CHARACTERISTICS OF PROTEINS EXTRACTED FROM FRESH ALFALFA (MEDICAGO SATIVA)

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This is to certify that the

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ABSTRACT

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By

Larry Lee Hood

Fresh alfalfa tissue (Medicago sativa) macerated in 0.1M TRIS-HCl buffer, pH 8.0, containing 10mM $K_2S_2O_5$ plus 5.8mM (0.1%) ascorbic acid, centrifugally clarified and and dialyzed against 0.02M TRIS HCl buffer, pH 8.0 yielded protein fractions with greater yield and superior solubility than preparations prepared by other schemes. The whole alfalfa extract (designated as fraction W) was fractionated according to protein solubility (1) in half-saturated ammonium sulfate, (2) at pH 4.4-4.5, or (3) after application of heat to 80°C for one minute. Fractions insoluble after these treatments were designated G (globulin-like), IP (isoelectric precipitate) and HP (heat denatured) while soluble protein mixtures were designated A (albumin-like), IS (isoelectric soluble) and HS (heat stable), respectively. All fractions were analyzed for content of protein, carbohydrate, lipid and ash. In general, protein mixtures precipitated by the above three procedures tended to be higher in protein and lipid than their soluble counterparts

which were higher in ash and carbohydrate. All fractions except the HP were soluble when taken up in aqueous buffer above pH 8.5. Gel electrophoresis on all soluble fractions revealed numerous protein zones which were quite similar for soluble protein fractions prepared by the three methods. The A, IS and HS fractions were unusual in that they possessed TCA-soluble components. Two of these fractions (IS and HS) possessed a composition unresolved by analysis for the four classes of biological materials examined.

The IP fraction was investigated for functional properties relating to possible commercial application. The fraction displayed excellent solubility below pH 2.3 and above 7.0 with a minimum at pH 4.0-5.0. IP proteins possessed good heat stability characteristics when in solution at pH 2.3 and 8.9. At pH 7.0 the system was heat sensitive and maximum precipitation occurred after 15 min at 70°C. The proteins of this fraction were further characterized by their sensitivity to added calcium ions at pH 8.9 but were relatively insensitive at pH 2.3. In the alkaline medium, maximum precipitation occurred at 50mM of calcium ion, whereas in the acid medium approximately 250mM calcium ion was required to achieve nearly total coagulation.

Most of the protein in unfractionated alfalfa extracts was of one type and was isolated by a combination of DEAE cellulose and Sephadex G-200 chromatography. It possessed ribulose 1, 5 diphosphate carboxylase (E.C.4.1.1.39) activity and sedimentated in veronal buffer (pH 8.6)

containing 12.5mM $MgCl_2$ with a $S^{\circ}_{20,w}$ of 25.3. An approximate molecular weight of 7.86×10^5 Daltons was determined. The electrophoretic mobility was -4.4 Tiselius units at infinite dilution in the same buffer. Thus, the major alfalfa protein is most likely the much investigated Fraction 1 protein of other plant species. Chemical analysis of this protein indicated the presence of 16.1% nitrogen and 1.1% hexose but the absence of hexosamine, sialic acid and nucleic acids. The protein contained 110 sulfhydryl groups per molecule with 64 of these occurring in disulfide bonds.

The protein was examined by gel electrophoresis in the presence of several dissociating agents, namely: 10mM mercaptoethanol, pH 11 phosphate buffer (with and without mercaptoethanol), 5M urea (with and without mercaptoethanol) and 0.1% SDS. Mercaptoethanol was not effective in dissociating the system suggesting that disulfide bonds do not occur between subunits. Both 5M urea and pH 11 buffer caused considerable dissociation of the system. SDS-gel electrophoresis revealed that the protein contained 3 major subunits with molecular weights of 5.6, 4.95 and 2.8×10^4 Daltons, as well as 3 minor species of undetermined size.

The amino acid analysis indicated that alfalfa Fraction 1 protein was slightly acidic and hydrophilic in nature and was characterized by high concentrations of Glu, Asp, Gly and Ala.

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INTRODUCTION

Casein, the traditional protein for addition to manufactured food products, is becoming increasingly expensive and the American food industry is rapidly developing technology and applications for proteins from other sources. The recent advances in extraction and utilization of protein from soybeans is an excellent example of this, and all indications are that this may be only the first of several "novel protein sources" to become available to the food processor.

The vegetative portions of other plants are high in extractable protein, but the applications of leaf protein concentrates (LPC) in the food industry is virtually non-existant. Traditional objections surrounding their flavor, odor, color and lack of functional properties are paramount deterents. The culture of alfalfa (Medicago sativa) is widespread, and appears to be a good choice for exploitation by the food industry. Alfalfa protein concentrates (APC) are presently used in the fortification of diets for swine, poultry and cattle, thus the technology relative to harvest and extraction is understood.

Present evidence indicates that APC can be prepared which is bland in flavor, light in color and which possesses

nutritional properties only slightly inferior to casein. The use of these concentrates, because of their customary insolubility, is relegated to products which can accept their addition in the form of flour-like isolates. Certain specialty food items require soluble protein additives since proteins with this property can most readily function as emulsifiers, foam stabilizers and water binders. Therefore, a study was undertaken to explore methods of extracting alfalfa protein to achieve their maximum solubility in the usual aqueous buffers. After attaining this, the next objective became the preparation of protein-containing fractions, employing fractionation with either ammonium sulfate, a change in pH or application of heat. The complex protein fractions from these treatments were examined for the distribution of protein components by gel electrophoresis and analyzed for contents of protein, carbohydrate, lipid and ash. The fraction insoluble at pH 4.4-4.5 was prepared in sufficient quantity to investigate several of its functional properties as they might relate to food product utilization.

Unfractionated alfalfa extracts were always characterized by one prominent protein component, which occurred in concentrations that would undoubtedly influence the properties of related APC fractions. This protein was isolated for chemical and physical characterization.

LITERATURE REVIEW

Plant Proteins

General

Pirie (1959) and Stahman (1963) reviewed the literature on plant proteins and concluded that proteins can be associated with either the seed or vegetative portions of the plant. The following review will be limited to the proteins normally associated with the rapidly growing plant cell rather than the mature seed.

Cellular disruption is a necessary first step to free the proteins contained within the plant cell. The efficiency of this procedure is the factor limiting the amount of protein extracted from a plant. The majority of the protein in a vigorously growing plant (40-50%) is located in the chloroplast while the remainder is associated with various cellular particles (nuclei, mitochondria, microsomes, etc.) or the cytoplasm of the cell (Pirie, 1959). The study of proteins originating from plant tissue presents special difficulties not encountered when working with animal or bacterial cell extracts (Stahman, 1963). The insolubility of plant proteins following their isolation is one of the most serious problems encountered. Stahman cites

several possible causes for this instability including one or more of the following: (1) binding of tannins and phytate to the protein during isolation, (2) release of vaculor acids that drop the pH of the cellular extract to the point of protein denaturation, (3) binding of the products of polyphenoloxidase to the protein, and (4) non-specific protein-protein aggregation leading to insolubility.

Anderson (1968) describes the improved efficiency of extracting soluble proteins and subcellular organellae when care is taken to (1) inhibit polyphenoloxidase activity and remove its substrates-tannins and phenols-during cellular disruption or (2) prevent the quinone products of this enzyme from condensing with the protein or subcellular particles by adding specific reagents to reduce the quinones to phenol-like compounds. The inhibition of polyphenoloxidase is achieved when copper chelators such as diethyldithiocarbamate or mercaptobenzothiazole or enzyme poisons such as cyanide or metabisulfite ions are added to the extraction media (Bonner, 1957; Pierpoint and Harrison, 1963; Anderson and Rowan, 1967; Anderson, 1968).

Many investigators used ascorbate to maintain plant protein stability during isolation but the results have been variable (Anderson, 1968). The function of ascorbate in preventing protein denaturation is to reduce quinones to phenolic precursors. The protection afforded by this technique is only temporary since ascorbate is gradually

destroyed and unless the enzyme is inhibited it will continue to produce quinones which will combine with the proteins. It seems likely that combinations of ascorbate and enzyme inhibitors may prove useful in isolating soluble proteins from plant sources.

Removal of the phenolic compounds by adsorption during protein isolation prevents their condensation to quinones by enzymatic activity. Loomis and Battile (1966) suggested that bothersome phenolic materials may be removed from cellular extracts by adding insoluble polyvinylpyrrolidone (PVP) which forms hydrogens bonds with the proton donating phenols. A similar approach was used by Lam and Shaw (1970) who reported the successful application of Dowex 1-X8 resin for isolating stable enzymes from the flax plant which is especially high in phenolic materials. They compared Dowex and PVP treatments of plant extracts and found greater protein recoveries and higher enzyme activities in isolates made with the Dowex resin.

The acidic contents of the vacuoles of many plant cells may lower the pH during maceration sufficiently to denature some proteins. This difficulty may be avoided by ammoniating the tissue during extraction (Knuckles, et al., 1970) or by extracting with a suitable buffer (Stavely and Hansen, 1967; McCowen et al., 1968; Betschart, 1971). Many plants also have acid-optimum proteolytic enzymes and it is possible to avoid undesirable protein hydrolysis by maintaining an alkaline pH during the extraction and isolation procedures (Singh, 1962; Stahman, 1963). Additionally,

the formation of phenol-protein complexes is inhibited at pH conditions above 7.5-8.0 (Loomis and Battile, 1966). Therefore, the application of gel filtration or dialysis with alkaline buffers should permit the separation of the undesirable smaller phenols from the protein mixture.

Non-specific aggregation of plant proteins is well documented and thought to be the result of sulfhydryl interaction (Stahman, 1963). Extraction buffers often include materials such as cysteine or mercaptoethanol which reportedly minimized protein interaction during isolation. Improved resolution of plant proteins during gel electrophoresis is cited as one example of the positive effects of such reducing agents in the buffer (Stavely and Hansen, 1967; Stahman, 1963). The proper amounts of such reagents would preserve the integrity of normally free sulfhydryl groups but excess amounts might result in the cleavage of disulfide bonds necessary for native protein structure.

Disruption of plant cells liberates most of the soluble protein as well as organellae which may be destroyed, adding their protein components to the mixture. Sedimentation analysis of cellular homogenates usually reveals 3-6 boundaries with the majority of the material (~50%) represented by an 18S component (Kawishima and Wildman, 1970). Singer et al. (1951) discussed the apparent homogeneity of the 18S material from tobacco and designated it as Fraction 1 protein. The remaining proteinaceous components (4S) were designated Fraction 2.

This nomenclature remains in use today even though zonal electrophoresis methods show most plant homogenates to be quite heterogeneous (McCowen et al., 1967; Stavely and Hansen, 1967; Wrigley et al., 1966; Romman et al, 1971). Electropherograms are characterized by the presence of a prominent band near the point of sample application which is interpreted to be similar to the 18S protein observed in sedimentation analysis.

Both electrophoresis and sedimentation analysis indicate that most of the protein associated with the plant cell is of one type which has been repeatedly demonstrated to be the enzyme 3-phospho-d-glycerate carboxlase (E.C. 4.1.1. 39) also known as ribulose 1, 5 diphosphate carboxylase (RuDP) or carboxydismutase (CDM). Kawishima and Wildman (1970) published an excellent review on this enzyme which has been extensively characterized from several plant sources. It is beyond the scope of this thesis to cover this subject in the detail of these authors, thus, only a brief summary on some of the more general characteristics will be presented.

