fraction was added 10 ml of isopropanol. Ten milliliters of acetone-haxane (1:1 v/v) were added to the other. The samples were mechanically shaken for one hour and then centrifuged 5 min in a clinical centrifuge. The supernatant was poured off and the precipitate dried under vacuum.

Modification I

Juice was obtained from fresh alfalfa by the standard extraction procedure. To a series of test tubes (8) containing 10 ml of fresh raw skim milk were added in duplicate either 0, 0.5, 1.0, or 2.0 ml of juice. One set was allowed to stand at room temperature and the other in a 37 C water bath, both for 6.5 hr.

The two samples which showed coagulation by the end of this time period (milk + 1 or 2 ml juice, incubated at 37 C) plus two controls--(1) raw skim milk and (2) alfalfa juice--were centrifuged at conditions

to yield a 60 S pellet (Beckman-Spinco L2-65 preparative ultracentrifuge with a No. 65 fixed angle rotor with cellulose acetate tubes, 202,000 g for 76 min). High voltage paper electrophoresis assays were made on the supernatants.

Yield Study and Pirie Extraction

The procedures followed for yield studies and the extraction by modification of the method of Morrison and Pirie (1961) are illustrated in Figures 10 and 11, respectively. Again the figure captions are to be read as text.

Figure 10. Flow diagram for Yield Study of standard extraction method.

The standard extraction method (or one of its modifications) was followed. Total weight of the fresh alfalfa and fiber residue and total volume of the juice were determined. Then 1500 ml of the juice were used as a starting volume for the remainder of the yield study. As illustrated in the flow diagram, each solid fraction was weighed and the volume of each liquid fraction measured. Representative samples were then removed for TS and N determinations. Sample sizes are given in this figure. The volumes of alkali and acid necessary for pH adjustment were noted.

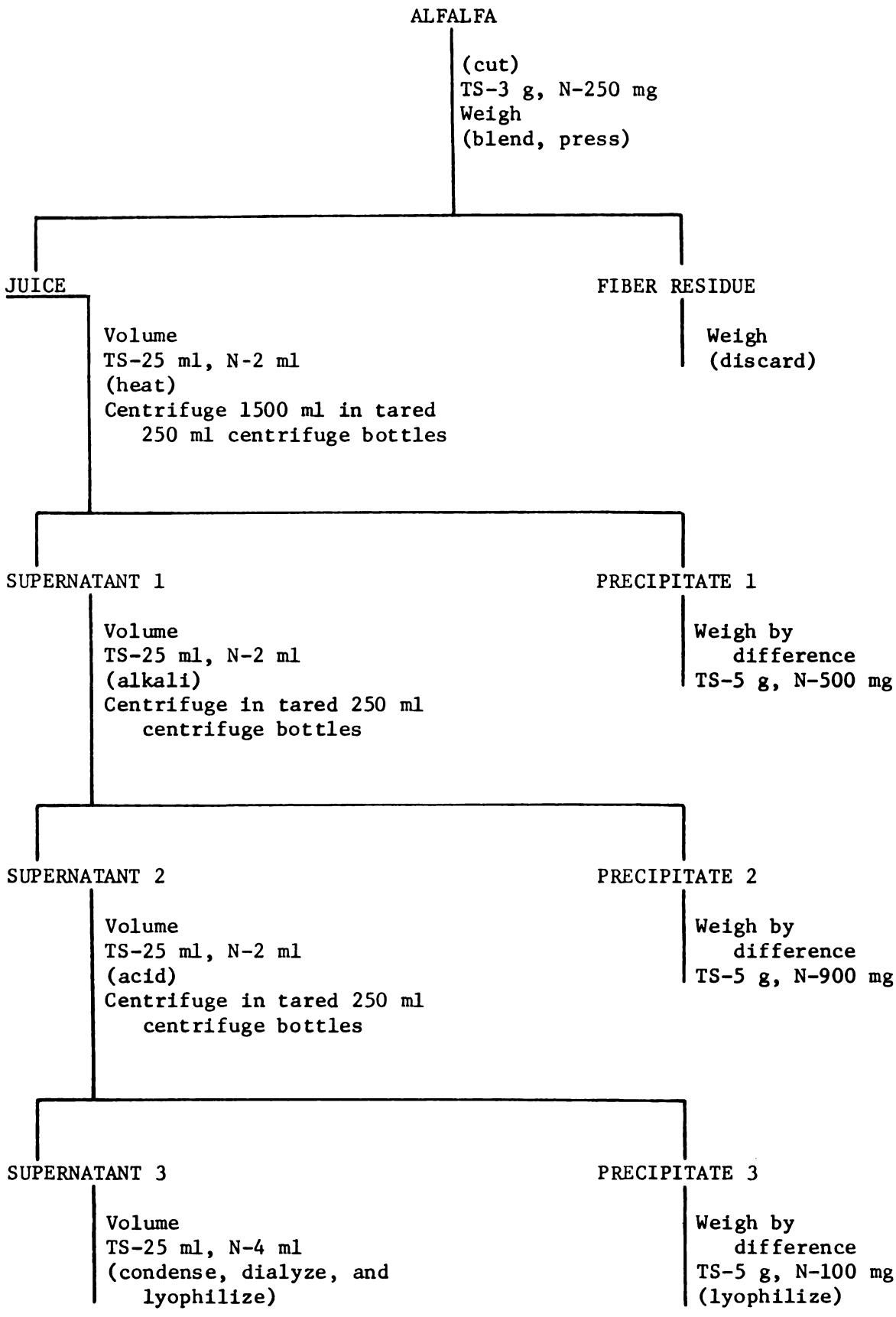
ALFALFA
(cut)
TS-3 g, N-250 mg
Weigh
(blend, press)
JUICE
FIBER RESIDUE
Weigh
(discard)
Volume
TS-25 ml, N-2 ml
(heat)
Centrifuge 1500 ml in tared
250 ml centrifuge bottles
SUPERNATANT 1
PRECIPITATE 1
Weigh by
difference
TS-5 g, N-500 mg
Volume
TS-25 ml, N-2 ml
(alkali)
Centrifuge in tared 250 ml
centrifuge bottles
SUPERNATANT 2
PRECIPITATE 2
Weigh by
difference
TS-5 g, N-900 mg
Volume
TS-25 ml, N-2 ml
(acid)
Centrifuge in tared 250 ml
centrifuge bottles
SUPERNATANT 3
PRECIPITATE 3
Weigh by
difference
TS-5 g, N-100 mg
(lyophilize)
Volume
TS-25 ml, N-4 ml
(condense, dialyze, and
lyophilize)

Figure 10

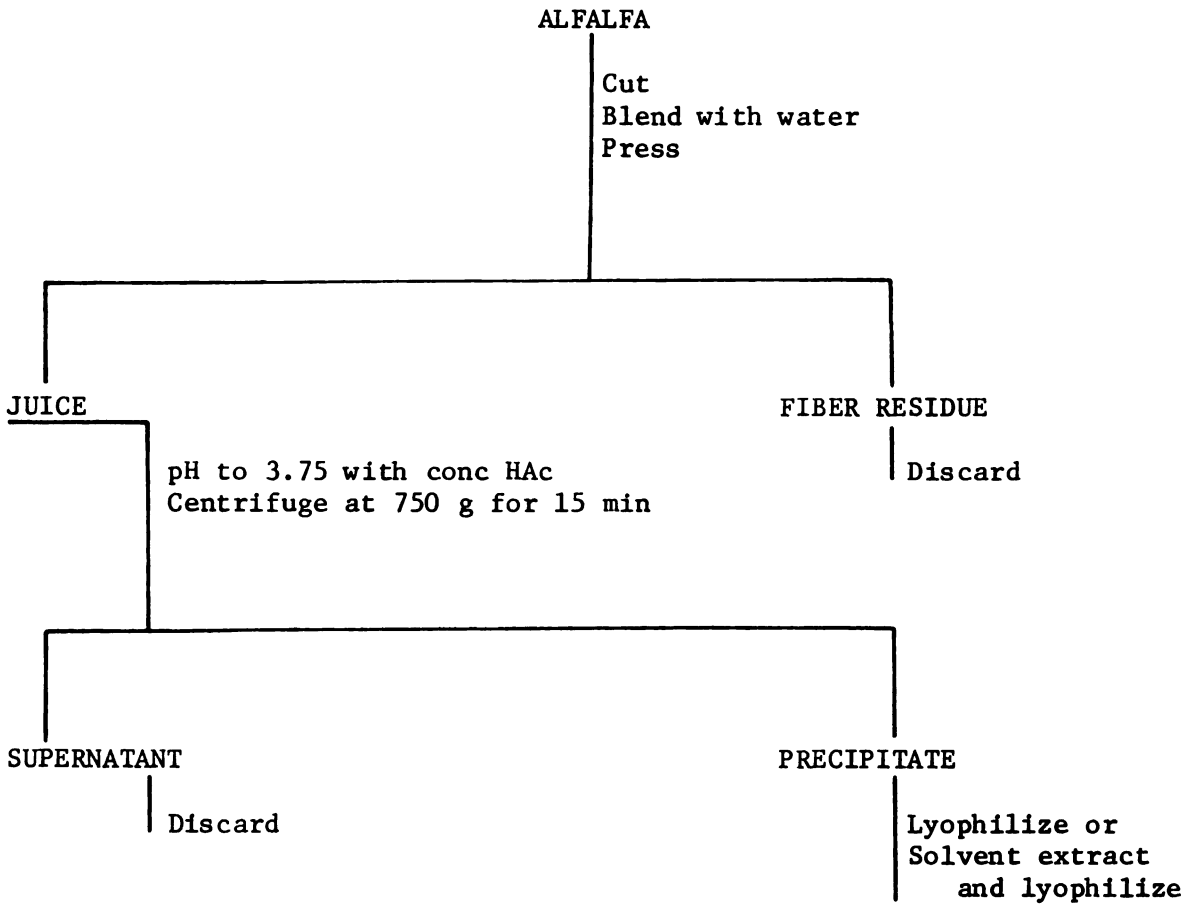

Figure 11. Extraction by modification of method of Morrison and Pirie (1961).

The method was essentially that described by Morrison and Pirie (1961). Fresh alfalfa was cut, blended with water, and pressed as in the standard extraction procedure. The juice was acidified, the suspension centrifuged, and the supernatant discarded. One third of the precipitate was lyophilized directly. The remaining portion was extracted with isopropanol or with ethanol followed by hexane prior to lyophilization. To one half (about 5 g) 100 ml of isopropanol were added. The alcohol-preparation mixture was mechanically shaken for 1 hr, centrifuged at 750 g for 20 min, and the supernatant discarded. This process was repeated three times. To the other half 100 ml of ethanol were added. The mixture was shaken, centrifuged, and the solvent decanted once as described for the isopropanol extraction. Then, a 100 ml portion of hexane was added to the precipitate which was again shaken 1 hr, centrifuged, and the supernatant decanted. Finally, the two solvent extracted precipitates were lyophilized.

Field Harvests

Three cuttings (June, July, October) were made of the 1970 Field A of individually planted low-saponin alfalfa (refer to materials section, p. 63, for detail). The first cutting consisted of six harvests (June 5, 6, 7, 9, 11, and 12) of 5 to 6 lb alfalfa each. The second cutting also included six harvests (July 17, 18, 19, 21, 22, and 24) of approximately the same size. Both cuttings were made at the one tenth bloom stage. Light frosts and the potential of a killing frost required the third cutting to be made at late bud-early flower stage (October 2 and 3).

The standard extraction method was employed for all collections. Precipitates 1 and 2 were discarded. At the end of each cutting the freeze-dried acid precipitates of all harvests were combined. Similarly, the freeze-dried acid supernatants of all harvests of a cutting were combined. Six fractions were thus obtained:

1P3 -- acid precipitate of the first cutting
1S3 -- acid supernatant of the first cutting
2P3 -- acid precipitate of the second cutting
2S3 -- acid supernatant of the second cutting
3P3 -- acid precipitate of the third cutting
3S3 -- acid supernatant of the third cutting

These were used for chemical analyses and biological assays. Concomitant yield studies were conducted with the first and fifth harvests of the first cutting, the second and fifth harvests of the second cutting, and the second harvest of the third cutting.

Eight harvests (about 5.5 to 6 lb alfalfa each) were made of the second cutting of Field B: August 27, 28, 29, 31, September 1, 3, 4, and 5. The one-tenth bloom stage again indicated the time of harvest. The standard extraction procedure was followed. As in the previous field harvests precipitates 1 and 2 were discarded. The acid precipitates from the eight harvests were combined; as were the acid supernatants.

The latter two fractions served as the sources of supplementary protein fractions for the organoleptic evaluation.

Chemical Methods

Nitrogen

Nitrogen was determined by the micro-Kjeldahl method. To each micro-Kjeldahl digestion flask were added about 15 mg of protein and 4 ml of digestion mixture. (The digestion mixture consisted of 5.0 g of $CuSO_4 \cdot 5\ H_2O$ and 5.0 g SeO_2 in 500 ml of concentrated sulfuric acid.) The digestion was carried out over a gas flame for 1 hr. After cooling, 1 ml of 30% hydrogen peroxide was added to each flask and the gas heated digestion was continued for another hour. Each flask was then cooled and rinsed with 10 ml water, after which the digestion flask was clamped directly on the micro-Kjeldahl distillation apparatus. The digested sample was then neutralized with 25 ml of 40% (w/w) sodium hydroxide solution. The released ammonia was steam distilled into 15 ml of 4% (w/v) boric acid containing 5 drops of mixed indicator (400 mg bromocresol green and 40 mg methyl red WS in 100 ml of 95% ethanol). The indicator is blue in alkaline media, green at the end point, and yellow in acid media. The distillation was continued until a final volume of 70 ml was collected in the receiving flask. The ammonium-borate was titrated with 0.02 N hydrochloric acid either manually or with a Fisher Automatic Titrimeter, Model 36. The hydrochloric acid was previously standardized with tris (hydroxymethyl) aminomethane using p-sulfa-o-methoxybenzeneazodimethyl-1-napthylamine. A reagent blank along with a tryptophan standard were analyzed to determine the average per cent recovery of nitrogen.

Nonprotein Nitrogen

Five ml of a 30% (w/v) trichloroacetic acid (TCA) solution were added to five ml of the sample. The mixture was shaken and allowed to stand at room temperature for 30 min. The diluted sample was centrifuged 5 min in an International clinical centrifuge, model CL. Five milliliter aliquots of the resulting supernatant were used for nitrogen determination by the previously described micro-Kjeldahl method.

Amino Acids

Amino acid analyses were performed on 22 and 70 hr acid hydrolysates of fractions from the first cutting, but on only 22 hr acid hydrolysates of fractions from the second and third cuttings. The amino acids were separated by column chromatography and quantitatively determined by automatically recording the intensity of the color produced by their reaction with ninhydrin (Moore and Stein, 1954, 1963; Moore, Spackman, and Stein, 1958; Spackman, Stein, and Moore, 1958) with a Beckman 120C Amino Acid Analyzer.

Samples containing 15 mg of protein were placed into 10 ml glass ampules. To each ampule 5 ml of once-distilled 6 N hydrochloric acid were added. Prior to deoxygenation the ampules were placed in a Sonogen ultrasonic cleaner by Branson Ultrasonic Corp. for 1-2 hr to break up the insoluble sample residues. Then the ampule contents were frozen in a dry ice-ethanol bath. Using a high vacuum pump, the ampule was carefully evacuated and warmed until all dissolved gases were removed from the liquid sample-acid mixture. It was necessary to add some antifoam to the neck of the ampule to prevent excessive foaming during this step. The ampule contents were then refrozen and the ampule sealed over an air-propane flame. The sealed ampules were placed in an oil bath

set in a 110 ± 0.1 C hydrolysis oven--Stabil-Therm Gravity Oven constructed by the Blue M Electric Co.--for 22 or 70 hr. The cooled ampule tops were broken open and 1 ml of norleucine standard (2.5 μmole/ml) was added to each as an internal standard to measure transfer losses. The hydrochloric acid was removed from the hydrolysate in a pear shaped 25 ml flask, connected to a Rinco rotary evaporator under vacuum, and partly immersed in a 50-55 C water bath. Antifoam in the stem of the connector prevented the sample from foaming out of the flask. The residue was washed with about 10 ml water and reevaporated. This was done three to four times in succession until hydrochloric acid could no longer be detected. The acid free hydrolysate residue of each sample was dissolved with 3-4 ml of diluter buffer (0.067 M sodium citrate-HCl, pH 2.2). Each sample solution was quantitatively transferred to a 5 ml volumetric flask and diluted to volume with buffer. To rid the samples of interfering humin they were centrifuged 10-15 min in an International clinical centrifuge. One tenth milliliter aliquots of the supernatant were applied to the analyzer columns. The resulting chromatograms were compared to those of standard amino acid calibration mixtures. The ratio of areas under the curve of each amino acid for the samples and the standard were compared and converted to give the amino acid composition of the sample. For those samples hydrolyzed both 22 and 70 hr, corrections for losses of threonine, serine, and tyrosine during acid hydrolysis were made using the equation given by Hirs, Stein, and Moore (1954). The other amino acids were determined as the simple average of the 22 and 70 hr results. All calculations were done by a computer program written in this laboratory for use in the Michigan State University computer laboratory.

Methionine and Cystine

Methionine and cystine undergo variable destruction during acid hydrolysis. Therefore, they were oxidized to methionine sulfone and cysteic acid by the procedure of Lewis (1966) using the performic acid reagent of Schram, Moore, and Bigwood (1954). Approximately 60-65 mg samples of protein were weighed into small sample bottles. These were placed in an ice bath and cooled to 0 C. Ten milliliters of 30% hydrogen peroxide and 90 ml of 88% formic acid were mixed and allowed to stand at room temperature for 1 hr. The performic acid was then cooled to 0 C in the ice bath. Ten milliliters of performic acid were then added to each sample. The sample bottles were kept in an insulated crushed ice bath inside a 4 C cold room for 17 hr. Twenty milliliters of ice cold water were added to each sample.

An unsuccessful attempt was made to freeze-dry the samples with a trap set up to prevent the performic acid from entering the lyophilizer. Each was then diluted with an additional 180 ml of water. The samples were split so that there were only 50 ml of the oxidized protein suspension per 250 ml bottle. Thus, the samples could be frozen in a very thin shell. However, the samples still did not remain frozen after being placed under vacuum.

As a workable procedure, samples were placed immediately on the lyophilizer without the intermediate trap, realizing that in the future a trap would have to be built between the lyophilizer and the high vacuum pump. Frequent oil changes and a cleaning of the pump after operation were necessary. The freeze-dried protein samples were then analyzed by the procedure described for amino acid analyses. Two tenths milliliter of the final supernatants was applied to the analyzer column. Standards

containing cysteic acid, methionine sulfone, and methionine sulfoxide were analyzed. Normalized values--corrected for sample weight, norleucine recovery, volume on the column, and incomplete oxidation--for cysteic acid and methionine sulfone of the oxidized samples were substituted for cystine and methionine, respectively, of the original unoxidized samples.

Tryptophan

This amino acid is destroyed by acid hydrolysis. Procedure W of Spies (1967) was used for its chemical determination separately. One to five milligram samples were weighed into 2 ml glass vials with screw caps. To each 0.1 ml of pronase solution was added. (The pronase solution was made by adding 10 ml of 0.1 M sodium phosphate buffer, pH 7.5, to 100 mg pronase. The suspension was shaken gently for 15 min and clarified by centrifugation for 5 min in an International clinical centrifuge. The pronase solution was prepared fresh every day that it was used.) The closed vials were incubated 24 hr in a 40 $\pm$ 1 C water bath. They were then quickly cooled to room temperature in a crushed ice bath. To each vial 0.9 ml of 0.1 M sodium phosphate buffer, pH 7.5, was added. In 50 ml Erlenmeyer flasks 30 mg of p-dimethylaminobenzaldehyde and 9.0 ml of 21.2 N sulfuric acid were mixed. The open vials were set in the Erlenmeyer flasks, tipped over, and mixed quickly by gentle swirling. The flasks were stoppered and placed in the dark at 25 C for 6 hr. Then, 0.1 ml 0.045% (w/v) sodium nitrite was added to each flask. After 30 min, the per cent transmittance through 1 cm silica cuvettes was determined at 590 nm on a Beckman DK-2A ratio recording spectrophotometer. Simultaneously, duplicate samples of the pronase solution, without protein, were treated and analyzed as above.

The tryptophan content of the pronase solution was subtracted from the total tryptophan value of the protein sample.

A standard curve covering the range of 0-200 μg of tryptophan was made according to Procedure E of Spies and Chambers (1948). Four milligrams of tryptophan were weighed into a 200 ml volumetric flask which was brought to volume with 19 N sulfuric acid containing 3 mg p-dimethylaminobenzaldehyde per ml. Zero, 1, 2, 4, 6, 8, and 10 ml aliquots were added to 25 ml Erlenmeyer flasks. The total volume was brought to 10 ml with the same 19 N sulfuric acid. The stoppered flasks were placed in the dark at 25 C for 6 hr. Then 0.1 ml 0.045% (w/v) sodium nitrite was added and the per cent transmittance at 590 nm read after 30 min.

Carbohydrate

Carbohydrate was determined by the method of Dubois *et al.* (1956). One half to two milligrams of the acid precipitate or 0.07 to 0.45 mg (3.5 mg/25 ml) of the acid supernatant were added to a 2.5 cm x 19.5 cm test tube with 3 ml water. Three milliliters of 5% (w/v) phenol (in water) solution were added and mixed with the sample by hand shaking. Fifteen milliliters of concentrated sulfuric acid were added as the tube was rotated by hand to give maximum mixing. The tubes were allowed to stand for 10 min at room temperature; then, after mixing again, the tubes were placed in a 30 $\pm$ 1 C water bath for 30 min. Per cent transmittance through silica cuvettes was read at 490 nm with a Beckman DK-2A spectrophotometer.

A standard curve was made over the range of 0.0 to 0.2 mg carbohydrate using a standard solution of 0.1 mg galactose-mannose (1:1 w/w) per ml.

Lipid

Total lipid was determined by modification of the procedure of Folch, Lees, and Sloane-Stanley (1957). One half to one gram samples were added with 100 ml of chloroform-methanol (2:1 v/v) into 400 ml beakers and stirred for 30 min. One hundred milliliters of 2% (w/v) potassium chloride solution were added and stirring continued for 15 min. The mixtures were then centrifuged--International, model V, size 2, with rotor #226--for 20 min at 1060 g. The lower lipid layers were removed with a syringe. Anhydrous sodium sulfate was added to remove any remaining residual moisture. The lipid extracts were filtered through Whatman No. 42 filter paper. Twenty-five or 50 ml aliquots were evaporated, first over steam and then in a 110 C hot air oven, in previously tared aluminum dishes. Control valves were determined for the solvent system.

Ash

Porcelain crucibles were heated in a "Hevi Duty" muffle furnace to 525 C, cooled, and weighed. One half to 1 g samples were placed in the tared crucibles and heated at 525 C until a white ash formed (about 48 hr). The crucibles were cooled and weighed. Then the samples were re-ashed at 525 C (about 48 hr) and the crucibles again cooled and weighed.

Moisture

Total solids of fractions obtained during extractions were determined. Representative samples were weighed into tared aluminum dishes, which were dried to a constant weight (usually 4-24 hr) in a 75 C Blue Line air oven manufactured by Blue M Electric Co.

Moisture of the freeze-dried fractions was determined by a different method. Two tenths to 0.4 g samples were weighed into small tared aluminum dishes. They were dried to constant weight over barium oxide in a desiccator under vacuum (one week).

Total solids of the vole diets were determined by drying the samples to constant weight in a 110 C air oven (4-5 days). In all three cases the per cent loss in weight was reported as moisture.

Saponin

Analyses of the vole diets for saponin was a modification of the method of Jones (1969). A two per cent human red blood cell solution was obtained from the local Red Cross. Two milliliters were added to a series of test tubes. About 100 mg of sample were added to each tube. The tubes were shaken and allowed to stand. One hundred per cent hemolysis (indicated by a bright red color) within 30 min implied a very high level of saponin; and after 30 min a high level of saponin. If after 2 hr no hemolysis had occurred, the red blood cells settled to the bottom, indicating low level of saponin. Intermediate levels were qualitatively identified by degree of hemolysis as indicated by color; i.e., cherry red equaled medium high levels and pink medium low levels. The saponin analyses were performed by Miss Vicki Marcarian of the Department of Crop and Soil Sciences.

Physical Methods

High Voltage Paper Electrophoresis

To evaluate the peptide content of the preparations, samples were spotted in 50 µl concentrations (five 10 µl applications) on Whatman 3M paper. The paper was wetted in buffer of pyridine-acetic acid-water

(1:10:189; v/v), pH 3.5. It was positioned in a laboratory made electrophoretic apparatus of Plexiglass over a horizontal supporting bar with the ends in the electrode (Pt) compartments (which were filled with 500 ml of buffer). The buffer and hanging paper were overlaid with Varsol, which served as a cooling substance. The apparatus was connected to a Savant Instruments, Inc., high voltage source. The determination was made at 2000 V for 1 hr giving a current density of ca 5-6 ma/cm of paper width. The paper was then removed and dried in an air oven at 90 C. Next, it was sprayed with ninhydrin reagent (0.35 g ninhydrin, 14 ml collidine, 135 ml acetic acid, 350 ml ethanol). Color was developed by replacing the paper in the air oven for 15 min. The peptides were identified by the purple color produced by their reaction with ninhydrin.

Biological Methods

Protein Efficiency Ratio

Composition of diets. The dry ingredients were weighed in the following proportions:

protein source	to give 7% protein
vitamin diet fortification mixture	2%
salt mix W	3%
alpha-cell, hydrolyzed	20%
carbohydrate	to bring total to 100%

The composition of the vitamin and mineral supplements is given in Tables 11 and 12, respectively. The carbohydrate mixture was made of 2 parts corn starch, 1 part sucrose, and 1 part dextrin. Vitamin free casein was used as the protein source for the control diet.

The original design was to make sufficient diet for an intake of 6 g/vole/day for 6 days per replication, for six replications. However,

Table 11. Composition of vitamin diet fortification mixture

Vitamin*	Amount (g)	Amount (mg)
Vitamin A concentrate (200 units/g)	4.5	
Vitamin D concentrate (400 units/g)	0.25	
Alpha-tocopherol	5.0	
Ascorbic acid	45.0	
Inositol	5.0	
Choline chloride	75.0	
Menadione (K)	2.25	
p-aminobenzoic acid	5.0	
Niacin	4.5	
Riboflavin	1.0	
Pyridoxine hydrochloride	1.0	
Thiamine	1.0	
Calcium pantothenate	3.0	
Biotin		20.0
Folic acid		90.0
Vitamin B_{12}		1.35

*Triturated in dextrose

Table 12. Composition of salt mixture W

Mineral salt	Percentage
Calcium carbonate	21.000
Copper sulfate ($\cdot 5H_2O$)	0.039
Ferric phosphate	1.470
Manganese sulfate (anhydrous)	0.020
Magnesium sulfate (anhydrous)	9.000
Potassium aluminum sulfate	0.009
Potassium chloride	12.000
Potassium dihydrogen phosphate	31.000
Potassium iodide	0.005
Sodium chloride	10.500
Sodium floride	0.057
Tricalcium phosphate	14.900

this was not possible due to inadequate amounts of certain protein fractions and actual formulations are given in Table 13.

Table 13. Formulations of control and experimental diets for weanling meadow voles

Ingredient	Diet						
	Casein	1P3*	1S3	2P3	2S3	3P3	3S3
Protein source	17.5	22.6	23.8	27.5	85.0	23.6	65.6
Vitamin mix	5.0	5.0	2.0	5.0	5.0	4.5	3.5
Salt mix	7.5	7.5	3.0	7.5	7.5	6.5	5.5
Alpha-cell	50.0	50.0	21.0	50.0	50.0	43.0	36.0
Carbohydrate	170.0	165.0	54.0	160.0	102.5	138.5	69.5
Total grams	250.0	250.1	103.8	250.0	250.0	216.0	180.1
Protein, %	7.00	7.00	7.02	7.01	7.00	7.00	6.99

*See methods section, p 79, for explanation of nomenclature

The dry ingredients were mixed with small amounts of water in a Hobart mixer, model N-50, to give a stiff dough. The dough was rolled out to a 1/4 in thickness and cut into rectangular 2 in by 6 in wafers which fit into the vole feeder (designed by Shenk and Elliott, 1969). Each such wafer was adequate for one animal for six days (one replication). The wafers were dried in an air cabinet dryer (100-110 F) for 24 hours. They were then allowed to equilibrate for 24 hr to the relative humidity of the animal room. The diets were then kept frozen until 8-12 hr before a feeding trial was begun. At this time the wafers were allowed to thaw at room temperature. One gram of corn oil was spread over and absorbed by each wafer during this time. (Preferably the corn oil should have been added at a level of 2% in the initial stage of mixing the diets.)

Test animals. Entire litters of 6-7 weanling meadow voles (*Microtus pennsylvanicus*) were used as the test animal in this study. The vole colony is maintained by Dr. F. C. Elliott of the Department of Crop and Soil Sciences at Michigan State University in a room with a fairly constant temperature of 50 F and 24 hr light. Shortly prior to weaning and afterwards, but prior to feeding the experimental diets, the voles were fed an intermediate diet of the following composition:

Milk (dried)	18%
Carbohydrate	35%
Alpha-cell	18%
Butter or corn oil	5%
Minerals	3%
Vitamins	2%
Honey	19%

Experimental procedure. The voles were put on the experimental diets two to four days after weaning (15-17 days of age). Whenever possible, only those voles which had attained a weight of 11 g were used. The voles were randomly assigned to individual disposable plastic laboratory cages with a crushed corn cob bed and some cotton. Each animal in a litter was fed a different experimental diet to reduce variance due to litter. The first two replicates (litters) included diets based on the acid precipitates of all three cuttings, the acid supernatants of the last two cuttings, and casein. The last five replicates included the above plus the diet based on the acid supernatant of the first cutting. The experimental diets and water were fed *ad libitum* for six days, with the exception of the seventh litter which was given the diets for only five days. Weight gain and feed intake were measured. With the last replicate the voles were given the experimental diets for 24 hr before the initial vole and feeder weights were made. Total solids were determined on a sample of each diet so that intake on a dry basis could be calculated. Protein efficiency ratios were calculated as the weight

gain of the vole divided by his intake of protein (on a dry basis).

Post-experimental Autopsies

The voles from the last two replications were sacrificed for autopsy. The first time the voles were examined for gross effects on the organs--size, color, general appearance. The second time histopathological examinations of the intestinal tract, liver, kidney, and brain were performed by the Veterinary Diagnostic Laboratories, Department of Pathology, Michigan State University.

Organoleptic Evaluation

Cookie Formulation

Twenty sugar cookies of each of the following formulations were prepared (see Table 14). The experimental fractions (previously stored one month) were substituted for flour in the control recipe.

Table 14. Formulations of the control and experimental cookies for organoleptic evaluation

Ingredient*	Control	Protein supplement				
		Acid precipitate			Acid supernatant	
Oleo	56.7 (1/4c)					
Sugar	99.1 (1/2c)					
Egg	27.4 (1/2)					
Sour cream	63.9 (1/4c)					
Baking soda	1.3 (1/4t)					
Baking powder	1.2 (1/4t)					
Salt	2.0 (1/4t)					
Nutmeg	0.33(1/8t)					
Vanilla	0.88(1/4t)					
Flour	148.0 (1 c)	142.15	133.4	118.8	125.5	56.2
Protein fraction	---	5.85	14.6	29.2	22.5	91.8
Alfalfa protein, %	0.0	1.0	2.5	5.0	1.0	2.5
Alfalfa fraction, %	0.0	1.5	3.7	7.3	5.6	23.0
Flour substituted, %	0.0	4.0	9.9	19.7	15.2	62.0

*amounts given in g

The cookie dough was prepared as follows: First the oleo and sugar were creamed. The egg was added and the mixture beaten until fluffy. Separately, the protein fraction, flour, baking powder, salt and nutmeg were mixed together. The baking soda was added to the sour cream. Alternately the dry and liquid ingredients were added to the creamed mixture. Lastly, the vanilla was added. The dough was refrigerated overnight. Then it was rolled to 1/4 in thickness and 2-1/2 in diameter cookies cut and lightly sprinkled with sugar. The cookies were baked at 375 F for 12-1/2 min.

Taste Panel

Untrained panelists were asked to independently rate color, flavor, texture, and general acceptability of the cookies. A closed hedonic scale was used. It was assigned score points from 1-9 with 1 representing dislike extremely and 9 like extremely. Figure 12 is a copy of the judging form given each panelist. Each cookie type was assigned a two-digit random number. For the first panel each of 20 panelists was given a plate with a control cookie and one each of the cookies supplemented with the three levels of acid precipitate. After rating these, each was given a second plate with a control cookie and one each of the cookies supplemented with two levels of the acid supernatant. A repeat panel of the acid precipitate supplemented cookies was conducted three days later with a second group of 20 panelists.

Statistical Analysis of Data

Mean values were calculated for each variable evaluated. Only results from the acid precipitate supplemented cookies and color values of the acid supernatant supplemented cookies were analyzed by analysis of variance (Amerine, Pangborn, and Roessler, 1965). Data showing

Name: _____ Date: _____

Plate No: _____

INSTRUCTIONS: Evaluate color, flavor, texture, and general acceptability of each sample according to the appropriate hedonic rating scale. Record results in the table below.

Color, flavor, texture and general acceptability	Additional comments about any or all samples
9 like extremely	
8 like very much	
7 like moderately	
6 like slightly	
5 neither like nor dislike	
4 dislike slightly	
3 dislike moderately	
2 dislike very much	
1 dislike extremely	

RESULTS:

Sample	color score	flavor score	texture score	general acceptability score

Figure 12. Judging form for the organoleptic evaluation.

significant sample differences were further subjected to Duncan's multiple range test (LeClerg, 1957).

RESULTS AND DISCUSSION

Extraction

Method

The extraction procedure was intentionally kept relatively simple so that it might be extended to a large scale commercial operation. The alfalfa was chopped to facilitate weighing and to create less of a strain on the blender. Also, water was added to accommodate the design of the blender. Singh (1967) noted that pre-chopping and extra water added during extraction improved the efficiency and increased yields. Equipment specifically designed for leaf pulping (such as that described by Davys and Pirie, 1960) does not require the addition of water. Buffers were not used as they would be inconvenient for a commercial operation. The blending time was kept short, for if extended to even 1.5 or 2 min the pulp becomes too fine and the fiber passes through the press slits into the juice.

Slow warming of the dark green juice (about 5 C/3 min) caused coagulation and precipitation of the chloroplasts and thus removed most of the green color. If the juice was heated rapidly in a steam-injected hot water bath this effect did not occur. This initial removal of the chlorophyll differentiates this procedure from the classical ones of Morrison and Pirie (1961) and Chayen *et al.* (1961). The juice was heated to temperatures below 50 C; for above 60 C there would be almost complete precipitation of the protein, resulting in a small yield of acid

precipitate at the final step. Continuous centrifugal filtration through Whatman No 2 filter paper was attempted but proved unsatisfactory for all centrifugation steps. Fine particles persistently passed through the filter paper and the larger particles rapidly plugged the pores.

Supernatant 1 was clear and golden in appearance. The pH was adjusted to 8.5 (from about 5.7), because this has been claimed to increase yields (cf. Morrison and Pirie, 1961; Pirie, 1966e). The last traces of green color were concentrated in the resulting precipitate.

Precipitation by acid was used initially because Morrison and Pirie (1961) recommended acid as the most convenient method for laboratory-scale processing. In Modification A (p. 68) both heat- and acid-induced precipitation were used. Characteristics of the fractions produced are given in Table 15.

Table 15. Characteristics of fractions prepared by Modification A

Description of fractions	Nitrogen (%)*	Percentage of original N	Crude protein (%)**
Precipitate 3A (acid)	15.0	12.1	93.8
Supernatant 3A	4.4	18.8	27.2
Precipitate 3B (heat)	8.0	0.5	49.9
Supernatant 3B	4.3	18.3	27.1
Precipitate 3A' (heat)	13.0	9.1	81.1
Supernatant 3A'	4.7	19.2	29.1
Precipitate 3B' (acid)	5.0	0.1	31.1
Supernatant 3B'	1.1	19.9	25.9

*on a dry basis

**per cent N x 6.25

Precipitate 3A, obtained by acid precipitation, contained 15% nitrogen. This constituted 12% of the original nitrogen. Further heat-induced precipitation resulted in another fraction containing 8% nitrogen--only 0.5% of the original nitrogen. When the procedure of heat-induced precipitation was employed as a first step, a fraction containing 13% nitrogen was obtained which accounted for only 9% of the original nitrogen. The addition of acid to the clarified supernatant produced only a small amount of precipitate (5% nitrogen), accounting for but 0.1% of the original nitrogen. Most of the protein thus appeared to be labile to both heat and acid. This was expected as acid and/or heat have been used by previous investigators to induce precipitation of the protein. However, the acid method gave a more complete precipitation and a fraction of higher protein content. This is in agreement with the observation of Pirie (1952) that acid-induced precipitation resulted in a higher yield and purer protein isolate, and of Singh (1960) that more nitrogen was precipitated by TCA than by heat; but in contrast to the latter's more recent reports (Singh, 1967, 1969). Morrison and Pirie (1961) reported that acid-induced precipitation denatures the protein less than heat-induced precipitation. Therefore, the procedure of acid-induced precipitation was adopted.