Carboxydismutase, e.g., Fraction 1 protein, has been identified in several species of plants and is one of several important enzymes that function in the CO_2 fixation pathway of organisms containing chlorophyll a. In higher plants, the protein is most often associated with the chloroplast where it comprises the majority of the soluble protein (Kawishima and Wildman, 1970). The purified enzyme

usually has an $S^{\circ}_{20,w}$ ranging from 19 to 21 while the molecular weight may vary from 3.75-6.00 x 10^5 Daltons (Paulsen and Lane, 1966; Pon, 1967; Kawishima and Wildman, 1970). The large size of this protein has been further confirmed by electron microscopy which often reveals a spherical form with a diameter of 100-200Å, frequently exhibiting one or more visible depressions (Haselkorn et al., 1955; Ridley et al., 1967; Steer et al, 1968). Haselkorn et al. (1955) described Fraction 1 protein from Chinese cabbage as a cube but more recent work on the protein from wheat (Steer et al., 1968) and spinach (Ridley et al., 1967) suggests that an octahedral structure is more representative. In 1971, Kawishima and Wildman reported the crystallization of the protein possessing 12 parallelogram-shaped faces arranged in a complicated fashion.

When examined by high resolution electron microscopy, the protein appears to have a subunit structure. Haselkorn et al. (1955) originally suggested that the molecule contained 24 identical subunits of about 2.25 x 10^4 Daltons. However, additional work on the protein isolated from spinach (Moon and Thompson, 1969; Rutner and Lane, 1967), five species of tobacco (Kawishima and Wildman, 1971c), a purple sulfur bacteria (Akazawa et al., 1972) and chlorella (Sugiyama et al., 1971) indicates that the molecule usually is composed of two non-identical subunits with molecular weights of 5.0-5.8 x 10^4 Daltons for the large unit and 1.2-2.4 x 10^4 Daltons for the small unit. Amino acid

analysis of the separated subunits revealed a remarkable similarity between the large fragment isolated from different species, whereas the smaller subunits showed considerable variation in composition (Rutner and Lane, 1967; Moon and Thompson, 1969). It has been suggested that the large subunit is the catalytically active portion of the molecule while the smaller subunit provides species specificity and possibly functions in a regulatory role (Rutner, 1970).

It is interesting that in spite of its large molecular size, Fraction 1 protein has been described as lacking disulfide bridges (Kawishima and Wildman, 1970). A review of the literature revealed a dearth of information on this point. However, several free sulfhydryl groups have been detected on the protein and some of these may be present at the active site of the enzyme (Rabin and Trown, 1964; Sugiyama and Akazawa, 1967; Sugiyama et al., 1968). Other aspects concerning the chemical nature of this protein are also uncertain. Kawishima and Wildman (1970) report that it remains unclear if Fraction 1 protein contains any carbohydrate. For instance, Ridley et al., (1967) reported that the protein isolated from spinach beet leaves contained glucose, xylose and traces of galactosamine and arabinose. Fraction 1 protein from spinach was found lacking in carbohydrate (Paulsen and Lane, 1967). There has been one report that the protein might also be conjugated with lipid because the protein yielded a positive reaction when treated

with Sudan black, a lipid stain, following gel electrophoresis (Ridley et al., 1967). Contamination of the protein by nucleic acids has frequently been reported but by exercising some precautions during the procedure protein preparations free of these materials are obtained (Kawishima and Wildman, 1970).

Alfalfa

The protein components of Medicago sativa have been studied in relation to three areas of interest, including (1) the protein content of concentrates for utilization in animal diets, (2) the protein content of the plant as a reflection of agronomic influences and (3) the relationship of specific proteins to rumminant "bloat syndrome." The first two areas of research will not be discussed in this review. However, aspects of the third area will be considered because it has been from the bloat syndrome research that much of the information on the biochemical nature of alfalfa proteins has emanated.

Osborne et al. (1921) extracted alfalfa proteins by grinding the frozen tissue in the presence of water. The resulting slurry was hydraulically pressed and the fiber-free extract collected. The liquid initially was green in color and possibly contained chloroplasts, but eventually became a clear brown fluid which was collected and adjusted to a final concentration of 18% ethanol. The voluminous "collodial" precipitate was collected and analyzed, yielding approximately 70% protein and calcium

phosphate and flavonal-like pigments. The proteins isolated by this procedure were insoluble in water but could be solubilized by heating in weak alkaline media. Chibnall (1923) described a method for separating "vacuolar" and "cytoplasmic" protein fractions from extracts of plant tissue. Later, Chibnall and Nolan (1924) used this method to investigate the chemical nature of these two fractions from alfalfa tissue, concluding that the cytoplasmic fraction corresponded to the material in the collodial precipitate of Osborne et al. (1921). Chibnall and Nolan (1924) also reported that the cytoplasmic protein mixture had an isoelectric pH of 4.0 to 4.6 after the contaminating calcium phosphate was removed.

Mertz and Matsumoto (1955) reported the first electrophoretic analysis of an alfalfa protein mixture. The protein solution was a clear amber fluid obtained by centrifugally clarifying a buffer extract of frozen leaves for 1 hr at 20,000 rpm in a Spinco Model L centrifuge (no. 20 rotor). Moving-boundary electrophoresis of this supernatant revealed 3 components with 75-80% of the material present in one boundary which corresponded with observations for the soluble protein mixture from other plant species (Singer et al., 1951; Lylleton, 1956). The protein associated with the major boundary during electrophoresis was reported to have a mobility of -5.37 Tiselius units (descending) in pH 7.0 cacodylate buffer. The protein was characterized by irreversible insolubility when equilibrated

to pH values below 6 and contained associated nucleic acids. Mertz and Matsumoto (1955) also reported that the concentration of the major protein component present in alfalfa extracts decreased 50% if isolated from sulfur deficient plants and concluded that the protein was sensitive to the mechanism of sulfur metabolism in the plant.

McArthur et al. (1964) reported the isolation of an alfalfa protein described as the foam stabilizing agent in the diet of animals suffering from the bloat syndrome. The protein appeared to comprise a considerable portion of the soluble protein mixture and, when isolated, displayed a single symmetrical boundary of about 18S. They reported an electrophoretic mobility in pH 7.0, phosphate buffer of -7.25 Tiselius units and suggested that the protein was similar to the Fraction 1 species observed by other works (Lyttleton, 1955; Lyttleton and Ts'o, 1957). In contrast to the work of Mertz and Matsumoto (1955), McArthur et al. (1964) observed no nucleic acids associated with the protein isolate. Several authors have since found positive correlation between the content of the 18S protein and the tendency of a forage plant to promote the bloat syndrome (Miltmore et al., 1969; Stifel et al., 1968a,b).

Only limited information has been reported about the nature of alfalfa proteins except for the Fraction 1 component. The polydispersed mixture of Fraction 2 proteins from alfalfa may contribute to foam stabilization during rumminant bloat conditions (Jones and Lyttleton, 1969).

However, aside from this single observation, little has been reported about the other properties of this complex fraction.

Kleczkowska (1969b) performed the first zonal electrophoresis on soluble alfalfa proteins using a starch-gel support and a borate-triethanolamine (pH 8.0) buffer system. The results revealed that most of the protein was associated with a single band. Furthermore, 16 zones were observed, 12 of which migrated toward the anode and 4 toward the cathode. Recently, Romman et al. (1971) extracted alfalfa proteins with a pH TRIS-HCl buffer (pH 8.0) and observed 13-15 protein bands by acrylamide electrophoresis according to the method of Davis (1964). These authors indicated that most of the bands occurred in the Fraction 2 mixture, and 3-5 zones displayed lipoprotein characteristics when stained with Sudan black. Subsequently, they correlated the presence of these lipoproteins with the tendency of six different alfalfa clones to cause bloat in cattle.

Few authors have given consideration to the affects of extraction conditions upon the type, quantity or the physical state of proteins following their separation from normal plant cell structure. Kleczkowska (1969b) performed starch-gel electrophoresis on alfalfa protein extracts prepared with water, 1M NaCl buffered at pH 7.0 and 0.1M K_2HPO_4. His results indicated that both NaCl and K_2HPO_4 extracted one more component then the unbuffered water. These data indicated that extraction of globulin-like components from alfalfa tissue requires additional ionic strength as compared

to water. These results are generally in agreement with those reported by previous workers (Chayen, et al., 1961; Stahman, 1963). Betschart (1971) reviewed the factors affecting protein extractability from green plants and found that the buffer of Stavely and Hansen (1967) consisting of 0.1M TRIS at pH 7.4 with 0.5M sucrose, 75mM ascorbate, 6.6mM cysteine hydrochloride and 14.2mM mercaptoethanol, improved the yield of alfalfa protein when compared to 0.5% SDS or plain TRIS-HCl or borate buffers. She attributed the improved extractability to the presence of reducing substances such as the ascorbate or mercaptoethanol in the extraction buffer. Additionally, she noted the greater maceration efficiency achieved with the "micro-mill" as compared to a laboratory blender dramatically increased the amount of protein extracted from the plant and concluded that the extent of maceration must affect the amount of protein extracted.

Large Scale Preparation of Alfalfa Protein Fractions

The great potential of leaf protein concentrates for helping relieve the present world dietary crisis has been suggested by several authors (Pirie, 1942a,b; Stahman, 1968; Betschart, 1971; Hamilton, 1971). Pirie (1971), Hamilton (1971) and Betschart (1971) also reviewed the agronomy, preparation, bulk extraction and nutritive value of protein concentrates from alfalfa and other legume plants. Stahman (1968) recognized that the alfalfa plant has the

greatest potential for exploitation in the United States. Present methods for commercial preparation of alfalfa protein concentrates (APC) usually require extensive tissue maceration, either in the presence or absence of water, followed by fiber separation from the cellular contents and a subsequent precipitation step to concentrate and dewater the proteinaceous material. The resulting APC is usually dark in color, highly flavored and devoid of important functional properties such as solubility, thermal and mechanical stability, mixing properties et cetera. Kinsella (1970) and Kohler (1972) support the belief that solvent extraction or additional fractionation of the typically green APC will be necessary to produce an acceptable protein source for the American food industry. The following discussion will be limited therefore, to procedures utilizing fractionation methods to produce alfalfa protein concentrates.

The production of a light colored, bland flavored APC is possible if the chlorophyll and associated lipids are removed by solvent extraction (Pirie, 1971; Kohler, 1972; Huang et al., 1971) or by removal of the chloroplasts through heat coagulation or centrifugation. Subba Rao et al. (1969) precipitated the chloroplastic material by heating and subsequently coagulated the proteins by heating the filtered solution to 80°C. Hamilton (1971) prepared a light colored APC by heat coagulation of the chloroplasts and concentrated the protein by acid precipitation. A unique

approach to the preparation of a colorless APC was reported by Wilson and Tilley (1965). These authors applied a centrifugal force of 23,000 x g for 1 hr to remove the chloroplasts from a water extract of alfalfa and then concentrated the bulk of the proteins with ethanol precipitation.

Acid precipitation of the whole cellular extract of alfalfa produced a green material which was decolorized by successive washes with water, ethanol, acetone and ether (Poppe et al., 1970). The preparation was a nearly white, completely tasteless protein concentrate. In 1971, Huang et al. suggested a direct precipitation of the proteins in an alfalfa extract with solvents. Their results indicated that either ethanol or acetone was effective in removing the proteins from solution. Additional solvent washes of the precipitate were required to produce a bland, light tan product.

"Cytoplasmic fraction" is the name given to the protein mixture precipitated from solution following the removal of the chloroplastic material (Kohler, 1972; Pirie, 1971). The term is convenient for describing the process but it is misleading because some organellae proteins are present in the mixture. Furthermore, Pirie (1971) indicated that the composition of the cytoplasmic fraction was not constant but varied with the physiological state of the plant and the method used to remove the chloroplasts. For instance, Lexander et al. (1970) heat coagulated the

chloroplasts at different pH values from 4.5 to 6.0 and found that the higher pH values tended to recover less protein in a co-precipitate with the chloroplastic materials.

The proteins of the cytoplasmic fraction of large scale alfalfa extractions are usually insoluble, thus little is known of their biochemical nature (Hamilton, 1971). It may be assumed, however, that the majority of the protein is the carboxydismutase enzyme previously discussed. Nothing has been reported on the protein components remaining in solution after the removal of the cytoplasmic fraction. The supernatant fraction from this treatment is often called the "whey," "brown serum" or "alfalfa solubles" (Pirie, 1971; Kohler, 1972). The soluble fraction from a few processes has been chemically analyzed and found to be a complex mixture of protein, carbohydrate, ash, and lipid (Spencer et al., 1971; Hamilton, 1971). In addition, laboratory feeding trials indicated that complicated and potentially toxic biological compounds such as saponins, alkaloids, and tannins were associated with the soluble fraction (Hamilton, 1971). For the present, therefore, this soluble fraction appears to be an economic loss and source of pollution to the APC processor and although one suggestion has been made that the material serve as a commercial growth medium for food microorganisms such as yeast (Pirie, 1971), nothing has been reported concerning this application.

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 specified. Distilled or deionized water was used in the preparation of all buffers used in this work.

Alfalfa

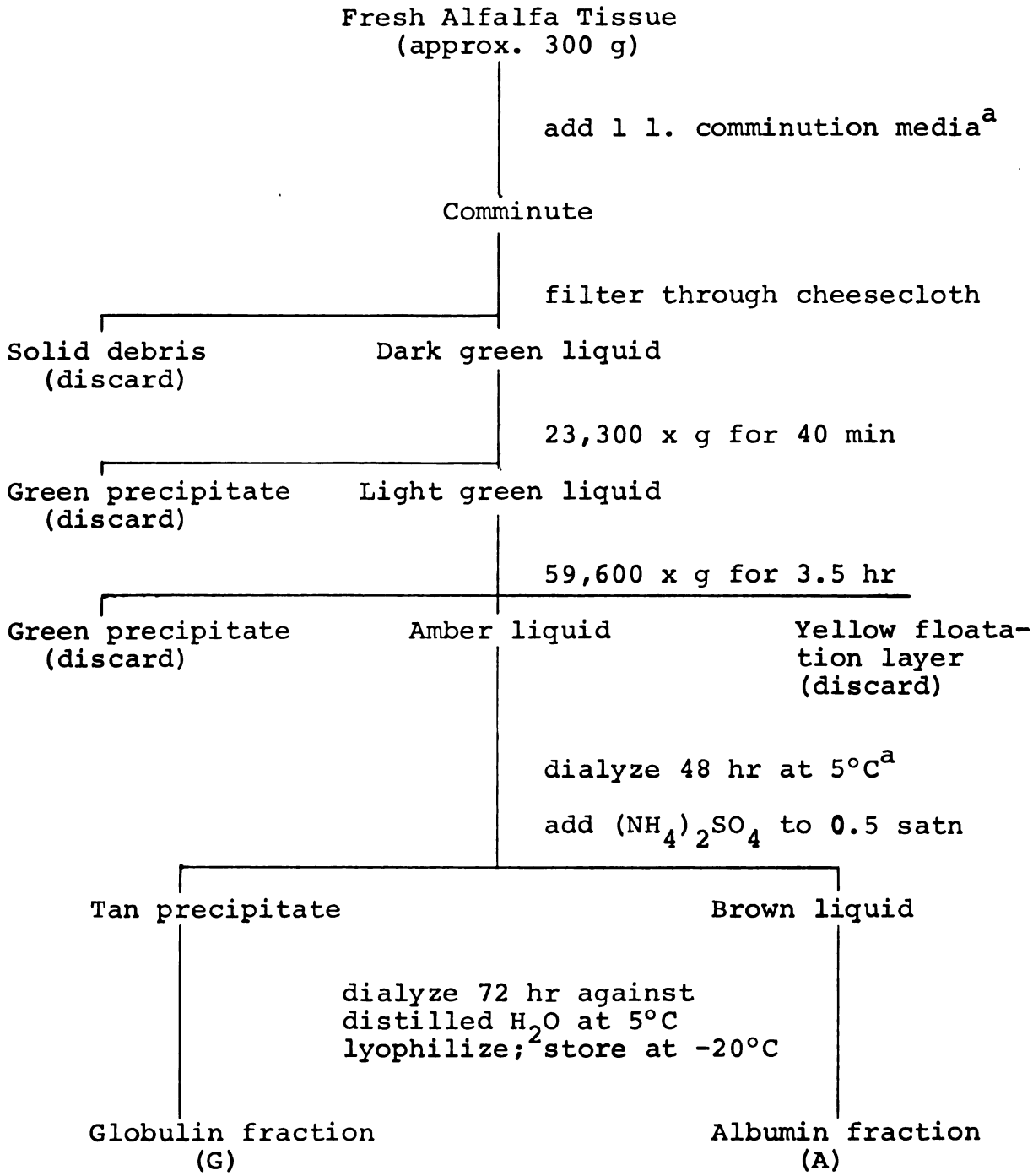
There were two primary sources for the fresh alfalfa used in this study. Plants were potted for greenhouse growth during winter and spring and reset in the soil for field growth during summer and fall. These plants were maintained by the Department of Crop and Soil Sciences at Michigan State University and included *Medicago glutinosa* and *Medicago sativa*. No attempt was made to prepare protein extracts from a specific variety of plants. A permanent stand of alfalfa (*Medicago sativa*) served as a secondary source of vegetation during the various periods in the summer and fall.

At or slightly before one-tenth bloom, the plant foliage was hand harvested and checked for botanical purity. Extraction procedures were initiated within 1-2 hr. Interim storage was at 4-8°C.

Preparative Procedures

Optimization of Extraction Conditions

Several different comminution and dialysis media (to aid in removal of phenolic materials) were examined for their affect upon protein yield and solubility-two results that required maximization in a study on alfalfa proteins. The basic extraction procedure is outlined in Figure 1. Plant tissue was chopped into 2-3 inch lengths, weighed and blended in a Waring blender with cold, freshly prepared comminution media using approximately 300 g tissue/liter. The different comminution media tried are presented in Table 1. The liquified material was filtered through four layers of cheesecloth, followed by centrifugation at 23,300 x g for 40 min in a Sorvall, Model RC-2B centrifuge at 5°C. The light green supernatant was freed of small floating particles by filtration through a kimwipe napkin and subsequently recentrifuged for 3.5 hr. at 59,600 x g on a Beckman/Spinco Model L-65 ultracentrifuge at 5°C. Small amounts of supernatant were also clarified at 105,600 x g for 1 hr. The centrifuge tubes usually contained a light green pellet, a yellowish-amber supernatant and a small amount of slightly congalled, yellowish floatation layer. The supernatant from each tube was removed with a 50 ml syringe and needle to avoid disturbing the floatation layer, pooled and dialyzed to equilibrium against various experimental media at 408°C (see Table 1). Follow dialysis, the extracted protein mixture was divided into two fractions based upon solubility



[a]Experimental variables--see Table 1, page 48.

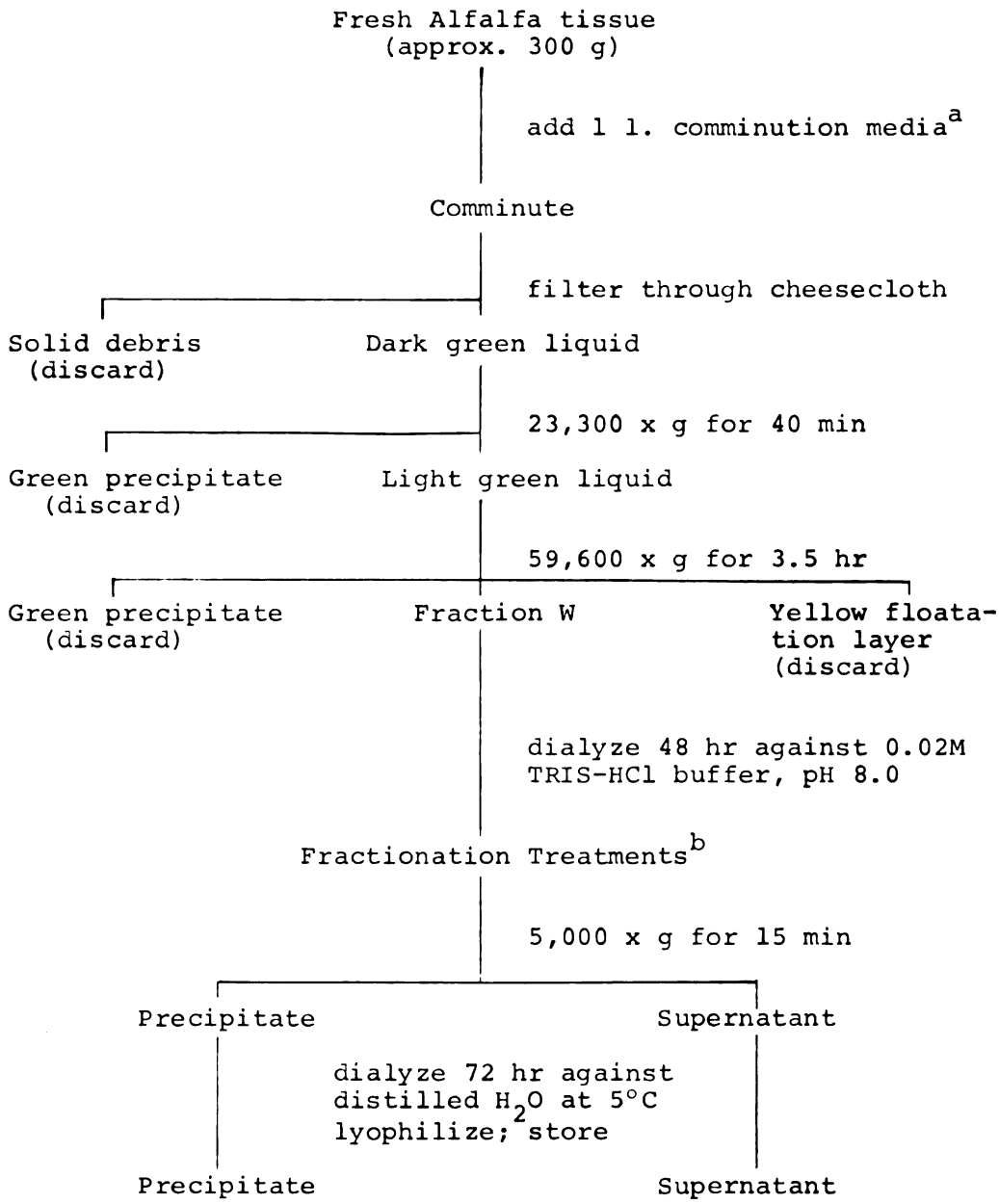
Figure 1.--Outline for the preparation of alfalfa protein fractions with different comminution and dialysis media.

in 0.5 saturated ammonium sulfate. The precipitate was collected by centrifugation for 15 min at 5000 x g, dispersed with water and dialyzed along with a supernatant fraction for 72 hr against deionized water at 4-8°C. The fractions were concentrated by pervaporation, shell-frozen in glass serum bottles and dried by lyophilization. Dried samples were held at -20°C unless otherwise indicated.