Characteristics of Fractions

Solubility. Unfortunately, the acid precipitate will not resolubilize upon neutralization in mildly alkaline solutions. This insolubility has been characteristic of leaf protein preparations (Pirie, 1957a, 1961a; Morrison and Pirie, 1961) and may be due to complex formation with tannins. According to Jennings *et al.* (1968), Van Buren and Robinson (1969), and Loomis and Battaile (1969) tannins and other aromatic

compounds interact with proteins to form both soluble and insoluble complexes by reactions often involving polymerization of the phenols and covalent bond linkages to the protein.

The nitrogen-containing components of the uncondensed supernatant fraction were not precipitated by TCA which, classically, would classify this fraction as non-protein nitrogen--amino acids, small peptides or other low molecular weight nitrogen containing compounds. Yet, there was no loss of nitrogen during dialysis. A small portion was accounted for by glucosamines and/or galactosamines (from the amino acid chromatograms), which possibly occur as moities of glyco-peptides or -proteins. The remainder was hypothesized to be peptides of molecular weight greater than 4000-6000, glycoproteins which are soluble in TCA or large NPN compounds (e.g., alkaloids).

Concentration of the supernatant fraction resulted in the formation of a heavy flocculant which was readily dispersed by shaking, but which quickly settled. This precipitate did not form in the uncondensed supernatant when held overnight prior to condensing. Apparently, the phenomenon was a ramification of the solubility characteristics of proteinaceous components.

Color and flavor. The acid precipitate was a cream colored and relatively bland (mild grassy odor and flavor) product. The color of the acid supernatant deepened as a result of concentration and appeared light brown in the lyophilized form. It had a pronounced grassy odor and a strong bitter taste. Color photographs (taken against a white background) of the fractions obtained from the three consecutive cuttings of Field A are shown in Figure 13 (see p. 79 for nomenclature). (The blue-green overtone is artifact.) Color seems to be related to the

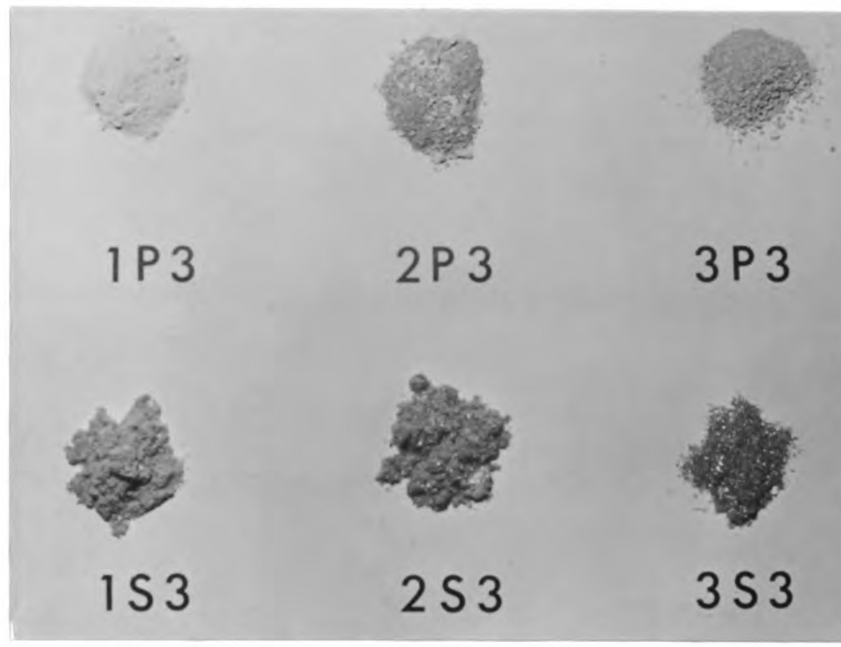

Figure 13. Acid precipitates and acid supernatants obtained by the standard extraction procedure from three cuttings.

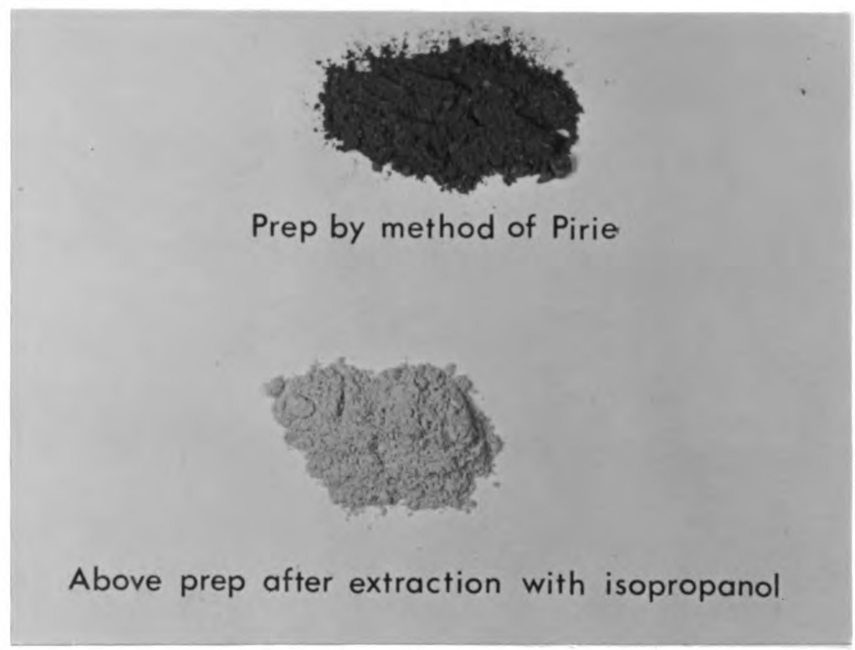

Figure 14. Acid precipitates obtained by Pirie extraction.

initial condition of the plant. The first cutting plants were green. The second and third cutting plants were yellow-brown; which produced a supernatant fraction that was a medium brown and a precipitate that appeared greyish. The color change was noticeable through the entire fractionation procedure. The juice from the latter two cuttings was yellowish-green instead of the deep green of the first cutting and all supernatants were brownish-yellow. Field B remained green through the entire growing season and the colors of the fractions obtained from all cuttings of this field were similar to those obtained from the first cutting of Field A.

Solvent extraction (see Modification H, p. 74) of the freeze-dried acid precipitate and of the acid supernatant resulted in no observable color loss in either fraction, althoughthe discarded solvent from the acid precipitate had a slight yellowish color. Extraction with isopropanol slightly reduced the "hay-like" flavor of the acid precipitate. However, there was no noticeable reduction in flavor of the supernatant, i.e., it retained its characteristically bitter flavor. Therefore, it was concluded that extraction of the protein isolates with isopropanol or acetone-hexane would not decrease the color or flavor defects sufficiently to warrant their use.

The classical LPC was obtained by direct precipitation of the protein from the juice (see Pirie extraction, p. 78), yielding a green powder which has received objections because of its color and grassy flavor (cf. Kamalamathan *et al.*, 1969; Buchanan, 1969a). Four extractions of this fraction with isopropanol resulted in a lighter colored precipitate, whereas an ethanol-hexane extraction did not remove the color as efficiently or completely. The precipitate, both before and after solvent extraction with isopropanol, is shown in Figure 14.

Compare these to 1P3 of Figure 13. Solvent extraction may prove to be an expensive process (cf. Pirie, 1969d) and therefore, simple low-speed centrifugation was emphasized as a means for removing the color-bearing components.

An attempt to remove the color by passing the supernatant over an ion exchange bed (see Modification A, p. 68) was unsuccessful because most of the protein remained on the column. This reduced the nitrogen contents of the supernatants 90% and the total solids contents 85% (see Table 16).

Table 16. Characteristics of supernatant fractions before and after passage over an ion exchange bed

Description of fractions	Total solids (%)	Nitrogen (%)*	Nitrogen (g)	Percentage of original N	Crude protein (%)**
Supernatant 3B	1.40	4.3	0.39	18.3	27.1
Filtrate	0.23	1.7	0.03	1.4	10.8
Supernatant 3B'	1.20	4.7	0.43	20.0	25.9
Filtrate'	0.20	2.7	0.05	2.1	16.9

*on a dry basis

**per cent N x 6.25

Proteolytic Activity in the Juice

Changes in NPN with time were measured both in the heated and unheated juice to determine if significant proteolytic activity occurred in the juice during extraction (see Modifications A and B, p. 68 and 69). Advantages of use of the NPN increment over other methods as an index of proteolysis have been discussed by Singh (1962). The results of these studies are presented in Table 17.

Table 17. Changes of alfalfa juice NPN from 0-24 hr after extraction

Time (hr)	NPN* (%) Modification A unheated	Modification A heated	Modification B unheated	Modification B heated
0	2.23 (4.65)**	2.73 (4.15)	1.16 (4.07)	1.46 (3.77)
0.5			1.21 (4.02)	1.51 (3.72)
1	2.24 (4.64)	2.83 (4.05)	1.11 (4.12)	1.46 (3.77)
2			1.16 (4.07)	1.56 (3.67)
3	2.32 (4.56)	2.87 (4.01)	1.26 (3.97)	1.61 (3.62)
4			1.36 (3.87)	1.56 (3.67)
6			1.46 (3.77)	1.71 (3.52)
8	2.53 (4.35)	2.74 (4.14)	1.56 (3.67)	1.65 (3.58)
24			1.71 (3.52)	1.76 (3.47)
nitrogen (%) of the juice	6.88		5.23	

*on a dry basis

**protein nitrogen given in parentheses

In both assays the original NPN of the unheated juice was about 80% of that for the heated juice. Although the data indicated a small degree of initial proteolysis for the heated sample prepared by Modification A, there was no significant net increase in NPN over the 8 hr observation period. However, the unheated juice showed an increase of 4.4% in NPN (or decrease in protein nitrogen), relative to the total nitrogen content of the juice, after 8 hr.

After about 2 hr an increase in the NPN content of the heated juice prepared by Modification B is observable. After 24 hr, an unrealistic holding time, the NPN content had increased only 5.7%. Unheated juice did not show an increase in NPN until after 3 hr. Thereafter, proteolysis increased at a faster rate than in the heated juice (see Figures 15 and 16).

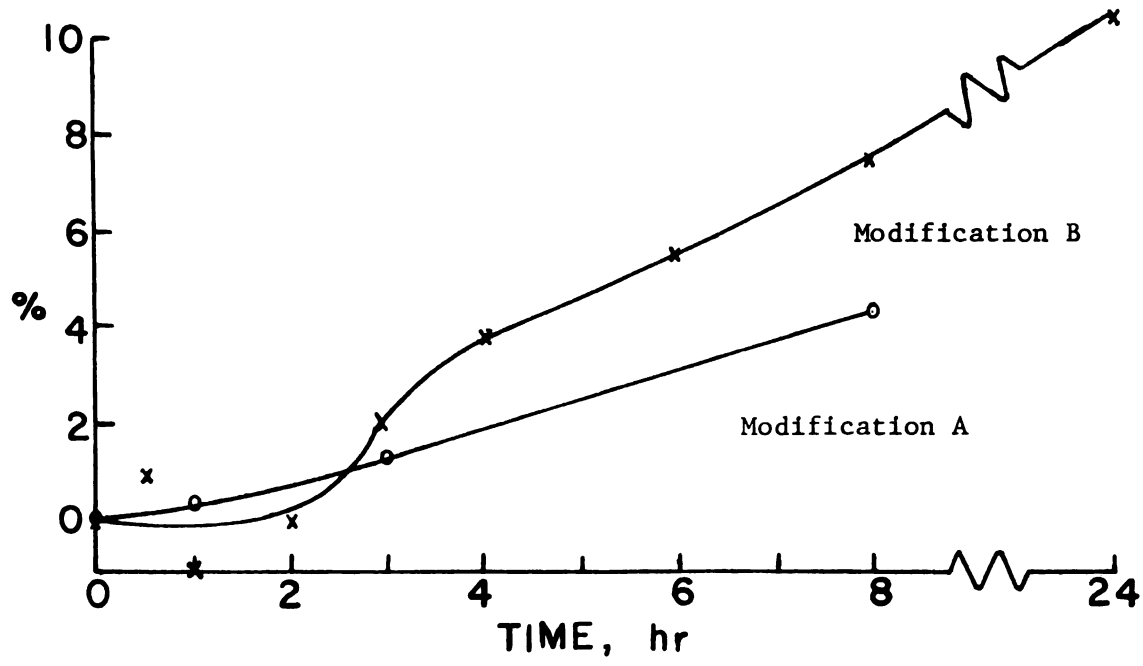

Figure 15. Increase in NPN as a percentage of the total nitrogen content of unheated alfalfa juice.

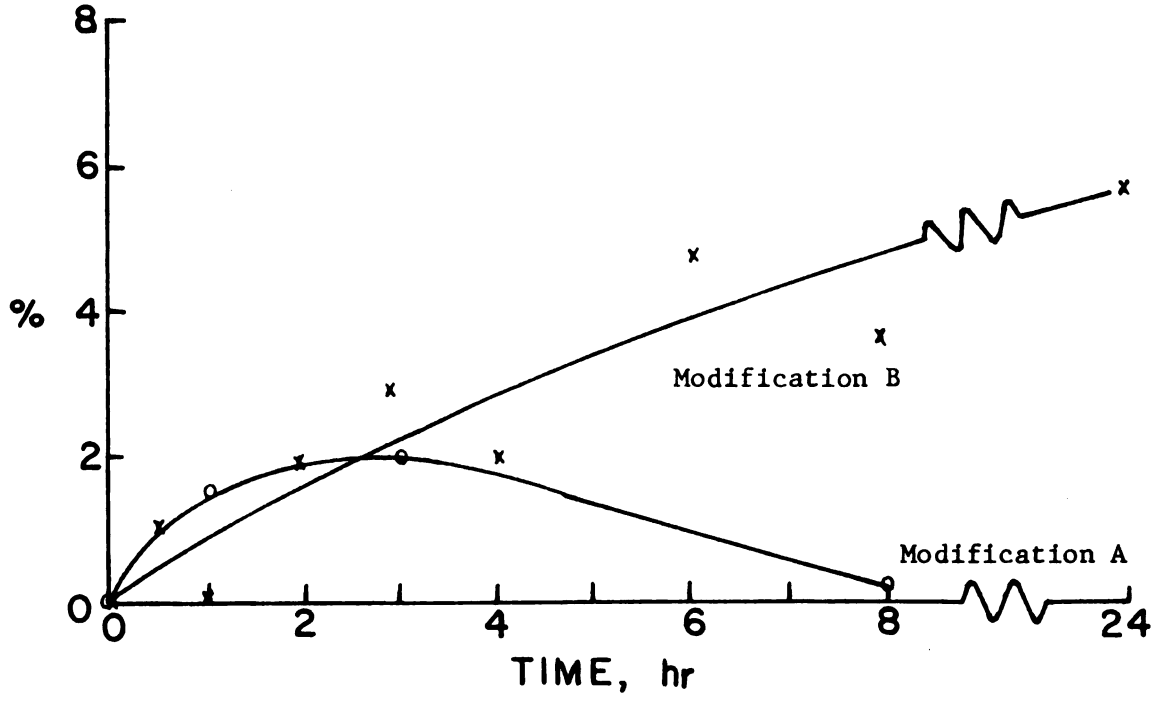

Figure 16. Increase in NPN as a percentage of the total nitrogen content of heated alfalfa juice.

Nazir and Shah (1966), who also investigated autolysis of unheated leaf extracts at room temperature, reported a loss in protein nitrogen of 3.5% in 2 hr and 26.5% after 24 hr. These values are over twice those obtained in this study. When working with large quantities of juice (about 12 l), the maximum time the unheated juice was held was 1 hr and, according to the results of the present study, there would be no detectable proteolysis. This agrees with the conclusion of Nazir and Shah (1966) that the juice can be kept up to 2 hr without significant protein destruction. Similarly, the heated juice was not held more than 1.5 hr.

A second approach used to evaluate proteolytic activity was incubation with raw skim milk followed by high-voltage paper-electrophoresis (HVPE) (see Modification I, p. 74). None of the tubes (milk plus juice) incubated at room temperature showed an increase in viscosity or clotting of the milk over the 6.5 hr time period. When incubated at 37 C, the milk sample containing 0.5 ml juice showed no coagulation, but the tubes with 1.0 and 2.0 ml of juice showed coagulation after 5 hr and 2.5 hr, respectively. This coagulation could be due to the proteolytic activity of the juice or to the complexing of the casein due to the presence of Ca^{+2} in the juice. In these two samples, no bands were observed by HVPE which were not present in the milk or juice controls (see Figure 17). Therefore, proteolysis of raw skim milk by fresh alfalfa juice could not be demonstrated by the appearance of peptides. Since extractions were conducted at room temperature, it was significant that no coagulation occurred in these experiments. Since the temperature was increased but once during the extraction (to 48 C), it was concluded that no significant proteolysis of leaf proteins occurred during the extraction procedure.

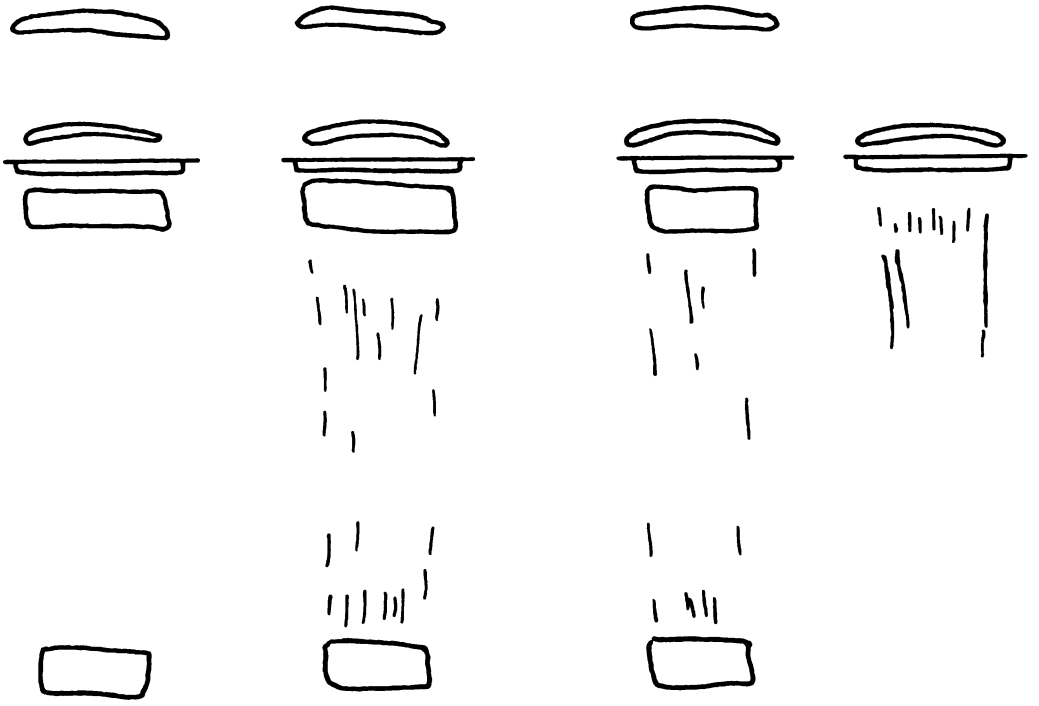

Figure 17. Schematic representation of high-voltage electrophoretogram showing peptide bands observed.

Enzyme Extractions

Cellulase. An attempt was made to increase the yields and purity of the final fractions by the use of carbohydrate hydrolyzing enzyme preparations. In the first experiment (Modification C, p. 71), Cellzyme was added during the blending step with the expectation that an increase in nitrogen content in supernatant 1 and a decrease in the nitrogen content of precipitate 1 would result. The results of this experiment are given in Table 18.

During the eight hours of incubation there was little change in the total nitrogen of supernatant 1 extracted from the enzyme-treated leaf but there was an increase of about 9% in NPN. Although there was an increase of almost 30% in NPN of the control, its final protein nitrogen content was greater than that of the supernatant of the

Table 18. Nitrogen contents of fractions obtained after blending with Cellzyme

Fraction	Time (hr)	Nitrogen* (%)	Non-protein nitrogen (%)	Protein** nitrogen (%)
Alfalfa		5.11	---	---
Juice				
control	0	6.01	---	---
Cellzyme	0	5.13	---	---
Supernatant 1				
control	0	4.47	2.03	2.44
Cellzyme	0	---	2.67	---
Cellzyme	4	4.51	2.46	2.05
Cellzyme	6	4.43	2.65	1.78
Cellzyme	8	4.50	2.90	1.60
control	8	4.96	2.62	2.33
Precipitate 1				
control	0	8.83	---	---
Cellzyme	0	8.62	---	---
Cellzyme	4	8.00	---	---
Cellzyme	6	8.16	---	---
Cellzyme	8	7.18	---	---
control	8	8.16	---	---

*on a dry basis

**%N - %NPN

enzyme-treated leaf. The compositional trends in the precipitates did not correspond to those observed in the supernatants. There appeared to be no increase in the protein nitrogen content of the supernatant of tne enzyme-treated preparation.

Holding the pulp in a large scale operation would not be practical because (1) loss in protein due to adsorption and proteolytic activity is two to three times as high at this stage as in the juice (Davys and Pirie, 1969) and (2) eventually one machine will be designed to handle both pulping and pressing in one operation. For this reason, and since

the blending takes less than a minute, in the following four experiments with cellulase, amylase, and pectinase (Modifications D, E, F, and G; p. 73 and 74) the juice was extracted before incubation with the enzyme. Precipitate 3 was the major protein fraction and therefore, an increase in the nitrogen content of this fraction would be desirable.

Nitrogen contents of the fractions isolated from the cellulase extractions are given in Table 19. Preparations obtained from the

Table 19. Nitrogen contents of fractions obtained after incubation of alfalfa juice with cellulase

Description of fractions	Nitrogen* (%)			
	modification D**		modification E	
Alfalfa	4.31		3.78	
Juice	5.14		4.61	
	control	cellulase	control	cellulase
Supernatant 1				
t = 0	3.67	3.53	3.52	3.43
t = 4		3.79		3.51
t = 6		---		---
t = 8	3.85	3.83	3.55	3.83
Precipitate 1				
t = 0	7.19	7.26	7.11	7.03
t = 8	7.86	7.25	6.11	6.65
Supernatant 3				
t = 0	2.35	2.37	2.32	2.29
t = 8	2.49	2.24	2.66	2.69
Precipitate 3				
t = 0	12.85	12.19	11.40	11.56
t = 4		11.50		10.89
t = 6		11.10		---
t = 8	11.14	13.10	12.54	11.86

*on a dry basis

**modification D: +0.465 mg cellulase/100 ml juice
modification E: +1.00 mg cellulase/100 ml juice

enzyme-treated leaf material showed an increase in the nitrogen content of supernatant 1. The data for precipitate 3 and supernatant 3 were inconclusive. In both experiments, there was a net increase with time in the nitrogen content of precipitate 3 (11.6-11.9% and 12.2-13.1%) obtained from the juice incubated with enzyme. However, at the lower enzyme concentration there was an increase in nitrogen of only 0.25% over that in the control preparation at time zero. At the higher enzyme concentration, precipitate 3 was lower in nitrogen than that of the control preparation after 8 hr of incubation and only slightly greater than that of the control at time zero. Apparently, treatment of the leaf material with cellulase enzyme preparations does not produce increased protein purity in the leaf isolate.

Amylase. The nitrogen contents of the fractions obtained from the extraction with alpha-amylase are given in Table 20. Relative to a normal extraction there was not a significant change in the nitrogen content of supernatant 1 from the enzyme-treated plant. Although there seemed to be a slight increase in the nitrogen content of precipitate 3 from the amylase-treated preparation after 8 hr of incubation (10.8-11.7%), the higher value was less than that of the control (12.1%).

Pectinase. The results from the pectinase-treated leaf extraction are presented in Table 21. This enzyme-treated preparation showed a decrease in the nitrogen content of precipitate 3 from 11.6-10.0% over the 8 hr of incubation. The control also showed a less pronounced decrease. Both preparations showed an increase in the nitrogen content of supernatant 3 during the 8 hr incubation period. Thus, there appeared to be some proteolytic activity in both preparations.

Table 20. Nitrogen contents of fractions obtained after incubation of alfalfa juice with alpha-amylase

Description of fractions	Nitrogen (%) control	α-amylase
Alfalfa	2.74	
Juice	4.04	
Supernatant 1		
t = 0	3.27	3.29
t = 4		3.23
t = 6		3.34
t = 8	3.38	3.31
Precipitate 1		
t = 0	6.00	6.64
t = 8	6.24	6.77
Supernatant 1		
t = 0	2.32	2.28
t = 8	2.60	2.64
Precipitate 1		
t = 0	12.11	10.84
t = 4		11.10
t = 6		9.64
t = 8	12.07	11.68

At the enzyme levels and conditions (room temperature and pH about 5.7) employed, there was no evidence that treatment of leaf materials with the various enzymes (cellulase, alpha-amylase, and pectinase) enhanced the purity of the protein recovered. However, because the amounts of protein recovered as well as its purity are of interest, more relevant data would have been realized if fractional yields had been recorded.

Table 21. Nitrogen contents of fractions obtained after incubation of alfalfa juice with pectinase

Description of fractions	Nitrogen* (%) control	pectinase
Alfalfa	3.60	
Juice	4.28	
Supernatant 1		
t = 0	3.50	3.53
t = 4		---
t = 6		3.45
t = 8	3.74	3.73
Precipitate 1		
t = 0	6.97	6.84
t = 8	6.98	6.09
Supernatant 3		
t = 0	2.53	2.61
t = 8	2.80	2.93
Precipitate 3		
t = 0	11.74	11.64
t = 4		11.31
t = 6		10.89
t = 8	10.97	10.00

*on a dry basis

Yields

Greenhouse alfalfa. Yield studies, based on nitrogen (N) and total solids (TS) determinations (see p. 77) were made of three extractions of greenhouse alfalfa: January 6, 1970 (I); February 17, 1970 (II); and April 6, 1970 (III). The results of these studies are recorded in Tables 22, 23, and 24. The nitrogen content of the fresh alfalfa ranged from 2.8% (II) to 3.6% (III) to 4.5% (I). This range is close to that reported for alfalfa by Guggolz, Herring, and Kohler (1967). The total solids content decreased with corresponding increases in nitrogen content (i.e., 25.3% TS and 2.8% N to 16.3% TS and 4.5% N). This negative

Table 22. Compositional characteristics of alfalfa leaf fractions obtained by yield study conducted concomitantly with Modification A of the standard extraction method (referred to as Extraction I)

Description of fractions	Weight (g)	Percentage of original wt	Total solids (%)	Total solids (g)	Percentage of original TS	Nitrogen (%)*	Nitrogen (g)	Percentage of original N	Percentage of extractable N	Crude protein (%)**	Crude protein (g)
Alfalfa	291.6		16.3	47.7	100.0	4.49	2.14	100.0	---	28.1	13.4
Water	1166										
Alfalfa + water	1457.6	100.0									
Juice	1191	81.7	1.4	16.9	35.5	6.88	1.16	54.2	100.0	43.0	7.3
Fiber residue	125.0	8.6									
Supernatant 1	1024	70.2	1.1	11.3	23.7	5.82	0.66	30.6	56.5	36.4	4.1
Precipitate 1	103.3	7.1	5.4	5.6	11.8	8.30	0.47	21.8	40.2	51.9	2.9
Supernatant 2	965	66.2	1.1	10.3	21.6	5.92	0.61	28.4	52.4	37.0	3.8
Precipitate 2	23.8	1.6	3.8	0.9	1.9	3.11	0.03	1.3	2.4	19.4	0.2
Supernatant 3	935	64.2	1.0	9.3	19.5	4.35	0.40	18.8	34.7	27.2	2.5
Precipitate 3	13.3	0.9	13.0	1.7	3.6	15.01	0.26	12.1	22.3	93.8	1.6

*on a dry basis

**N x 6.25

Yield: 2.52 g dry protein/lb wet alfalfa as acid precipitate (precipitate 3)
3.85 g dry protein/lb wet alfalfa as acid supernatant (supernatant 3)

Table 23. Compositional characteristics of alfalfa leaf fractions obtained by yield study of the standard extraction method (referred to as Extraction II)

Description of fractions	Weight (g)	Percentage of original wt	Total solids (%)	Total solids (g)	Percentage of original TS	Nitrogen (%)*	Nitrogen (g)	Percentage of original N	Percentage of extractable N	Crude protein (%)**	Crude protein (g)
Alfalfa	2868.1		25.3	724.8	100.0	2.81	20.4	100.0	---	17.6	127.3
Water	11473.4										
Alfalfa + water	14341.5	100.0									
Juice	11368	79.3	1.8	208.0	28.7	3.55	7.4	36.3	100.0	22.2	46.2
Fiber residue	2051.6	14.1									
Supernatant 1	10298	71.8	1.6	161.7	22.3	2.80	4.5	22.2	61.2	17.5	28.3
Precipitate 1	530.6	3.7	10.1	53.5	7.4	5.82	3.1	15.4	42.2	36.4	19.6
Supernatant 2	9845	68.6	1.6	152.6	21.1	2.57	3.9	19.5	53.7	16.1	24.6
Precipitate 2	155.9	1.1	8.4	13.0	1.8	1.56	0.2	1.0	2.7	9.8	1.3
Supernatant 3	9675	67.5	1.5	148.2	20.4	1.92	2.8	13.8	38.0	12.0	17.6
Precipitate 3	81.1	0.6	12.9	10.4	1.4	9.72	1.0	5.0	13.8	60.8	6.3

*on a dry basis

**N x 6.25

Yield: 1.00 g dry protein/lb wet alfalfa as acid precipitate (precipitate 3)
2.79 g dry protein/lb wet alfalfa as acid supernatant (supernatant 3)

Table 24. Compositional characteristics of alfalfa leaf fractions obtained by yield study conducted concomitantly with Modification B of the standard extraction method (referred to as Extraction III)

Description of fractions	Weight (g)	Percentage of original wt	Total solids (%)	Total solids (g)	Percentage of original TS	Nitrogen (%)*	Nitrogen (g)	Percentage of original N	Percentage of extractable N	Crude protein (%)**	Crude protein (g)
Alfalfa	997.6		21.8	217.8	100.0	3.61	6.8	100.0	---	22.6	42.7
Water	3992										
Alfalfa + water	4989.6	100.0									
Juice	4218	84.5	2.0	83.9	38.5	5.23	4.4	64.3	100.0	32.7	27.4
Fiber residue	577.1	11.6									
Supernatant 1	3874	77.7	1.5	56.6	26.0	4.04	2.3	33.5	52.1	25.3	14.3
Precipitate 1	344.6	6.9	8.5	29.4	13.5	6.80	2.0	29.3	45.6	42.5	12.5
Supernatant 2	3679	73.7	1.4	52.2	24.0	3.80	2.0	29.1	45.3	23.8	12.4
Precipitate 2	87.2	1.8	5.8	5.1	2.3	4.05	0.2	2.8	4.3	25.3	1.2
Supernatant 3	3603	72.2	1.3	46.8	21.5	2.42	1.1	16.5	25.7	15.1	7.1
Precipitate 3	55.5	1.1	19.2	10.4	4.8	10.79	1.1	16.5	25.7	67.4	7.1

*on a dry basis

**N x 6.25

Yield: 3.21 g dry protein/lb wet alfalfa as acid precipitate (precipitate 3)
3.21 g dry protein/lb wet alfalfa as acid supernatant (supernatant 3)

correlation has been reported between protein and fiber contents at all stages of growth (Van Riper and Smith, 1959; Ogden and Kehr, 1968). The low nitrogen specimen represented plants which had reached the seeding stage. This is in agreement with reports that the protein content of alfalfa decreases with maturity (Van Riper and Smith, 1959; Ogden and Kehr, 1968).

Only 36% of the leaf nitrogen was extracted from the more mature specimen. In contrast juice from the young plants contained more than 50% of the original leaf protein. That more protein can be extracted from young than from mature plants has been well documented (cf. Pirie, 1962; Singh, 1964; Nazir and Shah, 1966). However, specimen III, containing 3.6% nitrogen showed greater extractability than specimen I which had a higher nitrogen content (4.5%). This is in agreement with recent work indicating that high extractability and high nitrogen content in the leaf are not necessarily positively correlated (Byers *et al.*, 1956b; Byers and Sturrock, 1965; Nazir and Shah, 1966). The nitrogen content of the juice varied from 3.6-6.9% reflecting the nitrogen content of the leaf.

Precipitate 1 (chloroplastic) contained from 5.8-8.3% nitrogen, which also increased with the nitrogen content of the leaf. This fraction represented about 15-30% of the original leaf nitrogen or slightly less than half of the extracted nitrogen. The remainder of the extracted nitrogen remained as soluble nitrogen in supernatant 1. Here again, the supernatant nitrogen content varied, increasing with increasing nitrogen content of the leaf.

Only small amounts (1.0-1.3%) of the leaf nitrogen appeared in precipitate 2 (alkali-induced), containing from 1.6-4.1% nitrogen. Thus, supernatant 2, with nitrogen contents of 2.6-5.9%, retained from

19-28% of the original nitrogen in a soluble form.

This soluble nitrogen fraction was split between the two final fractions, i.e., acid precipitate and acid supernatant. About half, representing from 14-19% of the leaf protein, remained in the acid soluble supernatant (supernatant 3) which contained from 1.9-4.4% nitrogen. The precipitated fraction (precipitate 3) accounted for 5.0-16.5% of the leaf nitrogen. The low yield (5.0%) was obtained from the older plant extract. The nitrogen content of this fraction, representing the major protein fraction, varied from 9.7-15.0%.

Thus at the conclusion of the procedure, about 25% of the nitrogen extracted from young plants appeared in the major protein fraction (acid precipitate), whereas about 30% remained in the acid supernatant.

The higher the nitrogen content of the leaf the greater was the nitrogen content of all resulting fractions (except for precipitate 2). However, the yield of the original leaf nitrogen in each fraction (except for supernatant 3) increased with greater extractability of the leaf--extractability, as noted previously, did not always correlate with leaf nitrogen if other conditions, such as age, were similar. Consequently fractions obtained from extraction II (mature plants) always showed the lowest nitrogen contents and lowest yields of original nitrogen. Nitrogen contents of fractions from these plants were only about 45-75% as great as, and yields of leaf nitrogen only 35-80% that of, those from young plants. With one exception, fractions from extraction I showed the highest nitrogen contents, whereas fractions from extraction III showed the highest yields of original nitrogen.

All three supernatants from older plants contained a higher yield of extracted nitrogen (110-125%) than the same fractions from younger

plants. However, precipitate 3 (comparable to the various cytoplasmic fractions reported in the literature) from the older plants contained only half as much extracted nitrogen as that from the young plants. This is in contrast to Pirie (1963, 1964b) who reported that the chloroplastic to cytoplasmic protein ratio does not vary with maturity, and to Henry and Ford (1965) who claimed that mature leaves contain a higher proportion of cytoplasmic proteins because the reduction in extractable protein with maturity affects the chloroplastic more than the cytoplasmic fraction.

Summarizing, the young plants yielded 2.5-3.2 g dry protein as acid precipitate and 3.2-3.85 g dry protein as acid supernatant per lb of wet raw material. Plants in the seeding stage yielded only 1.0 g and 2.8 g dry protein as acid precipitate and supernatant, respectively, per lb wet alfalfa.