The solubility of these fractions in 0.1M TRIS-HCl buffer, pH 8.0 was determined as outlined in a following section.

Preparation of Alfalfa Protein Fractions

Alfalfa tissue was treated essentially as outlined in Figure 2. The extraction medium consisted of 0.1M TRIS-HCl buffer, pH 8.0 containing 10mM potassium metabisulfite, 5.8mM (0.1%) ascorbic acid and 0.04% sodium azide which was added as a preservative. The buffer solution was prepared 15 min prior to maceration of the plant tissue which was performed at 4-8°C. The liquified material was filtered through cheesecloth and clarified by two successive centrifugation steps previously described. The final supernatant was dialyzed 2 days against 0.02M TRIS-HCL buffer, pH 8.0 at 4-8°C. The retentate was a clear, golden-amber liquid which was fractionated in one of three ways: (1) adjustment to pH 4.4-4.5 with 2N HCl, (2) heated to 80°C at a rate of 2°C/min on a hot plate with a magnetic stirrer, or adjusted to 0.5 saturation with ammonium sulfate. The addition of acid or salt or the application of



[a]TRIS-HCl buffer, pH 8.0 containing 10mM Potassium metabisulfite, 5.8 mM (0.1%) ascorbic acid and 0.04% sodium azide.

[b]Included either a pH adjustment to 4.4-4.5, the addition of amonium sulfate to 0.5 saturation or the application of heat with stirring to 80°C.

Figure 2.--Outline for the preparation of alfalfa protein fractions by the application of acid conditions, heat or high salt concentrations to the dialyzed whole extract (fraction W).

heat resulted in a copious precipitate which was collected by centrifugation. Ammonium sulfate and buffer salts were removed from the fractions by dialysis against deionized water for 72 hr at 4-8°C. The samples were pervaporated shell frozen, lyophilized to dryness and held at -20°C for future analyses.

Isolation of the Major Protein Component

A major protein component was isolated from a non-lyophilized portion of the salted-out precipitate, designated here as Fraction G, employing a combination of ion-exchange and gel chromatography.

DEAE cellulose (Eastman) was prepared for use by the procedure outlined by Peterson and Sober (1962) and equilibrated with 0.1M TRIS-HCl buffer, pH 8.0. The slurried cellulose was poured into two 2.5 x 45 cm Sephadex columns and packed under nitrogen at 5-7 psi. The final dimensions of the columns were 2.5 x 35 cm. The columns were washed with 0.1M TRIS-HCl buffer, pH 8.0 until the pH was equilibrated. Ten to twelve milliliters of a 2-3% solution of fraction G was placed on the support. Samples of the G fraction had been previously dialyzed and equilibrated against three one-liter volumes of the same TRIS buffer for 24 hr at 4-8°C. A small precipitate was removed from the equilibrated solution at 2000 x g for 10 min. A stepwise elution of the cellulose columns was performed with a discontinous buffer system as described: approximately 100 ml of 0.1M TRIS-HCl buffer, pH 8.0, followed by

300 ml of 0.1M phosphate buffer, pH 7.0 and finally 150-180 ml of pH 7.0 phosphate buffer containing 0.3M NaCl. The eluate was monitored at 254 nm with a Model UA-2 Instrument Specialities detector and appropriate fractions collected. The columns were regenerated with 0.5N NaOH and re-equilibrated with the starting TRIS buffer.

Sephadex G-200 was equilibrated at room temperature in pH 8.0 TRIS-HCl buffer (0.1M) containing 12mM $MgCl_2$ and 0.02% sodium azide. The support-containing slurry was degassed for 15 min using a vacuum generated with a water aspirator and the necessary amount of support poured into a jacketed Sephadex column fitted with an upflow adapter and extension tube. The Sephadex beads were allowed to settle for 2-3 hr then packed under flow at 21 cm of hydrostatic pressure. When the final column height was established, a second flow adapter was fitted into the column which was then washed with several internal volumes of equilibrating buffer using reverse flow under 21 cm of hydrostatic pressure. The final column dimensions were 2.5 x 35 cm and the void volume was approximately 70 ml. Sample volumes were 8-10 ml of protein solution previously dialyzed against TRIS-HCl buffer, pH 8.0 containing 12.5mM $MgCl_2$, and was applied with the aid of a 4-way Pharmicia flow valve.

The column elution was monitored at 254 nm with an Instrument Specialites detector and appropriate fractions were collected.

Chemical Methods

Nitrogen

Nitrogen analyses were performed in duplicate. A semi micro-Kjeldahl apparatus with round bottom flasks and ball-and-socket ground glass joints was employed. The digestion mixture consisted of 5.0 g $CuSO_4 \cdot 5H_2O$ plus 5.0 g SeO_2 in 500 ml of concentrated sulfuric acid. Approximately 10 mg of dried sample was digested in 4 ml of the digestion mixture over a gas flame for one hour. The flasks were cooled at least 30 min followed by the addition of one milliliter of 30% hydrogen peroxide to each flask. Digestion continued for one hour. Each flask was cooled and rinsed with 10 ml of deionized water and allowed to stand 20 min at room temperature. The digestion mixture was neutralized with 25 ml of 40% sodium hydroxide and the released ammonia was steam distilled into 15 ml of 4% boric acid containing 3 drops of indicator. The indicator contained 400 mg of bromcresol green and 40 mg of methyl red in 100 ml of 95% ethanol. Distillation was complete when a total volume of 75 ml was collected in the receiving vessel. The ammonia-borate complex was titrated with weak HCl, the normality of which had been determined with trishydroxymethyl aminomethane (Sigma 121) as the primary standard. A reagent blank and a trypoophan standard were analyzed to determine the average per cent recovery of nitrogen which ranged from 95-98%.

Nonprotein Nitrogen

Samples were adjusted to a final concentration of 15% trichloroacetic acid (TCA). The mixture was agitated and allowed to stand at room temperature for 30 min, followed by centrifugation for 5 min at maximum speed on an International clinical centrifuge, Model CL. Measured aliquots of the resulting supernatant were used for nitrogen determination as previously described.

Hexose

Hexose analyses were determined by the colorimetric method of Dubois et al. (1956). A carefully weighed sample was dissolved in water or weak ammonium hydroxide solution. One milliliter was pipetted into a 15 x 150 mm test tube and one milliliter of 5% phenol (redistilled): water mixture added. Five ml of concentrated sulfuric acid was rapidly added in a manner to ensure thorough mixing and full heat development. The tubes were allowed to stand 10 min at room temperature and placed in a water bath at 25°C for 20 min. Transmittance was read at 490 nm on a Beckman DK-2A spectrophotometer.

A standard curve was constructed representing a range of 0 to 65 µg of a galactose-mannose (0.88:1.0 w/w) mixture dissolved in one milliliter of water. Blanks were prepared by omitting the protein from the reaction mixture.

Hexosamine

The method described by Johansen et al. (1960) was used to determine the total hexosamine content of the major protein component. Samples of 3-4 mg were weighed directly into 5 ml ampoules, mixed with one milliliter of 4N HCl, frozen in a dry ice-ethanol bath, evacuated, refrozen and sealed under vacuum. The samples were hydrolyzed for 6 hr at 100°C in a convection oven. After cooling, the hydrolyzed samples were transferred to distillation flasks. The empty ampoules were rinsed with one milliliter of 4N NaOH which was followed by two one milliliter rinses of deionized water.

Acetylacetone reagent was prepared by dissolving one milliliter of acetylacetone in 25 ml of 1M Na_2CO_3 solution and 20 ml of water. The pH was adjusted to 9.8 and the volume was made to 50 ml. This solution was used within 30 min. Erlich's reagent was prepared by dissolving 2 g of p-dimethaminobenzaldehyde in absolute ethanol containing 3.5% concentrated HCl to final volume of 250 ml. This solution was stored at 4°C.

Five and one-half milliliters of the acetylacetone reagent was added to each of the sample-containing flasks. The pH of this mixture was maintained between 9.5 and 10.0. The flasks were tightly stoppered and heated in a vigorously boiling water bath for 20 min, cooled in an icewater bath and connected to a micro-distillation apparatus. Each flask was heated with a micro-bunsen burner and the

steam-volatile chromogen distilled into a 10 ml volumetric flask containing 7.5 ml of ice cold Ehrlich's reagent. After mixing and a 30 min waiting period, transmittancy of the solution was read at 548 nm with a DK-2A spectrophotometer.

With a standard galactosamine and glucosamine solution (1:1, w/w) the average recovery of the duplicate determinations was 96-98%. A standard curve was constructed for a glucosamine-galactosamine mixture, ranging from 0 to 5 µg of carbohydrate. A reagent blank consisted of 1ml of 4N HCl, 1 ml of 4N NaOH and 2 ml of deionized water.

Sialic Acid

Warren's (1959) thiobarbituric acid method was adopted for the sialic acid determinations. A protein sample of approximately 7 mg was hydrolyzed in 0.1 N sulfuric acid for 1 hr at 80°C. Duplicate aliquots of 0.2 ml were mixed with 0.1 ml of sodium metaperiodate solution (0.2M sodium metaperiodate in 9M phosphoric acid). The mixture was agitated and permitted to stand for 20 min at room temperature. One milliliter of sodium arsenite solution (10% sodium arsenite in a solution of 0.5M sodium sulfate and 0.1N sulfuric acid) was added, followed by agitation until the yellow-brown color disappeared. Then, 3 ml of thibarbituric acid solution (0.6% in 0.5M sodium sulfate) were added, the tubes shaken, capped with marbles and heated in a boiling water bath for 15 min. The tubes were then placed in cold water for 5 min, followed by the

addition of 4.3 ml of cyclohexanone for extraction of the chromophore. The tubes were agitated and the contents transferred to 15 ml conically-shaped centrifuge tubes and centrifuged for 5 min. The clear, pink upper layer of cyclohexanone was monitored at 549 nm on the DK-2A spectrophotometer. A standard curve was prepared from authenic N-acetylneuraminic acid, ranging in concentration from 0 to 20 µg per ml.

Amino Acids

Amino acid analyses were performed on 24 hr protein hydrolysates employing the Beckman 120C automatic amino acid analyzer (Moore et al., 1958). Eight to 30 mg of sample was weighed and transferred to 10 ml glass ampoules. Five milliliters of 6N HCl were added and the mixture frozen in a dry ice-ethanol bath. The ampoules were evacuated, allowed to melt under vacuum to remove gases, refrozen, sealed and placed in an oil bath maintained at 110°C for 24 hr. The ampoules were removed, allowed to cool to room temperature, opened and one milliliter of standard 2.5 mM norleucine solution was added as an internal standard. The hydrolyzate was quantitatively transferred to a 25 ml pear-shaped evaporation flask and evaporated to dryness in a 55°C water bath. The residue was redissolved in deionized water and evaporated until no hydrochloric acid odor remained. Finally, the dried hydrolysate was made up to 5 ml with a sample buffer consisting

of 0.067M citrate-HCL buffer, pH 2.2. Aliquots of 0.2 ml were used for analyses. Two or more analyses of a standard amino acid mixture were conducted concurrently with the sample analyses, using the same buffer and ninhydrin solution.

Amino acid compositions were expressed as either moles of residues per 1000 moles of total amino acid residues or as relative molar ratios based on phenylalanine content.

Tryptophan

This acid-labile amino acid was determined by Procedure W in the method of Spies (1967). Three to four milligram samples were weighed into 2 ml glass screw-top vials, mixed with 0.1 ml of fresh pronase solution (10mg/ml of pH 7.5 phosphate buffer, 0.1M), agitated momentarily, the vials capped and incubated for 24 hr in a 40°C water bath. The vials were removed from the bath, cooled and placed into 50ml Erlenmeyer flasks containing 30 mg of p-dimethylaminobenzaldehyde and 9.0 ml of 21.2N sulfuric acid. The vials were tipped over and mixed quickly with the acid solution by gently swirling. The flasks were covered with Parafilm and incubated in the dark at room temperature for 6 hr. Following incubation, 0.1 ml of 0.045% (w/v) sodium nitrite was added to each flask, allowed to incubate for 30 min and the transmittancy of the supernatant determined at 590 nm.

The tryptophan contributed by autolysis of pronase was determined in simultaneous analysis and the value subtracted from that obtained from the sample protein.

A standard curve from 0 to 140 µg tryptophan was prepared according to procedure E of Spies and Chambers (1948).

Lipid

Total lipid was determined by a micro-modification of the method described by Mojonnier and Troy (1925). The specific details of the analysis are presented in the Appendix, Table A2.

Sulfhydryl Groups-Unexposed

The free sulfhydryl groups of the major protein component were determined by a modification of the procedure developed by Ellman (1959). The protein was equilibrated against 0.1M phosphate buffer, pH 7.7 by dialysis and had a final concentration of 3-4 mg/ml. One milliliter of protein solution was pipetted into a 10 x 150 mm test tube and 4 ml of 0.1M phosphate buffer, pH 8.0 containing 0.04% EDTA. Five milliliters of freshly prepared and filtered 8.5M urea in deionized water were added, the mixture agitated and allowed to stand 30 min prior to adding 0.04 ml of 5,5'-dithiobis-(2-nitrobenzoic acid) solution (DTNB). The DTNB solution was prepared by dissolving 39.6 mg DTNB in 10 ml 0.1M phosphate buffer, pH 7.0. The color was allowed 45 min to develop and absorbancy at 412 nm was monitored. Concentration of the sulfhydryl groups was determined using

an extinction coefficient of 12,000 as reported by Flavin (1962). A reagent blank was run concurrently with the protein sample.

Preliminary results indicated that the final urea concentration, exposure time to urea and time allowed for color development after the addition of DTNB were important variables. The final condition described above are those found to be most effective for this particular protein.

Sulfhydryl Groups-Exposed

The free sulfhydryl groups presumed to exist on the surface of the protein molecule were determined in the following manner. One milliliter of pH 7.7 phosphate buffer containing 8.5 mg protein was mixed with 3 ml of 0.1M phosphate buffer, pH 8.0 and 0.02 ml of DTNB solution added. The absorbance at 412 nm was determined after 45 min. Concentration of the sulfhydryl groups was determined using an extinction coefficient of 12,000.

Disulfide Groups

This analysis utilizes sodium borohydride to reduce disulfide groups to free sulfhydryl groups. The reducing agent was destroyed with acid and acetone and the concentration of sulfhydryl groups determined as described above. The method is a modification of the procedure developed by Cavallini et al. (1966).

The test is conveniently conducted in a 10 ml volumetric flask having a ground glass stopper. Approximately

3 mg of protein was equilibrated in 2 ml of 0.1M phosphate buffer, pH 7.7 and pipetted into the flask and 10 µl of octanol added to reduce foaming. Two milliliters of a urea-sodium borohydride solution was added and the flask stoppered and incubated 40 min in a 40°C water bath. The urea-borohydride reagent consisted of 10 g urea and 0.25 g sodium borohydride in 10 ml of deionized water. It is important that this reagent be made up and added to all flasks in less than 2 min to avoid pipetting errors due to evolution of hydrogen gas. It was convenient to first dissolve the urea in the water then weigh and add the borohydride and quickly pipette the mixture.

After incubation, the flasks were cooled at room temperature for 5 min and one milliliter of a low pH buffer added dropwise to each flask. The flasks were stoppered, inverted twice and allowed to stand 5 min at room temperature. The low pH buffer was prepared by dissolving 13.6 g KH_2PO_4 plus 1.66 ml concentrated HCl and made to a final volume of 100 ml with deionized water.

Two milliliters of acetone were added and the flasks stoppered and inverted twice. Each flask was then purged with nitrogen gas at 3 psi for 1 min followed by the addition of 0.04 ml DTNB solution and a second nitrogen purge of 30 sec. The flasks were permitted to stand at room temperature for 35 min at which time they were centrifuged for 5 min. Absorbance was determined at 412 nm 45 min after addition of the DTNB reagent. Water blanks were used to

test for sodium borohydride destruction since this reagent causes color development if present in the solution. The concentration of sulfhydryl groups was determined by the method described previously.

Biuret Procedure for Protein

A modification of the procedure described by Lane (1957) was used. Biuret reagent was prepared by dissolving 1.5 g $CuSO_4 \cdot 5H_2O$ and 6.0 g sodium potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$) in 500 ml of water followed by the addition of 300 ml of 10% sodium hydroxide. One gram of KI was added to the mixture and the volume diluted to one liter.

To determine the concentration of protein in solution, simply mix 1.0 ml protein solution with 4.0 ml biuret reagent, permitting the mixture to stand 30 min at room temperature. Read the transmittance at 540 nm on the DK-2A. Concentration of the protein was determined by reference to a standard curve constructed with bovine serum albumin previously corrected for nitrogen and moisture content. Blank determinations consisted of 1.0 ml buffer and 4.0 ml biuret reagent.

Ash

Porcelain crucibles were heated to 575°C in a muffle furnace, cooled and weighed on a five-place Mettler balance. Fifty to 100 mg of sample were weighed directly into the crucibles which were returned to the furnace. Samples reached constant weight after 48 hr and were cooled

in a desiccator for 4-5 hr and re-weighed. Ash was defined as the per cent of the original sample weight remaining as the residue.

Solubility of Alfalfa Protein Concentrate Fractions

Effect of pH

Solubility was defined as the percentage of Kjeldahl nitrogen originally present in a weighed sample that dissolved in buffers and in buffers receiving additional treatment.

The effect of pH on the solubility of alfalfa protein fraction IP (isoelectric) was studied, using buffers at constant ionic strength as described by Miller and Golder (1950). One percent solutions of the protein fraction was prepared in buffers ranging in pH from 2 to 10. The mixtures were agitated periodically for 30 min, centrifuged for 5 min at 2000 x g and filtered through No. 1 Whatman paper. The soluble Kjeldahl nitrogen was determined after appropriate blank values were subtracted.

Effect of Heat Treatment

One percent solutions of the IP fraction were prepared in pH 2.0, 7.0, and 9.0 buffers of Miller and Golder (1950) and examined for heat stability. Soluble Kjeldahl nitrogen was determined after heating 3 ml aliquots of the protein solution for 15 min at 55, 70, 85 and 100°C.

Effect of Calcium Ion Concentration

A modification of the method described by Zittle et al. (1959) was used.

Solutions of the IP fraction were prepared at approximately 1-2% concentration in buffers of pH 2 and 9 according to Miller and Golder (1950). Stock solutions of calcium chloride containing 500 mM/l were also prepared in these buffers. Small adjustments in pH were made with a few drops of concentrated HCl or a small amount of solid NaOH. Equal volumes of protein and calcium chloride solutions were mixed to yield final concentrations of Ca++ varying from 12.5 to 250 mM/l. These mixtures were agitated and placed in a water bath at 30°C. The coagulated material was removed by centrifugation for 5 min at 2000 x g. The supernatant was filtered through Whatman No. 1 paper and assayed for Kjeldahl nitrogen.

Physical Methods

Moving-boundary Electrophoresis

A Perkin-Elmer Model 38-A Tiselius electrophoresis apparatus was used to determine the electrophoretic mobilities of protein fractions in free solution.

Mobilities were calculated from ascending and descending patterns, using the following equation:

$$\mu = \frac{d\ a\ k}{t\ i\ m}$$

where μ is the electrophoretic mobility (10^5 cm^2 sec^{-1} $volt^{-1}$), d is the distance the boundary migrated from the initial boundary in cm, a the cross sectional area of the cell in cm^2, k the specific conductivity, t the time of electrophoresis in seconds, i the current applied in amperes, and m the magnification factor of the optical system.

Veronal buffer, pH 8.6 ($\Gamma/2 = 0.1$) was used in the electrophoretic mobility determinations. Protein samples had a final concentration of 1-2% after dialysis against approximately 600 ml of the buffer for 24 hr at 2-3°C with constant stirring. All electrophoretic analyses were conducted at 2-4°C. The electrophoretic mobility of the major protein component at infinite dilution (μ°) was determined by plotting the average of descending and ascending mobilities as a function of concentration and extrapolating to zero concentration.

Polyacrylamide Disc Gel Electrophoresis

Disc electrophoresis was performed as described by Davis (1964) with two modifications: (1) no sample gel was used, sample density was increased with solid sucrose and the sample which included 2 μl of bromphenol blue (0.5% in water) was applied on top of the spacer gel, and (2) the stock reservoir buffer was diluted 1:1 with deionized water instead of 1:9. Electrophoresis was conducted with a laboratory constructed apparatus having a buffer capacity of

about 600 ml in each of the electrode tanks. Electrophoresis was conducted initially at 2 mAmp per tube and increased to 5 mAmp per tube when the tracking dye entered the running gel. The addition of tracking dye was omitted for highly crude protein samples since a yellow band migrated with the ion front.

Gels were stained for 1-2 hr in a solution of Amido Black 10B, containing 250 ml water, 250 ml methanol, 50 ml glacial acetic acid and 5 g amido black or for 3-4 hr in a solution of Coomassie Blue as described by Weber and Osborn (1969). Amido black-treated gels were electrically destained in 7% glacial acetic acid whereas those stained in Coomassie Blue were treated according to the method of Weber and Osborn (1969).

Polyacrylamide-Urea Disc Gel Electrophoresis

The basic procedure of Davis (1964) with the above modifications was used. However, before adding the polymerizing reagents, solid urea was added to produce a final concentration of 5M in working solutions for both the running and spacer gels.

Polyacrylamide Disc Gel Electrophoresis in a High pH, Continuous Buffer System

The gel solution was prepared by dissolving 9 g of Cyanogum 41 in about 90 ml of 0.05 M phosphate buffer, pH 11 and the mixture filtered through Whatman No. 1 paper into a 100 ml volumetric flask. To this solution, 0.6 ml

N,N,N',N'-tetramethylethylenediamide (TEMED) was added and the volume adjusted to 100 ml with buffer. The buffer used for the gel solution was used in the electrode tanks and was prepared by dissolving 14.1 g Na_2HPO_4 in 2 l of deionized water with sufficient solid NaOH (about 1 g) to yield a final pH of 11.

To prepare the gels for electrophoresis, 20 ml of gel solution was mixed with 10 mg of solid ammonium persulfate and sufficient solution was added to each tube to yield a final gel column 5.5 cm long. The surface of the gel solution in each tube was layered with water as outlined by Davis (1964). Polymerization of the gel was permitted to proceed 20 min prior to electrophoresis. Electrophoresis was conducted for about 3.5 hr at which time the dye front had migrated approximately 4 cm under the influence of 4 mAmp of current per tube. Protein solutions prepared for electrophoresis were approximately 1% in concentration and were previously dialyzed 24 hr against the high pH buffer at 4-10°C. Following electrophoresis, the gels were removed, stained and destained as described above using amido black.