Field alfalfa. The results obtained from yield studies made during the summer field harvests are given in the following tables:

Table 25. First harvest of the first cutting (6/5/70)
Table 26. Fifth harvest of the first cutting (6/11/70)
Table 27. Second harvest of the second cutting (7/18/70)
Table 28. Fifth harvest of the second cutting (7/22/70)
Table 29. Third cutting (10/4/70)

The first cutting showed the typical negative correlation between nitrogen (5.2% and 3.0%) and total solids (15.6% and 24.3%). The crop condition (maturity, greenness, leafiness) was not visibly distinguishable between the two harvests, yet there was a noticeable difference in leaf nitrogen. Both specimens showed extractabilities greater than 50%; the lower nitrogen leaf having the greater extractability (62%). As previously noted in the experiments with greenhouse alfalfa, for all fractions except precipitate 2, the higher the nitrogen content of the leaf the

Table 25. Compositional characteristics of alfalfa leaf fractions obtained by yield study of the standard extraction method from the first harvest of the first cutting of field alfalfa

Description of fractions	Weight (g)	Percentage of original wt	Total solids (%)	Total solids (g)	Percentage of original TS	Nitrogen (%)*	Nitrogen (g)	Percentage of original N	Percentage of extractable N	Crude protein (%)**	Crude protein (g)
Alfalfa	2292		15.6	362.1	100.0	5.24	19.0	100.0	---	32.8	118.7
Water	8970										
Alfalfa + water	11262	100.0									
Juice	9979	88.6	1.7	169.1	46.7	5.76	9.7	51.3	100.0	36.0	60.9
Fiber residue	858.0	7.6									
Supernatant 1	9476	84.1	1.2	113.2	31.3	4.81	5.5	28.7	56.0	30.1	34.1
Precipitate 1	458.8	4.1	13.6	62.3	17.2	6.79	4.2	22.3	44.3	42.4	26.4
Supernatant 2	9199	81.7	1.2	109.9	30.4	4.97	5.5	28.8	56.1	31.1	34.1
Precipitate 2	162.1	1.4	5.7	9.2	2.5	3.15	0.3	1.5	3.0	19.7	1.8
Supernatant 3	9004	80.0	1.1	95.9	26.5	3.34	3.2	16.9	32.9	20.9	20.1
Precipitate 3	137.4	1.2	16.7	22.9	6.3	11.6	2.7	14.6	28.4	72.5	16.7

*on a dry basis

**N x 6.25

Yield: 3.30 g dry protein/lb wet alfalfa as acid precipitate (precipitate 3)
3.98 g dry protein/lb wet alfalfa as acid supernatant (supernatant 3)

Table 26. Compositional characteristics of alfalfa leaf fractions obtained by yield study of the standard extraction method from the fifth harvest of the first cutting of field alfalfa

Description of fractions	Weight (g)	Percentage of original wt	Total solids (%)	Total solids (g)	Percentage of original TS	Nitrogen (%)*	Nitrogen (g)	Percentage of original N	Percentage of extractable N	Crude protein (%)**	Crude protein (g)
Alfalfa	2235		24.3	543.1	100.0	3.01	16.4	100.0	---	18.8	102.3
Water	9400										
Alfalfa + water	11635	100.0									
Juice	9990	85.9	2.0	203.8	37.5	5.00	10.2	62.3	100.0	31.3	63.7
Fiber residue	1182.4	10.2									
Supernatant 1	9402	80.8	1.7	155.1	28.6	3.32	5.1	31.4	50.4	20.8	32.1
Precipitate 1	418.3	3.6	19.3	80.5	14.8	5.47	4.6	28.4	45.5	34.2	29.0
Supernatant 2	9168	78.8	1.3	121.9	22.4	4.00	4.9	29.9	47.9	25.0	30.5
Precipitate 2	121.1	1.1	7.6	9.2	1.7	3.84	0.4	2.1	3.4	24.0	2.2
Supernatant 3	8828	75.9	1.2	105.1	19.4	2.74	2.9	17.5	28.1	17.1	17.9
Precipitate 3	135.6	1.2	18.7	25.4	4.7	12.5	3.2	19.4	31.1	78.1	19.8

*on a dry basis

**N x 6.25

Yield: 4.02 g dry protein/lb wet alfalfa as acid precipitate (precipitate 3)
3.63 g dry protein/lb wet alfalfa as acid supernatant (supernatant 3)

higher the nitrogen content of the resulting fractions. There was one noteworthy exception; precipitate 3 from the 3.0% nitrogen leaf contained 12.5% nitrogen, whereas the same fraction from the 5.3% nitrogen leaf contained only 11.6% nitrogen. In all cases, each fraction from the lower nitrogen leaf represented a greater yield of the original nitrogen due to the enhanced extractability of this specimen. This latter harvest also showed slightly higher percentages of extracted nitrogen in the precipitates and lower percentages in the supernatants than observed for the first harvest.

The nitrogen contents of the various fractions were in the same range as those obtained from greenhouse plants at about the same stage of maturity (i.e., extractions I and III) although the average nitrogen contents of the "greenhouse" fractions were slightly greater than the "field" fractions. The "field" and "greenhouse" fractions also showed similar distributions of the extracted nitrogen. There was one difference; namely, field alfalfa precipitate 3 contained a greater yield of the leaf nitrogen (or extracted nitrogen) than the same fraction obtained from the greenhouse alfalfa--17.0% (or 29%) as compared to 14.3% (or 24%), respectively. Consequently, field harvests gave higher yields of protein in the acid precipitates averaging 3.65 g dry protein per lb wet alfalfa (greenhouse yields averaged 2.85 g per lb). There was little difference in the yield of acid supernatant protein between the two sets of plants.

The second cutting was lower in nitrogen (2.5% and 2.2% for second and fifth harvests, respectively) and higher in total solids (32.4% and 32.3%) than the first cutting. This was expected from the condition of the plants. Although they were the same plants at the same stage of maturity, the regrowth was yellowish, dry, and stemmy, whereas the first

Table 27. Compositional characteristics of alfalfa leaf fractions obtained by yield study of the standard extraction method from the second harvest of the second cutting of field alfalfa

Description of fractions	Weight (g)	Percentage of original wt	Total solids (%)	Total solids (g)	Percentage of original TS	Nitrogen (%)*	Nitrogen (g)	Percentage of original N	Percentage of extractable N	Crude protein (%)**	Crude protein (g)
Alfalfa	1940.4		32.4	629.1	100.0	2.52	15.9	100.0	---	15.8	99.2
Water	7960										
Alfalfa + water	9900.4	100.0									
Juice	7587	76.6	2.5	189.7	30.2	3.16	6.0	37.8	100.0	19.8	37.5
Fiber	1268.9	12.8									
Supernatant 1	7207	72.8	1.7	119.6	19.0	3.18	3.8	24.0	63.4	19.9	23.8
Precipitate 1	165.2	1.7	30.3	50.0	8.0	2.19	1.5	9.2	24.3	13.7	9.1
Supernatant 2	6954	70.2	1.7	115.8	18.4	2.98	3.5	21.7	57.5	18.6	21.6
Precipitate 2	131.7	1.3	7.0	9.2	1.5	3.58	0.3	2.1	5.5	22.4	2.1
Supernatant 3	6792	68.6	1.5	100.9	16.0	2.36	2.4	14.5	38.4	14.8	14.9
Precipitate 3	97.5	1.0	14.4	14.0	2.2	9.37	1.3	8.3	21.8	58.6	8.2

*on a dry basis

**N x 6.25

Yield: 1.92 g dry protein/lb wet alfalfa as acid precipitate (precipitate 3)
3.48 g dry protein/lb wet alfalfa as acid supernatant (supernatant 3)

Table 28. Compositional characteristics of alfalfa leaf fractions obtained by yield study of the standard extraction method from the fifth harvest of the second cutting of field alfalfa

Description of fractions	Weight (g)	Percentage of original wt	Total solids (%)	Total solids (g)	Percentage of original TS	Nitrogen (%)*	Nitrogen (g)	Percentage of original N	Percentage of extractable N	Crude protein (%)**	Crude protein (g)
Alfalfa	2017.4		32.3	650.8	100.0	2.22	14.4	100.0	---	13.9	90.2
Water	8070										
Alfalfa + water	10087.4	100.0									
Juice	8171	81.0	2.2	178.9	27.5	2.78	5.0	34.4	100.0	17.4	31.1
Fiber residue	1425.8	14.1									
Supernatant 1	7655	75.9	1.9	141.2	21.7	2.75	3.9	26.9	78.2	17.2	24.3
Precipitate 1	330.1	3.3	30.5	100.8	15.5	2.56	2.6	17.9	52.0	16.0	16.1
Supernatant 2	7529	74.6	1.8	136.3	20.9	2.68	3.7	25.3	73.5	16.8	22.8
Precipitate 2	123.3	1.2	7.2	8.9	1.4	3.59	0.3	2.2	6.5	22.4	2.0
Supernatant 3	7298	72.3	1.8	128.1	19.7	2.01	2.6	17.9	52.0	12.6	16.1
Precipitate 3	108.2	1.1	11.6	12.6	1.9	10.25	1.3	8.9	26.0	64.1	8.1

*on a dry basis

**N x 6.25

Yield: 1.81 g dry protein/lb wet alfalfa as acid precipitate (precipitate 3)
3.63 g dry protein/lb wet alfalfa as acid supernatant (supernatant 3)

growth was green, leafy, and tender. Extractability of these regrowths was low: 34% and 38%. The second harvest had a slightly greater (0.3-0.4%) nitrogen content in the leaf, juice, and three supernatants than the fifth harvest. However, the acid precipitate contained only 9.4% nitrogen as compared to 10.3% nitrogen in this same fraction obtained from the fifth harvest. The nitrogen contents of all fractions extracted from the second cutting, except precipitate 2, were significantly less (generally about 40-80%) than those observed for the same fractions obtained during the first cutting. Data of Hartman *et al.* (1967) indicated that the second cutting alfalfa and the juice extracted from it had protein contents only 90% of those of the first cutting alfalfa and resulting juice. Earlier Tilley *et al.* (1954) had reported second cutting alfalfa to have a higher crude protein content than the first cutting; although, the crude protein content of the LPC from the second cutting was lower than that from the first.

The lower percentages of leaf nitrogen in the second cutting fractions were expected because of the lower extractability encountered. Particularly striking was the observation that although supernatant 3 retained almost the normal amount of leaf nitrogen (16%), the acid precipitate contained only 8.6%. A comparable yield from the first cutting was 17%. Evidently, the reduction in leaf nitrogen in the plant consisted primarily of a reduction in the chloroplastic protein--as might be expected by the yellowish color of the plants--and in the amount of protein precipitated by acid. On the basis of the percentage of extracted nitrogen the percentages for the supernatant of the second cutting are greater by 25-50% than those found in the first cutting; although the percentages for precipitates 1 (chloroplastic) and 3 (acid) were 50% and 20% less, respectively. This increase in the ratio of soluble to

precipitable protein was also noted in the older greenhouse alfalfa which exhibited a low nitrogen content and low extractability. Whereas the age difference of the greenhouse alfalfa caused the greatest reduction to occur in the acid precipitable protein, differences in color and lushness of the field alfalfa resulted in the largest reduction in the chloroplastic protein.

The plants of the third cutting were similar in appearance to those of the second cutting, i.e., they were brown and dry. Nitrogen (2.5%) and total solids (31.7%) contents of the leaf were not greatly different from the second cutting. Nitrogen contents of the juice and precipitate 1 were slightly greater than, supernatant 3 and precipitate 3 within the range of, and supernatants 1 and 2 slightly less than, those of the second cutting. The extractability--50%--was much higher than the second cutting although slightly less than the first cutting. However, the percentages of leaf nitrogen in all extracted fractions were not necessarily greater than those of the second cutting. All three supernatants were within the same range as the second cutting; the increased extractability being exemplified by an increase in the recovery of leaf nitrogen in precipitates 1 and 3.

A comparison revealed that percentages of extracted nitrogen in the third cutting supernatant fractions were significantly lower than those of the second cutting, whereas percentages for precipitates 3 were about equal and the percentage for precipitate 1 from the third cutting was about twice that of the second cutting. Thus, except for precipitate 3, the third cutting showed a distribution of extracted nitrogen similar to the first cutting, although the nitrogen contents of the fractions obtained were not much different than those for the second cutting. It should be remembered that all three cuttings were

Table 29. Compositional characteristics of alfalfa leaf fractions obtained by yield study of the standard extraction method from the third cutting of field alfalfa

Description of fractions	Weight (g)	Percentage of original wt	Total solids (%)	Total solids (g)	Percentage of original TS	Nitrogen (%)*	Nitrogen (g)	Percentage of original N	Percentage of extractable N	Crude protein (%)**	Crude protein (g)
Alfalfa	1722.5		31.7	546.7	100.0	2.51	13.7	100.0	---	15.7	85.7
Water	6890										
Alfalfa + water	8612.5	100.0									
Juice	7067	82.1	2.9	201.4	36.8	3.41	6.9	50.1	100.0	21.3	42.9
Fiber residue	1147.2	13.3									
Supernatant 1	6535	75.9	2.0	128.7	23.5	2.68	3.5	25.2	50.3	16.8	21.6
Precipitate 1	335.4	3.9	22.8	76.5	14.0	3.75	2.9	20.9	41.7	23.4	17.9
Supernatant 2	6481	75.3	1.9	125.7	23.0	2.64	3.3	24.2	48.3	16.5	20.8
Precipitate 2	98.9	1.2	7.0	7.0	1.3	3.01	0.2	1.5	3.1	18.8	1.3
Supernatant 3	6289	73.0	1.9	117.0	21.4	1.89	2.2	16.1	32.1	11.8	13.8
Precipitate 3	124.6	1.5	12.3	15.3	2.8	9.98	1.5	11.1	22.2	62.4	9.5

*on a dry basis

**N x 6.25

Yield: 2.51 g dry protein/lb wet alfalfa as acid precipitate (precipitate 3)
3.64 g dry protein/lb wet alfalfa as acid supernatant (supernatant 3)

made at as nearly as possible the same stage of maturity (using the criteria of per cent bloom). Pirie (1966e) reported a study of regrowth patterns on the yield of extractable protein that gave conflicting results.

The nitrogen contents of fractions obtained from the greenhouse plants gathered at the post-seed stage were within the range of those of fractions extracted from the last two cuttings. Exceptions to this trend were encountered in the juice and the chloroplastic precipitate which contained higher contents of nitrogen, reflecting the green color of the greenhouse plants. The percentages of leaf nitrogen in the fractions were also of the same magnitude as those of the second and third cuttings. As noted previously the two groups of plants showed a similar extracted nitrogen distribution with the exception of the acid and chloroplastic precipitates.

Total solids. The TS content of the alfalfa varied inversely with the nitrogen content of the leaf, ranging from 15.6-32.4%. Generally, the TS content of the isolated fractions were proportional to the TS of the leaf. It was also noticed that (1) there was a gradual decrease of about 0.7% in the TS from the juice down to supernatant 3 and that, (2) of the precipitates, precipitate 2 was always lowest in TS and either precipitate 3 or precipitate 1 would be highest.

Ranges of TS content of the various fractions obtained from the several preparations are listed in Table 30. About 65% of the leaf solids was retained in the fiber residue. Another 17% was precipitated with the chloroplastic fractions, leaving about one-quarter of the leaf solids in supernatant 1. The alkali-induced precipitate contained slightly less than 2% of the original solids. On the average, the final

Table 30. Total solids characteristics of fractions extracted from alfalfa

Description of fractions	Total solids (%)	Percentage of original TS
Alfalfa	15.6 - 32.4	100.0
Juice	1.4 - 2.0	27.5 - 46.7
Supernatant 1	1.1 - 2.0	19.0 - 31.3
Precipitate 1	5.4 - 30.5	7.4 - 17.2
Supernatant 2	1.1 - 1.9	18.4 - 30.4
Precipitate 2	3.8 - 8.4	1.3 - 2.5
Supernatant 3	1.0 - 1.9	16.0 - 26.5
Precipitate 3	11.6 - 19.2	1.4 - 6.3

supernatant and precipitate accounted for 20.5% and 3.5%, respectively, of the leaf solids. This can be compared to 16.5% and 12%, respectively, of the leaf nitrogen. There were no particularly striking differences in the distribution of solids between the various preparations.

Summary. The extraction data seemed to fall roughly into two groups: (1) from plants in "good" condition--young and green (extractions I and III, first cutting), and (2) from plants in "poor" condition--mature or brown (extraction II, second and third cuttings). Some of the pertinent nitrogen data are recorded in Table 31. The previously reported crude protein contents of fresh alfalfa from which LPC was prepared have ranged from 19.5-33.1%* (Valli Devi *et al.*, 1965; Smith, 1966; Hartman *et al.*, 1967; Subba Rau *et al.*, 1969; Spencer *et al.*, 1969; Knuckles *et al.*, 1970), which is comparable to our "good" alfalfa. The average protein content of juice extracted from "good" alfalfa is within the range of 28.0-36.3% protein reported by Hartman *et al.* (1967), Subba Rau *et al.* (1969),

*Throughout the thesis compositional data (both from the literature and this study) will be given on a dry basis.

Table 31. Nitrogen characteristics of the fractions obtained from several extractions of alfalfa

Description of fractions	Nitrogen (%)*		Percentage of original N		Percentage of extractable N		Crude protein (%)**	
	good	poor	good	poor	good	poor	good	poor
Alfalfa	4.09	2.52	100.0	100.0	---	---	25.6	15.8
Juice	5.72	3.23	58.0	39.7	100.0	100.0	35.8	20.2
Supernatant 1	4.50	2.85	31.1	24.6	53.8	63.3	28.1	17.8
Precipitate 1	6.84	3.58	25.4	13.3	43.9	32.5	42.8	22.4
Supernatant 2	4.67	2.72	29.0	22.7	50.4	58.5	29.1	17.0
Precipitate 2	3.54	2.94	1.9	1.7	3.3	4.5	22.1	18.4
Supernatant 3	3.21	2.05	17.4	15.6	30.4	40.1	20.1	12.8
Precipitate 3	12.5	9.83	15.6	8.3	26.9	21.0	78.1	61.4

*on a dry basis

**N x 6.25

Yields: g/lb	good	poor
Precipitate 3	3.26	1.81
Supernatant 3	3.67	3.38

Spencer *et al.* (1969), and Knuckles *et al.* (1970) for alfalfa juice.

The nitrogen contents of the chloroplastic fractions obtained by Singh (1967) and Subba Rau *et al.* (1969) by heating lucerne juice, but to a slightly higher temperature, are comparable to our value obtained from "good" alfalfa. However, Singh (1967) found this fraction to contain 40% of the total precipitable protein; whereas our preparation contained nearly two-thirds of the precipitable protein.

Leaf protein concentrate prepared from alfalfa by various extraction methods has been reported to have a crude protein content of 49-68% (Smith, 1966; Singh, 1967; Spencer *et al.*, 1969, 1971; Subba Rau *et al.*, 1969; Poppe *et al.*, 1970) which is close to values obtained for the acid precipitate extracted from alfalfa in "poor" condition and considerably less (about 20%) than values for the acid precipitate from "good" alfalfa. The acid precipitate would be more comparable to the cytoplasmic fractions isolated by some investigators. The crude protein contents of cytoplasmic fractions obtained by Singh (1967) and Subba Rau *et al.* (1969) by heat precipitation are slightly less than the average crude protein content of acid precipitates extracted from "good" alfalfa. Singh (1967) also reported that his cytoplasmic fraction contained 45-50% of the precipitable protein. In contrast, our acid precipitate contained only about one-third of the precipitable protein.