Polyacrylamide-SDS Disc Gel Electrophoresis for Estimation of Polypeptide Molecular Weight

Gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was conducted according to the procedure of Weber and Osborn (1969). Protein samples ranging

from 1-4 mg were weighed into 5 ml vials and 1 ml of 0.01M phosphate buffer, pH 7.0, containing 0.1% SDS and 0.1% β-mercaptoethanol (ME) was added. These mixtures were permitted to equilibrate for 2 hr at room temperature, then a 50 µl aliquot was diluted with 50 µl of the same phosphate buffer, 5 µl of ME, 1 drop of glycerol and 3 µl of tracking dye (0.05% Bromphenol Blue in water). Thirty microliter portions were applied to the top of the gel columns and electrophoresis was conducted for 4 hr at 6 mAmp per tube. The gels were removed from the tubes and stained according to the method described by Weber and Osborn (1969).

Molecular weights for the subunits were estimated from a plot of relative mobility versus the log of the molecular weight for standard proteins. Standards used in this study were lysozyme (14,300), trypsin (23,300), pepsin (35,000), ovalbumin (43,000) and catalase (60,000). Relative mobilities were evaluated from measurements of the gel column, dye zone and protein zones as follows:

$$\text{Relative Mobility} = \frac{\text{distance protein migrated}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before staining}}{\text{distance of dye migration}}$$

Sedimentation Coefficient

Sedimentation velocity experiments were conducted in a Beckman-Spinco Model E ultracentrifuge, using a rotor speed of 39,460 rpm. All determinations were at 20°C and

conducted in a capillary-type single-sector, synthetic-boundary cell.

Sedimentation coefficient determinations of crude protein fractions were conducted in veronal buffer, pH 8.6 ($\Gamma/2 = 0.1$). The fractions had previously been lypholized and held at -20°C before equilibration in buffer for 24 hr at 4°C.

The sedimentation coefficient of the major protein component from alfalfa was determined on non-lyophilized samples. From preliminary experiments, it was considered necessary to examine only freshly isolated material since physical interactions occurred during storage which resulted in altered sedimentation patterns. The protein was dialyzed 2 days against veronal buffer, pH 8.6 containing 12.5 mM $MgCl_2$. Protein concentration was determined by the biuret method immediately before centrifugation.

The sedimentation coefficient is defined as the velocity of the sedimenting molecules per unit field as shown by the equation:

$$s = \frac{1}{\omega^2 x} \cdot \frac{dx}{dt}$$

where $\underline{x}$ is the distance of the boundary in centimeters from the axis of rotation, $\underline{t}$ is the time in seconds, and $\underline{\omega}$ is in radians per second (Schachman, 1957). Integration of the above equation yields the following relationship:

$$s = \frac{2.303 \log x}{\omega^2 t}$$

Upon plotting the logarithim of the distance sedimented against time of sedimentation, the sedimentation coefficient (S_{app}) may be obtained from the slope of the plot using the following formula:

$$S_{app} = \frac{2.303\ (10^{13})}{\omega^2} \cdot \frac{\log x}{t}$$

Sedimentation coefficients are usually reported as $S_{20,w}$ which is the value the protein would have in a solvent with the density and viscosity of water at 20°C. The observed sedimentation coefficients were corrected to this standard condition according to the following equation:

$$S_{20,w} = S_{obs} \left(\frac{\eta_t}{\eta_{20}}\right)\left(\frac{\eta_s}{\eta_o}\right)\left(\frac{1-\bar{v}\rho_{20,w}}{1-\bar{v}\rho_t}\right)$$

where the first term (η_t/η_{20}) is the ratio of the viscosity of water at the experimental temperature to that at 20°C, the second term (η_s/η_o) is the relative viscosity of solvent to that of water at any temperature and the terms $\rho_{20,w}$ and ρ_t are the densities of water at 20°C and the solvent at the experimental temperature, respectively. The partial specific volume, $\bar{v}$, of the protein was assumed a constant value (0.73) in all solvent systems employed.

The standard sedimentation coefficient in infinite dilution, $S^{\circ}_{20,w}$, was obtained by plotting the values of

$S_{20,w}$ versus the concentrations of protein and extrapolating to zero concentration.

Diffusion Coefficient

The diffusion coefficient was determined in a double-sector, synthetic-boundary cell at a speed of 3270 rpm with the An-J rotor. The following relationship was used:

$$D_{app} = (A/H)^2(1/4\pi t)(1-\omega^2 st)$$

where D_{app} is the apparent diffusion coefficient in cm^2 sec^{-1}, $\underline{A}$ the area enclosed by the schlieren pattern above its base line in cm^2, $\underline{H}$ the maximum height of the boundary in cm, $\underline{t}$ the time measured from the start of centrifugation in sec, and $\underline{\omega}$ the angular velocity of rotation in radians sec^{-1} (Lamm, 1929).

Measurement of schlieren patterns for height and area was accomplished and the data plotted as $\underline{(1/4\pi)A/H)^2}$ versus $\underline{t}$ to obtain the slope which is the apparent diffusion coefficient (D_{app}). The value of the observed diffusion coefficient (D_{obs}) was obtained as the intercept of the plot of D_{app} values versus protein concentration.

The effects of solvent upon the diffusion coefficient were corrected as follows:

$$D_{20,w} = D_{obs} \quad (293/(273 + t)) \cdot (\eta_s/\eta_w)(\eta_t/\eta_{20,w})$$

where $\underline{t}$ is the temperature of the diffusion coefficient measurement, (η_s/η_w) is the relative viscosity of solvent to that of water, and $(\eta_t/\eta_{20,w})$ is the relative viscosity of water at the temperature of the experiment to that at 20°C.

Miscellaneous Methods

Microbiological Analysis

Total and psychrophilic bacterial counts were performed at various stages in the isolation scheme for the preparation of crude protein fractions.

Difco Plate Count Agar (PCA) was used for enumeration of microorganisms. Dilutions were made with 0.1% Peptone blanks. Plates were incubated 72 hr at 32°C for total counts and 7 days at 7°C for psychrophilic organisms.

Representative colonies were examined in a wet mount under the oil immersion lens of an American Optical light microscope.

Preparation of Rabbit Anti-sera

A 2% solution of previously lyophilized whole alfalfa cellular extract (Fraction W) was prepared in 0.1M TRIS-HCl buffer, pH 8.0 and diluted 1:1 with Freund's complete adjuvent. The mixture was thoroughly emulsified by shaking in a syringe and 1.0 ml injections were given sub-cutaneously near lymph nodes on the lower neck and upper groin regions of a young adult Dutch Belted rabbit.

Follow-up injections consisted of smaller amounts of a 1% protein solution in TRIS buffer. The injection schedule is presented in the Appendix, Table A3.

Test bleeding occurred at 4 and 6 weeks after initiation of the injection schedule. Ten to twenty milliliters of blood were collected from the marginal ear vein, permitted to clot 1 hr at room temperature and placed overnight in the cold room. The clot was removed by centrifugation and the whole serum stabilized with 0.02% sodium azide or 0.01% thimerosal and held in the cold room for subsequent use.

Immuno-double Diffusion

A 2% solution of previously purified Bacto-agar (Difco) was prepared in veronal buffer, pH 8.6, stabilized with 0.02% sodium azide and held at 4°C until used. Small petri dishes (6.5 x 1.4 cm) were washed in weak detergent, rinsed in deionized water and dryed at room temperature. Before use, the dishes were pre-coated with 0.2% Bacto agar in distilled water and permitted to dry before addition of 10-12 ml of diffusion agar. After solidification, a five-hole pattern was cut into the gel with a Feinberg template.

For immuno-diffusion analysis, whole rabbit antisera was placed in the central well and the alfalfa protein fraction (1-4%), dissolved in veronal or TRIS buffers, were added to the outer wells. Diffusion was permitted to occur in a humid chamber at room temperature for 3-5 days. The

wells were recharged every second day during observation and precipitin patterns examined frequently in a diagonal light path.

RESULTS AND DISCUSSION

Alfalfa Protein Fractions

Optimization of Extraction Conditions

The primary purpose of exploring different extraction conditions was to maximize the solubility characteristic of alfalfa protein fractions. Stahman (1963) cites the lack of solubility as one of the most perplexing problems encountered in working with plant proteins. Studies on alfalfa protein fractions (Hamilton, 1971) indicated their tendency toward insolubility. Therefore, if any rational study on alfalfa proteins was to be conducted, the protein components had to be soluble in common laboratory media.

Table 1 summarizes the effect of maceration and dialysis media upon the percent nitrogen, solubility and yield of two complex protein fractions isolated from alfalfa tissue. Fraction G is the precipitate and Fraction A the supernatant material resulting from adjustment of the total cellular extract to 0.5 saturated ammonium sulfate.

The data on protein yields in Table 1 should be accepted guardedly because the yield of protein from plant

TABLE 1.--Effect of comminution and dialysis media upon yield and solubility of alfalfa protein fractions.

Sample designation	Fraction[1]	Comminution media	Dialysis media	Yield[2] (%)	Total Nitrogen (%)	Soluble Nitrogen (%)
S-24	G	H_2O	buffer a[3]	17.0	14.5	89.0
S-23	A			10.3	12.8	82.8
S-26	G	buffer b[4]	buffer a	18.6	14.0	88.5
S-25	A			11.4	12.9	94.0
S-32	G	buffer b + 20% PVP[5]	buffer a	18.0	13.5	95.1
S-31	A			4.6	8.3	94.1
S-28	G	buffer b + 17.4mM ascorbic	buffer a	16.1	13.8	95.4
S-27	A	acid		6.4	11.5	94.8
S-16	G	buffer b + 30mM $Na_2S_2O_5$	buffer a	13.6	14.1	95.3
S-15	A			4.0	12.8	83.5
S-14	G	buffer b + 30mM $Na_2S_2O_5$	buffer a	14.8	15.2	67.3
S-13	A	+ N_2 (10 psi)		7.0	11.8	83.0
S-8	G	buffer b + 15mM $Na_2S_2O_5$	buffer a + 15mM	8.1	14.8	42.5
S-7	A		$Na_2S_2O_5$	3.6	10.9	100.0
S-12	G	buffer b + 30mM $Na_2S_2O_5$	buffer a + 30mM	16.2	15.3	49.0
S-11	A		$Na_2S_2O_5$	7.3	12.9	77.2
S-10	G	buffer b + 30mM $Na_2S_2O_5$	buffer a + 30mM	15.0	15.2	36.6
S-9	A	+ N_2 (10 psi)	$Na_2S_2O_5$	8.5	13.5	79.0
S-20	G	0.35M NaCl	buffer a	16.5	13.8	100.0
S-19	A			4.5	11.8	85.0
S-36	G	buffer b + 10mM $K_2S_2O_5$	buffer a	20.7	13.9	100.0
S-35	A	+ 5.8mM ascorbic acid		7.8	10.6	100.0

[1]Obtained by adjusting dialyzed whole extract (fraction W) to 0.5 saturation with $(NH_4)_2SO_4$ and centrifuging to separate the precipitate (fraction G) from the supernatant (fraction A)

[2]Percent of original crude protein in the alfalfa plant computed on a dry weight basis as %N(0.75) x 6.25

[3]0.02M TRIS-HCl buffer, pH 8.0

[4]0.1M TRIS-HCl buffer, pH 8.0 plus 0.04% sodium azide

[5]Polyvinylpyrrolidone

tissue is a reflection of various agronomic influences during growth as well as the extent of cellular disruption during extraction (Pirie, 1971; Betschart, 1971). Microscopic examination of cellular homogenates revealed considerable cellular disruption, thus the complex protein fractions described in this thesis are probably representative of what is contained in the plant and the uncertainity is merely one of amount.

The data in Table 1 indicate that a slightly higher yield of protein fractions resulted when a simple alkaline buffer (S-26,27) was used as a comminution media as compared to unbuffered water or 0.35M NaCl (S-23,24, 19, 20). This was not surprising since improved extraction of plant protein under alkaline conditions has been reported by other workers (Stahman, 1963; Stavely and Hansen, 1968; Betschart, 1971).

The incorporation of reducing agents such as ascorbate, cysteine or metabisulfite to comminution media have been discussed previously (Anderson, 1968; Betschart, 1971). The results obtained in this study indicate that when 10mM metabisulfite and 5.8mM ascorbic acid (S-35,36) were incorporated into the comminution media, superior yields of soluble protein were obtained when compared with buffered media lacking these additives (S-25,26,31,32). However, higher levels of metabisulfite (i.e., 30mM-S-11,12) or longer exposure times (i.e., S-7,8,9,10,11,12) resulted in a reduction in the solubility of the two fractions studied.

This observation reflects the fragile nature of the disulfide bonds present in the proteins and indicates that some caution must be exercised when reducing substances are employed during preparation of alfalfa protein fractions. Stahman (1963) suggested that the maceration of plant tissue be conducted in a nitrogen atmosphere as one technique to inhibit polyphenoloxidase activity, thus improving the solubility of the extracted plant proteins. However, in this study, the bubbling of N_2 gas at 10 psi through the buffer during comminution (S-9,10,13,14) did not produce any additional effect on yield or solubility when compared to the simpler procedures using only buffers.

The incorporation of polyvinylpyrrolidone (PVP) into the buffered comminution media (S-31,32) yielded protein fractions in similar yields and with solubilities similar to those prepared with TRIS buffer (S-25,26). The type of interaction resulting in the insoluble alfalfa proteins reported by Hamilton (1971) was never elucidated but the data of this thesis indicate that if phenol-protein complexes were the problem, alkaline comminution and dialysis media eliminated it. The importance of the alkaline dialysis procedure to the preparation of soluble alfalfa protein fractions can not be quantitatively or qualitatively assessed but may be significant. Phenol-protein complex formation appears to be accelerated under acid conditions but retarded in alkaline media (Loomis and Battaile, 1966). Therefore, if the pH is correct for

inhibiting phenol binding, then dialysis could be expected to remove them from the protein containing solution.

The TRIS-HCl buffer, pH 8.0 containing 10mM $K_2S_2O_5$, 5.8mM (0.1%) ascorbic acid and 0.04% sodium azide constituted the comminution media used for the remainder of the studies. This buffer was prepared with pre-chilled distilled water (4-8°C) immediately before use and in no instance was the buffer prepared more than 30 min before grinding the tissue in the blender.

Microbiological analyses on routinely prepared alfalfa protein fractions revealed low populations of total and psychrophilic bacteria in the initial extract which continued to decrease during the isolation procedure. Apparently cold extraction conditions and the presence of sodium azide were effective inhibitors of undersirable microbial growth.

Chemical Composition of Alfalfa Protein Fractions

The chemical composition of fractions prepared by adjustment of the centrifugally clarified alfalfa extract to (1) pH 4.4-4.5, (2) 0.5 saturated ammonium sulfate, and (3) application of heat to 80°C are presented in Table 2. The fractions and their designations are as follows: HP (heat denatured at 80°C), HS (heat stable), IP (isoelectric precipitate), IS (isoelectric soluble), G (0.5 saturated ammonium sulfate precipitate), A (0.5 saturated ammonium sulfate soluble), and W (whole, unfractionated material).

TABLE 2.--Summary of chemical analyses on alfalfa protein fractions prepared by different methods.

	Total Ext.	Salt		Acid		Heat	
	W	G	A	IP	IS	HP	HS
% Protein (%N x 6.25)	68.5	87.0	63.3	86.8	47.5	95.6	32.5
% Carbohydrate	5.5	3.2	6.4	4.7	25.0	1.5	35.8
% Lipid	1.4	3.0	0.5	3.1	2.1	0.2	2.7
% Ash	21.0	1.2	21.1	1.1	3.8		3.4
Total	96.4	94.4	91.3	95.7	78.4	97.3	74.4

W = total, unfractionated alfalfa extract
G = insoluble fraction when fraction W was adjusted to 0.5 saturated ammonium sulfate
A = soluble fraction when fraction W was adjusted to 0.5 saturated ammonium sulfate
IP = insoluble fraction when fraction W was adjusted to pH 4.4-4.5
IS = soluble fraction when fraction W was adjusted to pH 4.4-4.5
HP = insoluble fraction when fraction W was heated to 80°C for 1 min
HS = soluble fraction when fraction W was heated to 80°C for 1 min

The precipitate fractions from the above treatments (fractions HP, IP, and G) contained more protein (%N x 6.25), less carbohydrate (as hexose), less ash but more lipid than the corresponding supernatant fractions.

Hamilton (1971) subfractionated the cytoplasmic fraction from alfalfa by acidification to pH 4.5. The resulting two fractions (precipitate and supernatant) had chemical compositions similar to the acid precipitate (IP) and acid soluble (IS) fractions obtained in this study. Her precipitate fraction contained 66-81% protein, 6.5-9.4% carbohydrate, 1.0-2.4% lipid and 1.2-2.5% ash. Her acid soluble fraction corresponded closely to the above IS material and contained 20.5-33.5% protein, 31.0-35.0% carbohydrate, 1.0-3.2% lipid and 4.67-6.10% ash.

Subba Rau et al. (1969) subfractionated the cytoplasmic fraction from alfalfa by heating to 80°C. Their precipitate contained 77% protein, 5% lipid, 1.5% mineral matter and 16% carbohydrate by difference. The HP fraction obtained in this study contained more protein (95.6%) and less carbohydrate (1.5%) and no detectable ash. Some of the differences between the chemical composition of the two heat precipitates might reflect the pH of the solution at the time of heating. In this study the dialyzed whole extract was at pH 8.0 while in their work the pH appeared to be closer to the indigenous pH, i.e., 5.8-6.0. Spencer et al. (1971) and Lexander et al. (1970) demonstrated that

the pH at the time of heating alfalfa extracts affects the chemical composition of the fractions.

Subba Rau et al. (1969) did not describe the composition of their 80°C supernatant. However, Spencer et al. (1971) induced coagulation in the entire green extracted alfalfa juice (adjusted to pH 8.5) by heating to 82°C and found that the supernatant fraction contained 20.5% ash, 0.5% lipid and 20.8% protein. The supernatant was not assayed for carbohydrate content. The HS fraction obtained in this study contained less ash (3.4%) but more protein (32.4%) than did the supernatant fraction reported by Spencer et al. (1971). Differences in the composition of the supernatant between the present work and that of Spencer et al. (1971) may reflect their use of an undialyzed, whole juice extract as the starting material.

Fraction W represents the total soluble material present in a centrifugally clarified extract of alfalfa tissue. It's protein content (68.5%) is intermediate between those of the three precipitates (IP, HP, G) and their corresponding supernatants (IS, HS, A). The ash content of fraction W was high (21.0%) and similar to the ash content of fraction A. Other workers have reported high ash contents in unfractionated plant extracts. For instance, Hartment et al. (1967) spray dried the juice of alfalfa and found that it contained 12.1-12.7% ash while pea vine juice contained 12.8% ash. These workers also found that dried alfalfa juice had 31.3-34.9% protein and 40.2-45.7%

carbohydrate (carbohydrate determined by difference). The larger carbohydrate and lower protein contents of their extracts, when compared to fraction W, could be ascribed to the chloroplast associated carbohydrate which was not removed before spray drying. Their higher carbohydrate content obviously causes the level of protein to drop proportionally in the analysis.

The unusually high ash content of the W and A fractions was of interest. Atomic absorption analysis (see Appendix, Figure F3) on the ash from a typical A fraction revealed that it contained a high calcium content (15%). It was observed that the ash of both fraction A and W could be removed from these fractions by dialysis against weak acid. Apparently, the mineral matter concentrates in the soluble fractions after removal of the major portion of the protein and was tentatively believed to be collodial calcium phosphate. It is possible to explain the absence of ash from the IP and IS fractions due to its solubility at the acid conditions of fractionation. However, there is presently no explanation for the relatively low concentration of ash in both the HP and HS fractions which were derived by heating the W fraction to 80°C.

All protein fractions except W were examined for non-protein nitrogen (NPN) as previously described. In general, the NPN contents of the G, IP, and HP fractions were less than 1%. But the supernatant fractions IS, A and HS contained significant amounts of NPN material as

indicated by the data in Table 3. That some of this NPN material was retained following 48 hr dialysis against distilled water suggests that proteins or other large nitrogen containing polymers reside in the TCA supernatant. The presence of TCA-soluble protein in alfalfa protein fractions has not previously been described. Additional discussion concerning the physical properties of the TCA-soluble portion of the IS, HS and A fractions will follow.

Solubility of Alfalfa Protein Fractions

Effect of Isolation and Storage

When initially isolated by the procedure presented in Figure 2, fractions W, G, A, IP, IS, and HS were essentially soluble in 0.1M TRIS-HCl buffer, pH 8.0. The heat precipitated fraction (HP) was not solublized. Following several months of storage at -20°C, fractions G, A, IS and HS retained their complete solubility at pH 8.0. However, after two months storage at room temperatures, fractions W and IP were 10-15% less soluble than their freshly prepared counterparts.

Effect of pH

In 1970, Kinsella reviewed the potential of leaf protein concentrates for human consumption and suggested that more information concerning the functional properties of plant proteins was needed. The lack of solubility in normal aqueous media has traditionally hampered this type

TABLE 3.--Effect of dialysis upon the nonprotein nitrogen (NPN) content of alfalfa protein fractions.

Fraction	NPN[a] (%)	Non-dialyzable NPN[a] (%)
W	NT[b]	NT
G	1.0	NT
A	87.9	5.4
IP	0.2	NT
IS	82.2	37.9
HP	NT	NT
HS	69.7	35.4

[a]Based upon the original nitrogen content of the fraction.

[b]Not tested.

of research on alfalfa protein fractions. However, the soluble fractions obtained in this study made possible a study directed toward properties relating to their use in formulated food systems.

For a model protein system, the IP fraction was selected because acid precipitation appears to be the method of concentration most adaptive to industrial applications.