The literature generally makes little mention of the final liquor. Spencer *et al.* (1969, 1971) reported a protein content (ranging from 16-29%) for this fraction comparable to that obtained for the final supernatant from "good" alfalfa; although Poppe *et al.* (1970) found the protein content of the liquor to be not higher than 17%.

In conclusion, in agreement with Festenstein (1961) and Byers and Sturrock (1965), yields were noticeably dependent upon the age and physical

state of the leaf and were considerably reduced after the plant had developed seeds. Yields from plants which were dry and yellow were lower than those which were tender and green. The lower yields appeared to be a combined result of less nitrogen in the leaf, reduced extractability, and less acid precipitable material. The percentage of alfalfa leaf nitrogen extractable into the juice varies with the mechanics of the extraction process. The values obtained by this small scale laboratory method for "good" alfalfa are comparable to those obtained by some specially designed leaf extractors, e.g., Hartman *et al.* (1967), Singh (1967), and Knuckles *et al.* (1970). Although workers may often report the percentage of nitrogen extractable into the juice, they generally fail to give the percentage of nitrogen precipitable by heat or acid. One exception was Chayen *et al.* (1961) who reported that by their industrial impulse rendering process 22-64% of lucerne nitrogen is obtained in the protein precipitate. A summation of the chloroplastic and acid precipitates from the "poor" alfalfa would give a value equal to the lower end of the range reported by Chayen *et al.* (1961). The "good" alfalfa gave a value in the middle of that range. From one pound of wet alfalfa in good condition, 3.3 and 3.7 g of dried protein were obtained as precipitate 3 and as supernatant 3, respectively.

Quality and Nutritive Value

Chemical Evaluation

Gross composition. The proximate composition of the acid precipitate and acid supernatant from each cutting are given in Table 32. All values were corrected for moisture in the freeze-dried samples. Protein was calculated as nitrogen times 6.25; 6.25 being the classical conversion factor for an ideal, non-conjugated, 16% nitrogen, protein. Unfortunately no 100% satisfactory method has been formulated for determining the

Table 32. Proximate compositions of alfalfa leaf protein fractions

	Fraction					
	1P3*	2P3	3P3	1S3	2S3	3S3
Protein	81.50	68.34	66.14	33.54	22.41	20.57
Carbohydrate	6.54	9.10	9.43	35.51	32.63	30.64
Lipid	1.08	1.66	2.44	0.95	3.11	3.23
Ash	1.24	1.46	1.52	5.20	6.10	4.67

*see methods section, p. 79, for explanation of nomenclature.

total carbohydrate content of LPC (Byers, unpublished). Lipid includes a wide variety of compounds and consequently concentrations reported will depend upon the particular solvent system employed. Chloroform-methanol (2:1 v/v) was used, as many workers have found this solvent system to be satisfactory for removing most lipid materials from LPC (Byers, unpublished; Lea and Parr, 1961; Lima, Richardson, and Stahmann, 1965; Shah, 1968).

The acid precipitate contained approximately 72% protein, 8% carbohydrate, 2% lipid, and 1.5% ash. The protein contents were compared with literature values for fractions extracted from alfalfa in the previous section on yields. In review, the protein contents are greater than the whole LPC preparations; and slightly greater than (in the case of the first cuttings) and slightly less than (in the case of the second and third cuttings) the cytoplasmic fractions isolated from alfalfa juice. Nonprotein constituents are not frequently reported. Smith (1966) reported a slightly greater value for carbohydrate (12.5%) in LPC obtained by the impulse rendering of alfalfa than that obtained for the acid precipitates. Subba Rau *et al.* (1969) reported an even greater carbohydrate content (16%) for their cytoplasmic fraction.

However, this was obtained by difference rather than by analysis. Most preparations of LPC have high contents of lipid--12-25% (Pirie, 1960; Lea and Parr, 1961; Smith, 1966; Shah, 1968; Subba Rau *et al.*, 1969)--because the concentrate, unless solvent extracted, contains chlorophyll and associated lipids. However, Spencer *et al.* (1969, 1971) reported the fat content of their protein-chlorophyll-xanthophyll concentrate (PRO-XAN) to be only 7-9%. Subba Rau *et al.* (1969) reported their cytoplasmic fraction to be low in lipid (5%). However, this is still slightly greater than contents obtained for the acid precipitates; although the ash contents of the two are similar. On the other hand, the PRO-XAN concentrate was reported by Spencer *et al.* (1969, 1971) to have a much higher ash content (10-13%).

In contrast, the acid supernatant contained only about 25% protein, 33% carbohydrate, 2.5% lipid, and 5.5% ash. The only literature comparison that can be made for the acid supernatant is with the alfalfa solubles obtained as a by-product of the wet fractionation procedure. Spencer *et al.* (1969, 1971) reported comparable protein contents. The supernatant contained a somewhat greater lipid content and about four times less ash than the alfalfa solubles.

Both fractions showed changes in composition between cuttings (see Figures 18 and 19). Most striking was the decrease in protein content from the first to second cuttings; the acid precipitates dropped from 82-68% and the acid supernatants from 34-22%. The decrease was only about 2% between the second and third cutting. However, commensurate increases in carbohydrate, lipid, or ash contents were not encountered. There was a small increase in the carbohydrate content of the acid precipitate from the first to third harvest. The carbohydrate content of the acid supernatant, on the other hand, gradually decreased from 36-31%.

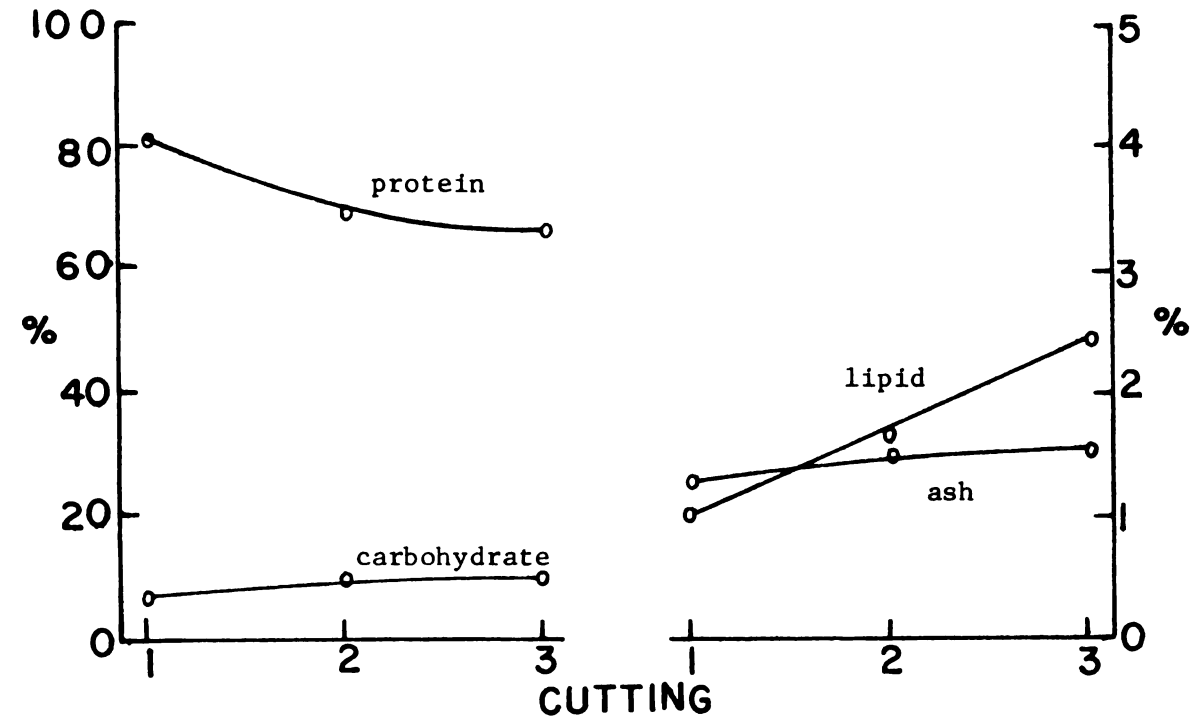

Figure 18. Changes in protein, carbohydrate, ash, and lipid contents of the acid precipitate from the first through the third cutting.

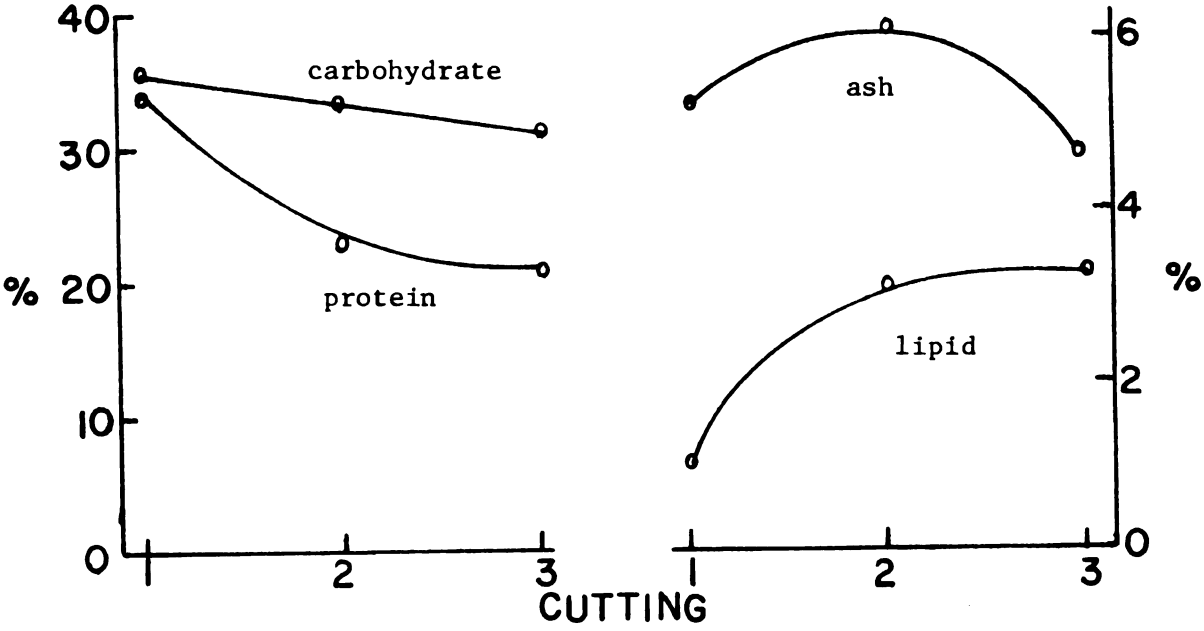

Figure 19. Changes in protein, carbohydrate, ash, and lipid contents of the acid supernatant from the first through the third cutting.

Both fractions exhibited increases in total lipid. Pirie (1961c) reported that lipid content is independent of the number of cuttings. The ash content was essentially constant for all regrowths.

These four components accounted for from 80-90% of the precipitate fractions but for only from 60-75% of the supernatant. It is conjectured that the supernatant fraction contains significant amounts of phenolic and polycyclic type compounds, e.g., tannins, saponins, etc., which have been found in plant extracts (cf. Singleton and Kratzer, 1969; Birk, 1969).

Amino acid composition. The amino acid compositions of the acid precipitate and acid supernatant from each cutting are given in Table 33 and are compared with the essential amino acid composition of whole egg protein. Composition expressed in moles of amino acid per 1000 moles amino acids and in grams of amino acid per 100 grams of sample are given in Tables A2 and A3, respectively, of the appendix.

The precipitate and supernatant, as might be expected, show somewhat different distributions of amino acids. In general, the precipitates have greater contents of Met, Leu, Tyr, phenylalanine (Phe) and Arg, and lesser amounts of Cys, aspartic acid (Asp), serine (Ser) and proline (Pro) than the supernatants. Neither fraction exhibits unusually high or low concentrations of any particular amino acid residue.

Amino acid compositions which have been published are generally of LPC which was prepared by direct precipitation from the juice; and therefore contains chloroplastic proteins. Nevertheless, the acid precipitate shows a distribution of amino acids very similar to that reported by Gerloff *et al.* (1965) for alfalfa LPC obtained by impulse rendering, except that the Met contents are twice those given by Gerloff *et al.* (1965). This is understandable since they did not determine Met

Table 33. Amino acid composition of alfalfa leaf protein fractions expressed as grams of amino acid/100 grams protein

Amino acid	Protein fraction						
	1P3*	2P3	3P3	1S3	2S3	3S3	Egg**
Lysine	6.60	6.89	9.21	8.24	7.53	6.76	6.4
Threonine	5.17	5.14	5.17	6.08	6.10	5.74	5.1
Valine	6.94	7.17	6.43	6.41	5.49	5.65	7.3
Methionine	3.11	2.47	2.39	1.82	1.93	1.07	---
Cystine	1.15	1.19	1.12	3.37	3.05	3.75	---
(met + cys)	4.26	3.66	3.51	3.64	3.86	2.14	5.5
Isoleucine	5.16	5.73	4.92	5.00	4.52	4.55	6.6
Leucine	8.78	9.10	8.35	6.61	5.63	5.74	8.8
Tyrosine	4.35	4.45	5.03	2.83	3.51	3.16	---
Phenylalanine	6.38	6.69	5.99	4.14	4.18	4.16	---
(phe + tyr)	10.73	11.14	11.02	6.97	7.69	7.32	10.0
Tryptophan	2.48	2.91	2.51	1.85	2.94	2.72	1.6
Histidine	3.31	2.68	3.01	2.19	2.43	2.17	---
Arginine	7.93	7.46	7.15	3.87	4.19	3.72	---
Aspartic acid	9.92	10.46	9.86	12.93	13.76	14.91	---
Serine	2.58	3.07	3.19	5.51	6.02	5.41	---
Glutamic acid	11.33	11.65	10.63	11.97	12.51	13.73	---
Proline	4.45	4.54	4.11	5.49	6.00	5.96	---
Glycine	4.72	4.87	4.44	5.71	5.27	5.60	---
Alanine	5.64	3.51	6.49	5.97	4.93	5.22	---

*see methods section, p. 79, for explanation of nomenclature.

**FAO/WHO, *Protein Requirements*, 1965.

on samples oxidized prior to acid hydrolysis. The acid precipitate also shows a similar composition, excepting Met and Trp, to that reported by Smith (1966) for alfalfa cytoplasmic protein. Again this difference is probably due to differences in analytical techniques. However, the acid precipitate does not show such close agreement with the amino acid composition of lucerne cytoplasmic protein determined by Wilson and Tilley (1965). The acid precipitate had higher concentrations of Met and Phe and a much lower content of Arg than the cytoplasmic fraction.

The protein liquor is generally discarded and the author is not aware of any published amino acid compositions of this fraction. Hartman *et al.* (1967) published the amino acid composition of alfalfa juice extracted from two cuttings of alfalfa. The amino acid distribution for the juice is, with the exception of Met, similar to that obtained for the acid precipitate. There is a much larger variance noticeable between the juice and the acid supernatant; particularly with Lys and Cys in the first cutting, leucine in the second cutting, and Phe and Arg in both cuttings.

There is little difference in the amino acid composition of similar fractions prepared from different cuttings. Although significant work is lacking on amino acid composition of alfalfa LPC extracted from regrowths, leaf protein generally shows a more or less constant amino acid distribution irrespective of species or maturity (cf. Gerloff *et al.*, 1965; Pirie, 1966c). However, a few trends are apparent. The acid precipitates prepared from successive cuttings during the season showed an increase in Lys of nearly 40% with smaller increases in Ser (24%) and Tyr (16%) and decreases in Met (23%) and Arg (10%). The supernatant exhibited small increases in two nonessential amino acids--Glu (15%) and Asp (15%)--with a decrease in Lys (18%). The amino acid

distribution in alfalfa juice from the first and second cuttings also showed reasonable consistency (Hartman *et al.*, 1967). The major change from the first to second cutting was a 30% increase in Cys; smaller variations were in Lys (+16%), Arg (+13%), Met (-18%), and Asp (-18%). The only correspondence between the present study and these literature values appears to be the increase in Lys and decrease in Met in the acid precipitate from the first to the second cuttings.

To evaluate the potential nutritional value of a protein, a comparison can be made of its essential amino acid pattern relative to a standard protein of known "high" nutritive value. The chemical score (CS) expresses the quality of a protein in relation to its limiting amino acid, i.e., that essential amino acid present in smallest amount in relation to its requirement, and thus, becomes a means of predicting maximum theoretical efficiency of protein utilization. This value is derived by computing the concentration ratios of the individual essential amino acids between the test protein and a reference protein (Mitchell and Block, 1946). The lowest ratio (x 100) is the chemical score. Unfortunately, no perfect reference protein exists (Cresta *et al.*, 1969). In 1957 FAO published a provisional essential amino acid pattern which has been used as a reference protein. The other most frequently used reference proteins are milk (either cow's or human) and egg. In 1965 FAO/WHO adopted the essential amino acid pattern of whole egg protein in preference to the 1957 provisional pattern. Cresta *et al.* (1969) concluded that when the CS is used to estimate the ability of a food protein to maintain the nitrogen balance the pattern of milk protein is more suitable than egg protein. However, when the CS is used as an estimation of the capacity of the proteins to meet growth requirements (protein efficiency ratio), the egg protein pattern appears to be more satisfactory.

Chemical scores for the acid precipitates and supernatants calculated against all four reference proteins are listed in Table 34. For the purpose of these calculations, Cys and Tyr were included at a level not to exceed that provided by Met and Phe, respectively.

Table 34. Chemical scores of alfalfa leaf protein fractions

Reference protein*	Leaf fraction					
	1P3	2P3	3P3	1S3	2S3	3S3
1957 FAO provisional pattern	100[a**]	87[a]	84[a]	87[a]	92[a]	51[a]
Egg	78[a]	67[a]	64[a]	66[a]	71[a]	39[a]
Cow's milk	81[b]	88[c]	77[b]	67[d]	57[d]	58[d]
Human milk	81[b]	85[a]	77[b]	69[e]	63[d]	50[a]

*essential amino acid patterns from FAO/WHO, Table 6 (1965)

**limiting amino acid: [a]S-aa; [b]ile; [c]lys; [d]leu; [e]aromatic aa

Chemical scores calculated in reference to the provisional pattern and egg in all cases were based on the sulfur-amino acids as the limiting amino acid. Methionine has been stated to be the limiting amino acid in LPC by several investigators (cf. Gerloff *et al.*, 1965; Singh, 1967; Sentheshanmuganathan and Durand, 1969). Against the provisional pattern, all fractions with the exception of 3S3 have relatively high CS, i.e., between 84 and 100. These values exceed those for cereal grains, oilseeds, casein, and are approximately equal to beef liver (FAO/WHO, 1965). With egg protein as a reference, the relative order of CS is the same but the absolute values are lower (64-78, excepting 3S3), but are still not sufficiently low to be classed as poor. Smith (1966) reported the chemical scores of alfalfa LPC and cytoplasmic protein to be 57 and 65,

respectively, with the sulfur-amino acids limiting. Since milk (cow and human) protein is also considered limiting in the sulfur-amino acids, CS calculated relative to milk are generally slightly higher than those based on egg. Thus, in 1P3 and 3P3 Ile becomes limiting and in 2P3 either Lys or the sulfur-amino acids are limiting. The supernatant fractions, when referred to cow's milk, show Leu to be limiting, and when referred to human milk, show the aromatic-amino acids, Leu and the sulfur-amino acids to be limiting for the first, second, and third cuttings, respectively.

With a CS range of 77-88 against milk and 64-78 against egg, the acid precipitated protein might be better for maintenance than for growth according to the criteria of Cresta *et al.* (1969). However, CS values for the supernatant fraction relative to milk are within the range of egg, thus it would be considered as a protein source equivalent for maintenance and growth. Ranking of the six samples in order of decreasing protein quality as evaluated by CS values obviously depends on which protein is used as a reference.

The CS is an adequate first approximation for screening protein for nutritional quality. However, the entire essential amino acid picture should be considered for more meaningful evaluation. The ratios (as percentages) of each essential amino acid for each fraction to each standard--FAO, egg, cow's milk, and human milk--are given in Tables 35, 36, 37, and 38, respectively.

Each of the non-limiting essential amino acids is present in an amount greater than the 1957 FAO provisional pattern for all fractions studied. This was also noted by Buchanan (1969b) for wheat LPC and by Sentheshanmuganathan and Durand (1969) for LPC extracted from various Indian plants. Relative to egg, Val and Ile represent the second and

Table 35. Percentage of each essential amino acid as compared to that in the 1957 FAO provisional pattern

Amino acid	Fraction					
	1P3	2P3	3P3	1S3	2S3	3S3
Lys	100	→	→	→	→	→
Thr	100	→	→	→	→	→
Met + (Cys)	100	87.1	83.6	86.7	91.9	51.0
Val	100	→	→	→	→	→
Ile	100	→	→	→	→	→
Leu	100	→	→	→	→	→
Phe + (Tyr)	100	→	→	→	→	→
Trp	100	→	→	→	→	→

Table 36. Percentage of each essential amino acid as compared to that in egg

Amino acid	Fraction					
	1P3	2P3	3P3	1S3	2S3	3S3
Lys	100	→	→	→	→	→
Thr	100	→	→	→	→	→
Met + (Cys)	77.5	66.5	63.8	66.2	70.2	38.9
Val	95	96.8	88.1	87.8	75.2	77.4
Ile	78.2	86.8	74.5	75.8	68.5	68.9
Leu	100	100	94.9	75.1	64.0	65.2
Phe + (Tyr)	100	→	→	69.7	76.9	73.2
Trp	100	→	→	→	→	→

Table 37. Percentage of each essential amino acid as compared to that in cow's milk

Amino acid	Fraction					
	1P3	2P3	3P3	1S3	2S3	3S3
Lys	85.4	88.3	100	100	96.5	86.7
Thr	100	→	→	→	→	→
Met + (Cys)	100	→	→	→	→	64.8
Val	100	100	93.2	92.9	79.6	81.9
Ile	80.6	89.5	76.9	78.1	70.6	71.1
Leu	88.7	91.9	84.3	66.8	56.9	58.0
Phe + (Tyr)	100	100	94.9	75.1	64.0	65.2
Trp	100	→	→	→	→	→

Table 38. Percentage of each essential amino acid as compared to that in human milk

Amino acid	Fraction					
	1P3	2P3	3P3	1S3	2S3	3S3
Lys	100	→	→	→	→	→
Thr	100	→	→	→	→	→
Met + (Cys)	99.1	85.1	81.6	84.7	89.8	49.8
Val	100	100	97.4	97.1	83.2	85.6
Ile	80.6	89.5	76.9	78.1	70.6	71.1
Leu	99.8	100	93.8	74.3	63.3	64.5
Phe + (Tyr)	100	→	→	69.0	76.1	72.5
Trp	100	→	→	→	→	→

third, respectively, limiting essential amino acids in the acid precipitate. The other residues exist in concentrations comparable with egg. An analysis of the supernatant fraction shows that, in addition to the sulfur-amino acids, Val, Ile, Leu, and the aromatic residues are present in lower concentrations than in egg (64-88%). It is especially important to note that in both fractions threonine (Thr), Lys, and Trp, which are often limiting in real diets, particularly cereal, are in concentrations greater than that of egg. Therefore, these two fractions should be considered as important potential supplements to diets low in these amino acids--e.g., corn meal, limiting in Trp; rye, limiting in Thr; oat, rice, limiting in Lys (FAO/WHO, 1965). It must be remembered that leaf protein would never be used as the sole source of nitrogen. Therefore, its ability to complement the amino acid composition of other common dietary proteins should also be considered. Chayen *et al.* (1961) stated that supplementation of other protein foodstuffs appears to be the greatest potential value of leaf protein preparations.

Compared to milk, the concentrations of the non-limiting essential amino acids of the acid precipitate vary from 82-100% of those in the reference; whereas for the supernatant fraction, they range from 64-100% of those in the reference.

The precipitate appears to have a more desirable amino acid distribution and possesses a lower ratio of non-essential to essential amino acids. The ratios (N/E) are as follows:

1P3	1.00	1S3	1.23
2P3	0.93	2S3	1.29
3P3	0.96	3S3	1.46

The total essential amino acid pattern is considered when calculating the essential amino acid index (EAA-I) which is a more sophisticated type of chemical score. This value is defined as the geometric mean of

the ten essential amino acid (includes Arg and His) egg ratios; the egg ratio being the ratio of an essential amino acid in a protein relative to its concentration in whole egg protein (Oser, 1951). EAA-I values for the leaf fractions are listed with some standard proteins calculated by Oser (1951):

1P3	94.6	1S3	81.1	Egg	100
2P3	94.4	2S3	80.4	Casein	89
3P3	91.2	3S3	74.2	Wheat	67

As was the case for the CS, the EAA-I is more valuable in distinguishing wide variations than narrow ones, but gives the same general conclusion, i.e., "good" for the precipitate, "fair" for the supernatants from the first and second cuttings, and "poor" for the supernatant from the last cutting.

The general conclusion reached in the literature is that LPC has a favorable balance of essential amino acids and non-essential amino acids--except Met; therefore, it should be a well balanced source of dietary protein if supplemented with Met. It would also be a better supplement than most of the seed proteins but not as good as casein or egg (cf. Gerloff *et al.*, 1965; Stahmann, 1968a,b; Pirie, 1969b).

A similar conclusion was reached here for the acid precipitate except that Met did not appear to be as limiting; in fact, the Met contents were similar to that of milk. Since Smith (1966) has shown the amino acid compositions of alfalfa chloroplastic and cytoplasmic fractions to be similar, the slightly better nutritive value, as indicated by composition, for the acid precipitate is probably due to the identification of Met as Met sulfone rather than to the absence of the chloroplastic proteins.

Composition alone can be a misleading indicator of nutritive value. Chemical evaluations of protein quality are primarily based on the premise

that the pattern of amino acids absorbed during the process of digestion reflect the total amino acid composition of the ingested protein. Thus, they do not evaluate digestibility, absorption, and utilization of the protein or other components in the protein source.

Biological Evaluation

Protein efficiency ratio. The nutritive value of the fractions can be evaluated better by biological assays than by examining the amino acid composition alone; for bioassays provide data reflecting the sum total of the protein's utilization. One common method is measuring the rate of growth of an animal under defined conditions. If there is a deficiency of one or more essential amino acids, growth will be reduced (McLaughlan and Campbell, 1969).

The particular assay chosen for this study was the protein efficiency ratio--PER (Osborne, Mendel, and Ferry, 1919). In this assay, growth is related to protein intake. Although enjoying widespread use, the PER assay elicits one major criticism; namely, that no allowance is made for maintenance. However, no matter what method of protein quality evaluation is used, the various proteins are placed into the same relative quality ranges in regard to protein nutrition (Bergen, 1971). Therefore, despite its limitations, the PER method appears to be as precise and simple as any protein evaluation presently available (McLaughlan and Campbell, 1969).

The rat is the classical animal used for biological assays of protein quality. However, in this study sufficient quantities of the leaf protein preparations were not available to conduct conventional feeding studies with rats. Therefore, the much smaller meadow vole (*Microtus pennsylvanicus*) was chosen as the experimental animal. Only 5 g of diet

at a 7% level of protein per day are required and a PER can be obtained in five or six days. Elliott and associates (Elliott, 1963a; Markarian and Elliott, 1968; Shenk, Elliott, and Thomas, 1970) demonstrated that the meadow vole can be used successfully for bioassays. It has been used primarily in studies with forage plants (Elliott, 1963a; Schillinger and Elliott, 1966a).

The PER for each diet obtained from replications 1, 2, 4, 5, and 7 are reported in Table 39. The mean value and its standard error for each protein source are also included. Analysis of variance could not be done meaningfully due to insufficient replications and heterogeneous variance. However, five replications do give relatively reliable mean values. Replication 3 was not useable as the three animals on the acid precipitate fractions emptied their water bottles. The resulting dehydration was no doubt partially responsible for the observed loss of weight or reduced gain. Protein efficiency ratios of replication 6 were somewhat erratic, including the control (PER of less than 1), indicating a poor growing litter. The control for replication 5 killed

Table 39. Protein efficiency ratios of alfalfa leaf protein fractions and casein

Replication	Protein source						
	Casein	1P3*	2P3	3P3	1S3	2S3	3S3
One	1.22	2.03	1.85	1.38	---	died	1.58
Two	2.50	2.05	2.40	2.62	---	died	-0.24
Four	2.72	1.79	2.82	3.18	---	died	0.27
Five	2.46	1.05	2.32	1.08	2.12	died	-0.84
Seven	3.55	3.43	2.93	3.37	1.61	died	died
Mean	2.49	2.07	2.46	2.33	1.87	died	0.19
Standard error	0.34	0.35	0.17	0.42	0.18	0.0	0.45

*see p. 79 for explanation of nomenclature.

itself on the fourth day by climbing up into the top of his feeder. Therefore, an average of the control values for replications 1, 2, 3, 4, and 7 was used for this litter. There is no value for 1S3 in the fourth replication because the animal escaped.

The differences in growth rates between litters (Whitmoyer, 1956) is immediately apparent, emphasizing the requirement for placing one animal of each litter on a different diet rather than one diet per litter; and for including a control for comparison in each litter. The casein PER mean of 2.49 agrees with the accepted PER value of 2.5 (Elliott, personal communication). This result gives validity to the PER means obtained for the experimental diets.

Consider first the acid precipitates. The fraction prepared from the second cutting had a PER of 2.46 which is comparable to casein; and the PER from the third cutting fraction was 2.33 which is only slightly lower than that for casein. Thus these two fractions appear to possess protein of good biological quality. The acid precipitate obtained from the first cutting gave a lower PER (2.07) than casein or the same fractions from latter cuttings, but still was greater than 2.0.* Thus, the biological assays reflected the general conclusion reached by amino acid analyses, i.e., that the acid precipitate appears to be a protein with a nutritive value close to casein. Both are limiting to a similar degree in the sulfur-amino acids.

In the literature most workers claim LPC, undamaged by inappropriate drying methods, to have a nutritive value as great as or better than the best seed proteins or fish meal but not as effective for the

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*PERs greater than 3, from 2-3, from 1-2, and less than 1 indicate protein of excellent, good, fair, and poor nutritive value, respectively.

nutrition of young animals as casein or egg (cf. Pirie, 1968; Henry and Ford, 1965). Shurpalekar *et al.* (1967) reported freeze-dried lucerne LPC, unsupplemented with vitamins or minerals, to have a PER of only 1.54, significantly less than skim milk powder. This value was increased to 2.77 with methionine supplementation. Singh (1969) reported a PER range of only 1.3-1.8 for lucerne LPC, which is less than that obtained from the acid precipitates. Subba Rau *et al.* (1969) reported a PER (1.36) for lucerne LPC in the range of Singh (1969). However, their cytoplasmic fraction appeared to have a higher nutritive value as evidenced by a PER of 2.02. This is still somewhat lower than values obtained for our comparable acid precipitate fractions.

The reasons for the differences in PER between the first and last two cuttings were not obvious from amino acid composition. Variance in nutritive value, as evaluated by biological assay, between regrowths has not been systematically investigated. Byers (1967b) reported in her *in vitro* digestibility study that the results from regrowths were erratic. Both Woodham (1965) and Singh (1969) noted that different batches of protein preparations from the same species of plants may vary in nutritive quality. In this study the last two cuttings gave lower yields and precipitates with lower protein contents than the first cutting, yet yielded a protein of slightly higher nutritive value when evaluated biologically. This agrees with the statement of Byers (1961) that high extractability does not necessarily mean the product isolated is of high nutritive value and vice versa.

Although the PER for leaf protein fed as the sole source of nitrogen to young animals is not necessarily a satisfactory estimate of nutritive value for humans, any bioassay is probably a better index of nutritive value for man than any *in vitro* method (Buchanan, 1969b). However, the

value of a protein supplement, which leaf protein would be, depends not only on its limiting amino acid but on the excesses of essential amino acids in the supplement and how well this excess makes up deficiencies in the diet being supplemented. Therefore, although it was necessary and important to know the PER values of the protein fractions individually, their true worth as a dietary supplement can only be assessed when incorporated into "practical" diets. Subrahmanyan and Sur (1949), Sur and Subrahmanyan (1954), Shurpalekar *et al.* (1967), and Doraiswamy *et al.* (1969) have shown that lucerne powder and lucerne LPC are good supplements to the poor Indian rice and ragi diets.

Meaningful conclusions, based on only two replications, are difficult to draw for the supernatant fraction obtained from the first cutting (1S3). Yet, these limited data indicate that this fraction is only fair in quality.

Quite obviously, the animals did not prosper on the second cutting supernatant fraction (2S3); usually dying within 24-48 hr after initiation of the experimental diet. Their feed intake generally was somewhat reduced (3-21 g) but proportional to the length of time they survived. Therefore, one can conclude that they did not die from starvation due to rejection of the diet, but probably from a toxic component in the diet. This toxic factor appears to be present in a lower concentration in the supernatant fraction from the last cutting (3S3). With the exception of the last litter, these animals appeared to be normal although they were not responding in growth (PER = 0.19). And, unlike the animals on 2S3, their intake of food was not reduced below normal; see Table 40. The acid precipitate values from replication 3 are not included in this table because absence of water would exert an effect on diet intake. Intake was considered normal for all experimental diets, except for 2S3.

Table 40. Feed intakes (in g/day) of voles

Replication	Protein source						
	Casein	1P3	2P3	3P3	1S3	2S3	3S3
One	3.8	3.9	3.9	4.9	---	---	4.5
Two	3.3	3.3	3.6	5.0	---	---	3.8
Three	3.8	---	---	---	---	2.8[a]	4.5
Four	5.0	3.9	4.2	4.2	---	2.6[a]	4.5
Five	---	4.1	4.8	3.8	3.3	2.4[b]	4.3
Six	3.3	3.9	5.1	5.1	4.0[e]	4.2[e]	4.8
Seven	4.3[e]	4.2[e]	4.3[e]	5.0[d]	5.9[c]	3.5[b]	---
Mean	3.9	4.0	4.3	4.7	4.4	3.1	4.4

[d] length of replication: [a]one day; [b]two days; [c]three days; [d]four days; [e]five days; all others 6 days

Toxicity. In early feeding experiments, Cowlishaw *et al.* (1956a,b) reported that lucerne LPC contained a water soluble toxic factor (which was claimed to be saponin); its effect could be counteracted by adding cholesterol to the diet or by washing the precipitate with hot water. They also noted (Cowlishaw *et al.*, 1956b) that the dried whole juice had a low nutritive value. In contrast, Hartman *et al.* (1967) claimed that spray-dried alfalfa juice had a nutritive value equal to that of LPC. However, their evaluation was based on chemical assays and *in vitro* digestions. Later, Subba Rau *et al.* (1969) determined the PER of spray dried lucerne juice to be only 0.34. One of the eight animals on this diet died and one half of them lost weight; thus, their results were similar to those obtained in this study for 3S3. Also, in both cases differences in amino acid composition were not sufficient to account for the nutritional variation between the soluble extracts (whole juice or acid supernatant) and the precipitated fractions.

Aqueous extracts of alfalfa have been shown to contain saponins, to which a variety of diverse physiological effects have been attributed (cf. Scardavi and Elliott, 1967; Birk, 1969). Therefore, saponin toxicity is a possible answer to the poor performance observed with animals on the supernatant diets. In the semiquantitative analyses for saponin level of the vole diets, the acid precipitates showed negligible to zero red blood cell hemolysis, whereas the acid supernatants exhibited an abnormally high degree of red cell hemolysis, indicating high saponin level. Quantitating the degree of red cell hemolysis caused by the supernatant fraction diets was difficult due to their natural brown color. Although only low saponin alfalfa plants were used, the water soluble saponins may have concentrated in the supernatant fraction during the extraction process. Saponin levels in the plant are generally highest in the second cutting (Elliott, personal communication; Hanson *et al.*, 1963) which may be the reason for the increased toxicity of that supernatant fraction. Also, since the latter two cuttings had protein contents of only two-thirds that of the first cutting, only two-thirds as much of the first cutting fraction was required to achieve equivalent protein levels in the diets. Consequently, these animals (1S3) could have received a lower concentration of the toxin, which may account for the fair performance the voles showed on 1S3 as compared to the obvious toxicity of 2S3 and 3S3.

Autopsies were performed in a further attempt to elucidate the cause of the toxicity of these diets and to determine the extent of their pathological effects on the voles. With replications five (vole fed 2S3) and six (voles fed casein, 1S3, 2S3, 3S3, and 3P3) only gross abnormalities were looked for. The vole fed casein exhibited a full body cavity with no fluid, a full and firm intestinal tract, a cecum

12.5 cm long with a greenish-yellow color, and a visible liver. Voles carry out some digestion in the cecum, therefore its size is of importance. The two voles which had been fed the very toxic 2S3 showed an absence of body fat, edema of the mesentery and intestinal wall and red fluid in the body cavity (ascites). The intestinal tract contained only a small amount of mucus (plus in one animal some white flecks of unknown origin similar to mucus). The cecums were of small diameter, shorter than normal (6 and 10.5 cm), and not completely full--containing only some semi-solid, brown, fecal-like material. The lungs, heart, kidney, and liver showed normal color, position, and shape. However, the liver of one of the voles was slightly smaller than normal and hidden under the thoracic cage. These symptoms are indistinguishable from those of starvation. However, feed intakes had indicated the latter not to be the cause of death.

The voles fed 1S3 and 3S3 exhibited a few of the above symptoms but to a much lesser degree. Both showed a small amount of ascites. Again lungs, heart, kidney, and liver were normal except that the vole fed 1S3 had a smaller liver. This vole's intestinal tract was filled with the previously mentioned semi-solid material but it had a slight yellowish color (more normal). The tract of the vole fed 3S3 was fuller and showed better tone. Both cecums were full, but they varied in length--13 and 10 cm for the voles fed 1S3 and 3S3, respectively.

The vole which had been fed the diet based on the acid precipitate from the third cutting showed no abnormalities--i.e., the body cavity was full with no fluid, the liver was normal color and size, and the cecum was 12 cm long and had a yellowish-green color.

Histopathologic examination of those voles of the last replication showed similar lesions in the voles fed 2S3 and 3S3. These

consisted of necrosis of the mucosa of the duodenum. The epithelial cells were desquamated with only a few cells at the base of the mucosa. There appeared to be an excess of mucus production in the intestinal tract. The vole fed 2S3 also had an unidentified precipitate in the tubules of the medulla in the kidney. However, there was no necrosis of tubular epithelium. No significant lesions were found in the liver, kidney, or brain. In summary the histopathological examination indicated the primary lesion in the voles fed the supernatant fractions from the last two cuttings to be enteritis.

Most of the published physiological effects of alfalfa saponins are concerned with growth depression in chicks and ruminant bloat (cf. Scardavi and Elliott, 1967; Birk, 1969). However, Hanson *et al.* (1963) did note that saponins were reported to have an irritating effect on mucous membranes. Sollman (1957) reported that saponins are not absorbed from the intestine so that acute oral doses produce only local effects--marked increase of mucus and moderate increase of fluid; death resulting from inflammation of the alimentary canal. This conforms to the autopsy results, and in addition to the other characteristics which saponins and the supernatant fractions exhibit--growth depression, bitter taste, non-dialyzable, foaming in aqueous solution, and hemolysis of red blood cells--suggest that saponins probably exerted an important role in the toxicity of the supernatant fractions. But before any significant property is unequivocally attributed to a saponin, it should be isolated in pure form and an investigation of its composition, structure, and activity carried out (Birk, 1969).

There are also other possible toxic agents which must be considered. An important group is products resulting from the "browning reactions." The reactants--amine, sugar, and phenolic groups--are present in the

supernatant. Browning can result in the following characteristics also possessed by the acid supernatant: brown discoloration, bitter flavor, tendency to foam, loss of nutritive value, and most important--possible toxic effects (Friedman and Shibko, 1969). Although the voles eating 2S3 usually became listless and shivered and often became hunch-backed, one (replicate 5) exhibited hyperexcitability and continuously ran in circles--similar to symptoms exhibited by mice fed browning reaction reductones (Cutting *et al.*, 1960; Ambrose *et al.*, 1961).

The supernatant fraction was observed to contain non-coaguable and non-dialyzable peptides and/or protein. Ramirez and Mitchell (1960) believed alfalfa trypsin inhibitor to have these characteristics. Additional compounds which have been extracted from plant materials, including alfalfa, and shown to be toxic include alkaloids, phenols, tannins, and other unknown factors (Keeler, 1969; Elliott, 1963a,b; Singleton and Kratzer, 1969; Schillinger and Elliott, 1966b). Undoubtedly several of these factors could have contributed to the toxicity of the supernatant fractions. Before further speculation is made as to the use of this by-product as a feed supplement (cf. Pirie, 1966c; Spencer *et al.*, 1969) further testing with various species of animals and characterization and separation of the toxic component(s) will obviously be necessary.

Acceptability

Several investigators (cf. Guha, 1960; Byers *et al.*, 1965; Oke, 1966a) have claimed that green LPC can be successfully incorporated into several foods. Others (cf. Smith, 1966; Kinsella, 1970) have claimed the green color and grassy flavor to be objectionable. Since nutritive value has little meaning if the product is objectionable for aesthetic

or other reasons, the protein fractions were incorporated into plain sugar cookies with no attempt to hide flavor or color. The mean values (hedonic scale) and statistical significance for each of the characteristics evaluated--color, flavor, texture, and general acceptability--are given in Tables 41 and 42.

Table 41. Mean values and statistical significance for organoleptic variables for the acid precipitate

Level of alfalfa protein (%)	Color*	Flavor*	Texture*	General* acceptability
0.0	7.23^{A}	7.10^{A}	$6.65^{a,A}$	7.00^{A}
1.0	5.72^{B}	5.75^{B}	$6.00^{b,B}$	5.85^{B}
2.5	5.55^{B}	4.45^{C}	$5.23^{c,C}$	4.83^{C}
5.0	4.60^{C}	3.05^{D}	$4.43^{d,C}$	3.53^{D}

*means with same superscript form a statistically homogeneous group: lower case letter, P = .05; upper case letter, P = .01

The cookies made from the acid precipitate had a peppery appearance which varied with the amount of supplementation. In comparison to a plain white cookie, it was not as acceptable but was not considered objectionable. Texture was rated similarly to color. With increasing supplementation a more noticeable grainy or branny texture appeared. General acceptability appeared to be most closely related to the flavor rating, but was always slightly higher (0.1-0.5 point). Both varied about one unit between levels, i.e., control about 7 (like moderately), 1% supplementation about 6 (like slightly), 2.5% supplementation about 4.5-5 (neigher like nor dislike), and 5% supplementation about 3-3.5 (dislike moderately to slightly). For these two variables, each of the concentration levels was statistically significantly different from each

other. It was necessary to add 5% alfalfa protein (i.e., substitution of 20% of the flour) to induce unacceptability. The principal objection was usually referred to as a mild grassy flavor, the degree of which increased with greater amounts of supplementation. This is in agreement with Kamalanathan *et al.* (1969), who found that the color and texture were relatively minor problems when they incorporated LPC into various preparations. The leafy flavor, however, had an adverse influence on the preparations. Pirie (1959c) and Morrison and Pirie (1960) maintain that LPC has little or no flavor of its own and that it blends well with moderate levels of spice or other flavoring agents.

Table 42. Mean values and statistical significance for organoleptic variables for the acid supernatant

Level of alfalfa protein (%)	Color*	Flavor*	Texture*	General* acceptability
0.0	7.20^{A}	7.40^{A}	7.25^{A}	7.33^{A}
1.0	5.45^{B}	1.75^{B}	5.20^{B}	2.40^{B}
2.5	4.60^{B}	1.25^{B}	3.30^{C}	1.55^{B}

*means with same superscript form a statistically homogeneous group: lower case letter, P = .05; upper case letter, P = .01.

The acid supernatant was unacceptable at both levels of fortification. However, the medium to dark brown color was not objectionable. The texture at the 1% level was acceptable, but became hard and unacceptable at the 2.5% level. Flavor appeared to be the major factor in determining general acceptability. Comments indicated that the flavor at all levels of fortification was objectionable due to its extreme and sharp bitterness. In both panels, the judges did not agree absolutely

on the numerical ratings but almost always rated the various samples in the same relative order.

In conclusion, the acid precipitate can be substituted for flour (up to a level of 10%) in a plain cookie without objection. The acceptable level would undoubtedly increase if incorporated into a food with a stronger flavor, e.g., chocolate, peanut butter, or highly spiced dishes. Kamalanathan *et al.* (1969) noted that the flavor could be successfully masked by spices, banana being particularly effective. Their conclusion was that for better acceptability LPC should be incorporated into highly spiced or flavored preparations. Also acceptability appears to depend on the particular cultural food habits or prejudices of the consumer (cf. Buchanan, 1968; Pirie, 1969b,d).

CONCLUSION

A relatively simple method was used to isolate two protein fractions--one acid insoluble and the other acid soluble--from juice extracted from fresh alfalfa. In brief: the alfalfa was blended with water and pressed, the resulting juice heated to 48 C followed by centrifugation to remove the chloroplasts, the pH of the supernatant raised to 8.5 and the precipitate removed by centrifugation, and lastly, this second supernatant was acidified and the two final fractions separated by centrifugation. No significant proteolysis was noted in the juice under the conditions of the extraction. Incubation of the juice with cellulase, amylase, or pectinase did not increase the purity (i.e., protein content) of the acid precipitate, the fraction of major interest. Extractability of nitrogen was largely dependent on the condition of the alfalfa at the time of harvest; being highest if the plant was young (1/10 bloom), green and tender. (However, biological assays showed the protein from the plants which exhibited lower extractability to have the higher nutritive value.) With alfalfa in good condition, nearly 60% of the nitrogen of the leaf was extracted into the juice and of this extracted nitrogen about 27% appeared in the acid precipitate and 30% in the acid supernatant. On the other hand, for partially yellowed or mature alfalfa relatively high in dry matter extractability was only about 40%, with only 21% of the extracted nitrogen being precipitable by acid and 40% remaining in the acid supernatant. Yields increased with higher nitrogen content in the leaf; but this increase resulted

from the greater amounts of nitrogen in the final fractions, not necessarily from increased extractability.

The acid precipitate contained approximately 72% protein, 8% carbohydrate, 2% lipid, and 1.5% ash on a dry basis. In contrast, the dry matter of the acid supernatant contained only about 25% protein, 33% carbohydrate, 2.5% lipid, and 5.5% ash. There were compositional changes between fractions obtained from the three cuttings made during the summer. Most noticeable was the decrease in protein contents of both the acid precipitates and acid supernatants from the first to second cuttings. However, there was little variation in amino acid composition between fractions from different cuttings. The acid precipitate had a slightly more favorable balance of essential amino acids than the acid supernatant; although methionine was the limiting amino acid in both fractions from all three cuttings. Resulting chemical scores, based on egg protein, with the exception of the acid supernatant from the third cutting, ranged from 64-78. Particularly significant is the fact that in all fractions threonine, lysine, and tryptophan--often limiting in many human diets--are present in concentrations greater than that of egg.

As evaluated by protein efficiency ratio, the nutritive value of the acid precipitate varied slightly with cutting (PER = 2.1, 2.5, 2.3 for the first, second, and third cutting fractions, respectively), that of the second cutting fraction being equal to that of casein. However, the supernatant fractions appeared to be toxic, the degree of toxicity highest with the second cutting fraction and lowest with the first cutting fraction. Necropsy indicated the primary lesion to be inflammation of the intestinal tract. Chemically these diets were shown to contain high levels of saponins. This is a probable cause of the toxicity; although other unknown factors, e.g., browning reaction products, should be considered.

The acid precipitate is light colored and fairly bland, and therefore was acceptable in cookies when substituted for 10% of the flour. The acid supernatant was unacceptable, not because of its brown color, but due to its extreme bitterness.

In conclusion, a protein concentrate, which has a nutritive value similar to casein with excellent potential as a supplement to cereal diets and is acceptable organoleptically, has been extracted from alfalfa. The mother liquor, however, has been shown to be toxic to weanling meadow voles and unacceptable in human food.

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APPENDIX

Table A1. The principal chemicals used in this study and their sources

Chemical*	Source
In the extraction procedure:	
Sodium hydroxide	Mallinckrodt Chemical Works, St. Louis, Mo.
Acetic acid	" " "
Isopropanol	Allied Chemical, New York, N.Y.
Acetone	Mallinckrodt Chemical Works
Hexane	Fisher Scientific Co., Fair Lawn, N.J.
In the nitrogen and nonprotein nitrogen determinations:	
Sulfuric acid	Fisher Scientific Co.
Cupric Sulfate	Mallinckrodt Chemical Works
Selenium dioxide	K & K Laboratories, Plainview, N.Y.
Hydrogen peroxide, 30%	Mallinckrodt Chemical Works
Sodium hydroxide, technical grade purified scales for N determinations	" " "
Boric acid	Fisher Scientific Co.
Bromocresol green	Matheson, Coleman, & Bell, Norwood, Ohio
Methyl red (WS)	Nutritional Biochemicals Corp., Cleveland, Ohio
Hydrochloric acid	J. T. Baker Chemical Col, Phillipsburg, N.J.
p-sulfa-o-methoxybenzeneazo-dimethyl-1-naphthyl-amine	Sigma Chemical Co., St. Louis, Mo.
Tris (hydroxymethyl) amino-methane, primary standard	" " "
Trichloroacetic acid	Mallinckrodt Chemical Works
In the amino acid analyses:	
Sodium citrate	Bio-Rad Laboratories, Richmond, Calif.
Thiodiglycol	" "
Detergent BRIG-35	" " (especially prepared
Ninhydrin	" " for use in amino acid
Cello-solve	" " analyzers)
Pentachlorophenol	Eastman Organic Chemicals, Rochester, N.Y.
Standard amino acid calibration mixture	Bio-Rad Laboratories
Norleucine	Nutritional Biochemicals Corp.
L-cysteic acid monohydrate	" " "
L-methionine DL sulfoxide	Sigma Chemical Corp.
L-methionine sulfone	" " "
Antifoam AF emulsion	Dow Corning Corp., Midland, Mich.
Potassium hydroxide	Mallinckrodt Chemical Works
Formic acid, 88%	" " "

Table A1 (cont'd.)

Chemical*	Source
In the tryptophan determination:	
DL-tryptophan	Nutritional Biochemicals Corp.
Pronase, B grade	Calbiochem, Los Angeles, Calif.
p-dimethylaminobenzaldehyde	Eastman Organic Chemicals
Sodium phosphate, monobasic	Fisher Scientific Co.
Sodium phosphate, dibasic heptohydrate	Mallinckrodt Chemical Works
Sodium nitrite	J. T. Baker Chemical Co.
In carbohydrate determination:	
Phenol	J. T. Baker Chemical Co.
D(+)mannose	Fisher Scientific Co.
D galactose	Pfanstiehl Laboratories, Waukegan, Ill.
In lipid determination:	
Chloroform	Mallinckrodt Chemical Works
Methanol	J. T. Baker Chemical Co.
Potassium chloride	Mallinckrodt Chemical Works
Anhydrous sodium sulfate	J. T. Baker Chemical Co.
In biological assays:	
Vitamin diet fortification mixture	Nutritional Biochemicals Corp.
Salt mix W	" " "
Alpha-cell, hydrolyzed	" " "
Casein, hammerstein	" " "
Dextrin, white technical	" " "
Sucrose	Big Chief, Monitor Sugar Co., Bay City, Mich.
Corn starch	A. E. Staley Mfg. Co., Decatur, Ill.
Corn oil	" " " " "
In organoleptic evaluation:	
All ingredients were brand names and purchased from a local grocery store.	
In high voltage paper electrophoresis:	
Raw skim milk	Mixed milk of Holstein cows that were part of the Michigan State University dairy herd

Table A1 (cont'd.)

Chemical*	Source
Pyridine	Mallinckrodt Chemical Works
Varsol	Standard Oil, Okemos, Mich.
Ninhydrin	General Biochemicals, Chagrin Falls, Ohio
2,4,6 collidine	Eastman Organic Chemicals

*if a chemical was used more than once, it will be listed only under the first analysis for which it was needed.

Table A2. Amino acid composition of alfalfa leaf protein fractions expressed as moles of amino acid/1000 moles amino acids

Amino acid	Protein fraction					
	1P3*	2P3	3P3	1S3	2S3	3S3
Lysine	57.17	60.23	79.56	68.13	63.35	56.40
Threonine	56.75	56.94	56.66	63.72	65.03	60.73
Valine	77.76	81.13	71.81	68.58	59.71	61.00
Methionine	26.30	21.14	20.20	14.74	15.89	8.71
Cystine	12.40	12.90	11.99	34.69	31.89	38.94
Isoleucine	50.67	56.77	48.18	46.88	43.01	43.01
Leucine	86.20	90.19	81.68	61.89	53.60	54.26
Tyrosine	29.63	30.57	34.15	18.42	23.19	20.70
Phenylalanine	48.18	50.99	45.09	29.80	30.64	30.22
Tryptophan	14.78	17.54	14.93	10.51	17.04	15.64
Histidine	26.80	21.91	24.34	16.94	19.07	16.94
Arginine	56.40	53.53	50.71	26.29	28.88	25.51
Aspartic acid	95.69	101.90	94.83	119.04	128.87	138.62
Serine	32.95	39.54	40.54	67.13	74.46	66.47
Glutamic acid	97.43	101.18	91.17	98.25	104.43	113.76
Proline	50.91	52.40	46.82	59.97	66.59	65.63
Glycine	91.80	95.72	86.24	106.04	99.62	104.95
Alanine	88.18	55.41	101.09	88.98	74.72	78.52

*see methods section, p. 79, for explanation of nomenclature

Table A3. Amino acid composition of alfalfa leaf protein fractions expressed as grams of amino acid/100 grams sample

Amino acid	Protein fraction					
	1P3*	2P3	3P3	1S3	1S3	3S3
Lysine	5.38	4.70	6.09	2.76	1.69	1.39
Threonine	4.21	3.51	3.42	2.04	1.37	1.18
Valine	5.66	4.90	4.25	2.15	1.23	1.16
Methionine	2.53	1.69	1.58	0.61	0.43	0.22
Cystine	0.94	0.81	0.74	1.13	0.68	0.77
Isoleucine	4.21	3.91	3.26	1.68	1.01	0.94
Leucine	7.16	6.22	5.52	2.22	1.26	1.18
Tyrosine	3.55	3.04	3.33	0.95	0.79	0.65
Phenylalanine	5.20	4.57	3.96	1.39	0.94	0.86
Tryptophan	2.02	1.99	1.66	0.62	0.66	0.56
Histidine	2.70	1.83	1.99	0.74	0.54	0.45
Arginine	6.46	5.09	4.73	1.30	0.94	0.77
Aspartic acid	8.08	7.15	6.52	4.34	3.08	3.07
Serine	2.11	2.10	2.11	1.85	1.35	1.11
Glutamic acid	9.23	7.96	7.03	4.02	2.80	2.82
Proline	3.63	3.10	2.72	1.84	1.34	1.23
Glycine	3.84	3.33	2.94	1.92	1.18	1.15
Alanine	4.60	2.40	4.29	2.00	1.10	1.07

*see methods section, p. 79, for explanation of nomenclature

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