The IP fraction was solubilized in constant ionic strength buffers ranging from pH 2.0 to 10.0. The pH-solubility curve is presented in Figure 3 and indicates that the fraction has a minimum solubility between pH 4.0-5.0. This observation is in agreement with the pH required to maximize the precipitation of alfalfa proteins from whole extracts and cytoplasmic functions (Subba Rau and Singh,

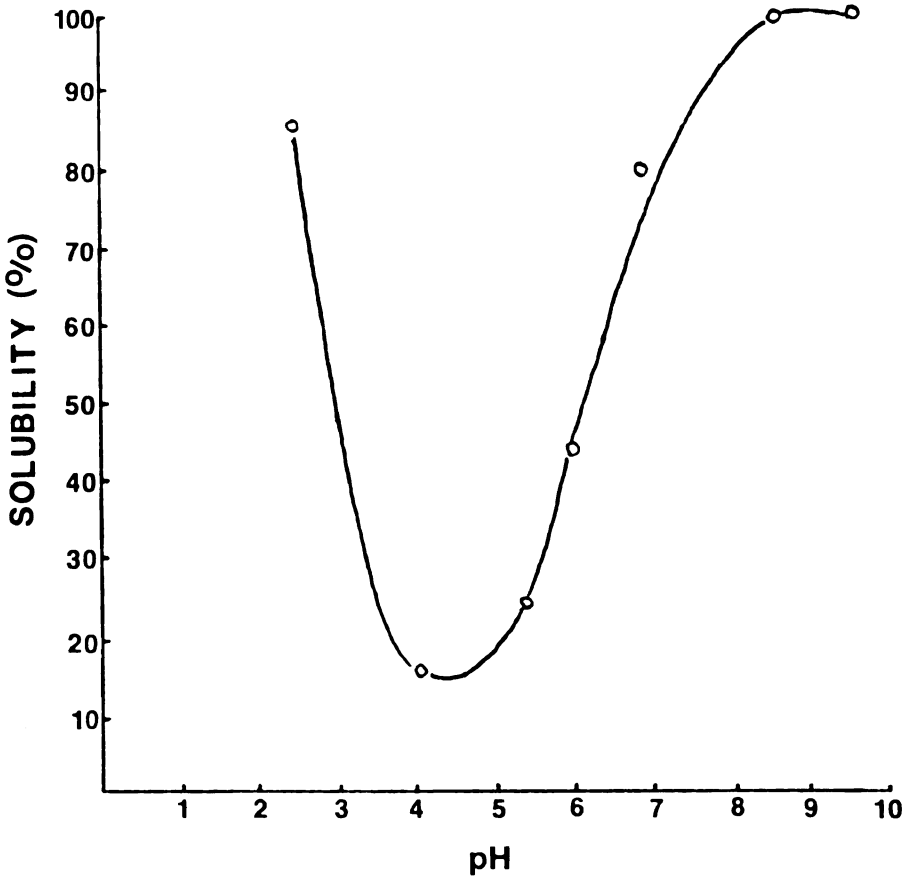


Figure 3.--Solubility of the IP fraction in buffers with different values of pH.

1970; Hamilton, 1971). Lu and Kinsella (1972) isolated a protein mixture from alfalfa meal and reported the isoelectric pH to be 3.5-5.0. However, the presence of buffer salts affect the isoelectric pH of the mixture.

When at the pH of minimal solubility, the IP fraction was observed to retain approximately 17% of the original protein mixture in solution. The material remaining soluble at pH 4.0-5.0 was not elucidated but could have been derived from acid-soluble components (protein or carbohydrate) adsorbed to the IP fraction during isolation. The surprising solubility of the IP fraction below pH 3.0 and above pH 7.0 would appear to offer considerable potential for its incorporation into a variety of food products.

Effect of Heat Treatment

The IP fraction was dissolved in buffers prepared according to Miller and Golder (1950) to yield 1% solutions with final pH values of 2.3, 6.9 and 8.6. The solutions were heated at various temperatures for 15 min after which the percentage of the fraction remaining soluble was determined. The results of these studies are presented in Figure 4.

Surprisingly, the samples which dissolved at pH 2.3 and 8.6 were nearly totally soluble after heating for 15 min at 100°C. The sample dissolved near neutrality displayed a rapid drop in solubility at temperatures above 55°C. About 25% of the original material remained stable at the higher temperatures.

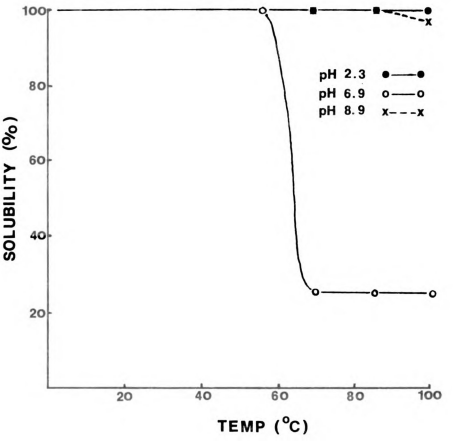


Figure 4.--Effect of heat treatment upon the solubility of the IP fraction in solution at three different values of pH.

Samples of the IP fraction heated to temperatures of 70-100°C at pH 8.6 precipitated only slightly but were "hazy" in appearance. When in solution at pH 6.9, the IP fraction contained both a precipitate and haziness when heated to 55° and higher. The highly acid solutions (pH 2.3) remained clear at all temperatures. Haze formation in protein solutions following the application of heat was interpreted as thermal denaturation. Protein solutions heated to temperatures that caused haze formation developed precipitates upon standing at 4°C for one week. Unheated, control samples remained clear during storage. These results suggest that although proteins have not been precipitated initially by heat treatment they have been "sensitized" and are subject to gradual changes leading to aggregation during storage. This observation may be of importance in the preparation of light colored alfalfa protein fractions by the procedure of coagulating the chloroplasts with a mild heat treatment. Even though proteins may not be directly coagulated by the low thermal treatment, they may be destabilized thus, when precipitated, i.e., by acid treatment to pH 4.0-5.0, they may be rendered permanently insoluble. Heat denatured alfalfa protein resisted all attempts to achieve resolubilization.

The data of this study suggest that the affect of heat treatment upon alfalfa proteins is related to the pH at the time of heating. Previous work by Lexander et al. (1970) supports this contention. Additionally, the work by

Lu and Kinsella (1971) indicated that proteins can be extracted from alfalfa meal heated previously to 75 and 150°C. Proteins accounting for 25% of the IP fraction of this study were not coagulated at 100°C when in solution at pH 6.9. These may constitute members of the heat stable protein mixture present in dried alfalfa meal.

Effect of Calcium Ion Concentration

Approximately 1% solutions of the IP fraction were prepared at two values of pH and solubility of the protein was studied in relation to calcium ion concentration. Figure 5 illustrates the results of this study.

At pH 8.9, the proteins were less stable to Ca++ than when in solution at pH 2.3. The proteins in alkaline buffer were not totally precipitated, even at calcium concentrations of 250mM. However, when the IP fraction was solubilized at pH 2.3, essentially all of the protein was precipitated by 250mM calcium ion. This difference in Ca++ sensitivity may reflect lack of calcium ion binding at the low pH or a difference in composition of the protein mixture soluble at the two pH values tested. Reference to previously discussed data on the solubility of the IP fraction at different pH values indicates that approximately 20% of the proteins soluble near pH 9 are absent at pH 2.3.

Calcium-sensitive proteins were reportedly present in the soluble protein mixture extracted from alfalfa meal dried at 75°C (Lu and Kinsella, 1972). However, the extent of sensitivity of alfalfa proteins to divalent cations has

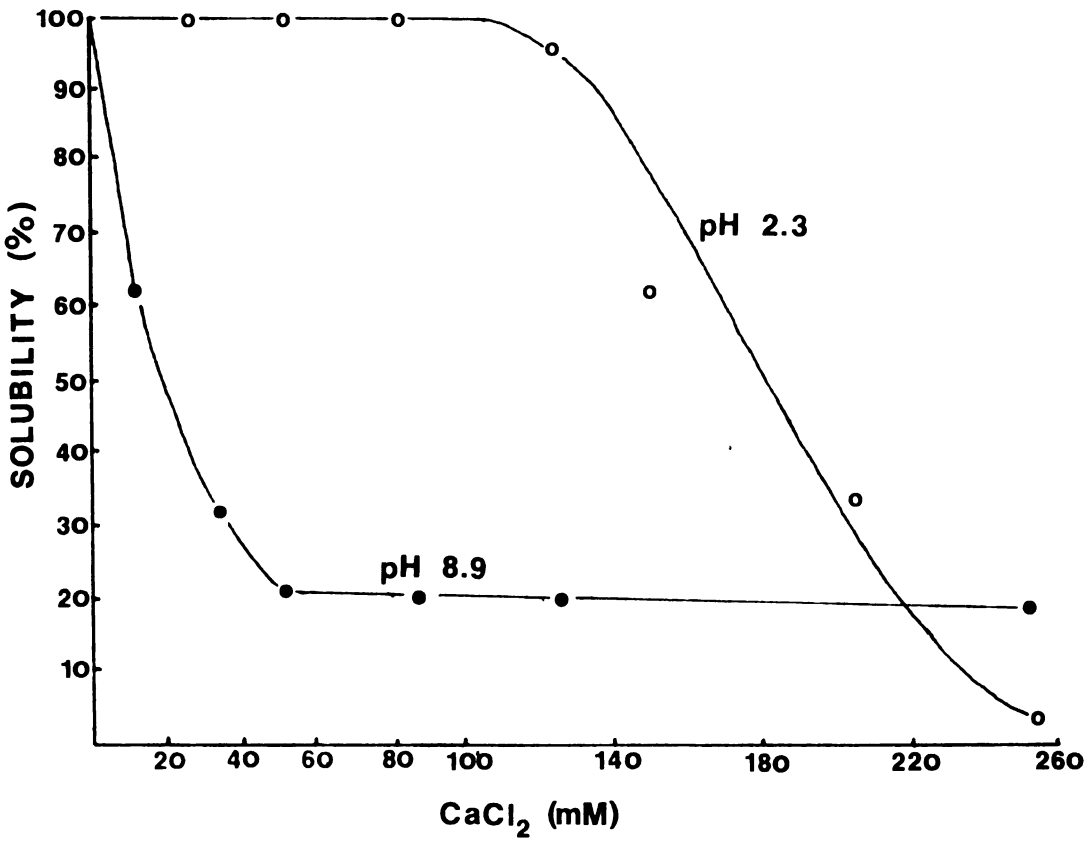


Figure 5.--Effect of $CaCl_2$ concentration upon the solubility of the IP fraction in solution with buffers at two different values of pH.

not been described previously. The complex protein mixture soluble at pH 8.9 was believed to be more sensitive to low Ca++ concentrations because proteins possess a greater net negative charge than when in solution at pH 2.3. Greenberg (1944) states that dicarboxylic and hydroxyl amino acids are largely responsible for calcium binding in proteins. Acid and phenolic side groups on amino acids are highly charged in alkaline media and may promote increased binding of the calcium cation. The data of other workers support the conclusion that pH values above the isoelectric point of proteins tend to increase the binding of calcium ions (Edsall et al., 1950; Gurd and Wilcox, 1956; Zittle and Pepper, 1958).

The test utilized in this study reflects the calcium-protein interaction leading to an insoluble aggregate. This phenomenon is reportedly the result of intramolecular salt briding (chelation) or charge neutralization by the positive metal ion that results in precipitation similar to that observed at the isoelectric pH (Gurd and Wilcox, 1956). In certain instances, however, calcium concentrations were encountered in this study that produced stable collodial suspensions of the proteins remaining after the removal of the coagulated material. Calcium concentrations of 25mM for the alkaline conditions and 125 and 83mM for the acid solution produced hazy suspensions, indicating further the greater degree of Ca++ sensitivity of the IP proteins in alkalaline media. Additionally, the

precipitated proteins which accompanied the collodial suspension suggests that the IP fraction is composed of protein components having differential calcium sensitivity.

Physical Properties of Alfalfa Protein Fractions

Electrophoretic Analyses

The distribution of protein components in the various soluble fractions, as revealed by disc-gel electrophoresis, is represented in Figure 6. Fraction W (Figure 6, gel 1) shows 4 distinct components and 5 diffuse zones. Kleczokowska (1969a) identified 16 components in vertical, starch-gel electropherograms of a total cellular extract from alfalfa. Several of these migrated toward the cathode and would not have been apparent in the disc electropherograms as obtained in the present study.

Romman et al. (1971) applied disc-gel electrophoresis to the unfractionated extract of alfalfa and reported 13 components with most of the material present as one migrating species which was classified as Fraction 1 protein (carboxydismutase). Most of the material staining as protein in fraction W of this work was also present in one zone.

Alfalfa proteins separated into G and A fractions (Figure 6, gels 2 and 3 respectively) demonstrated the heterogeneity expected but not resolved in the W fraction. A total of 24 components were separated, 11 of which were

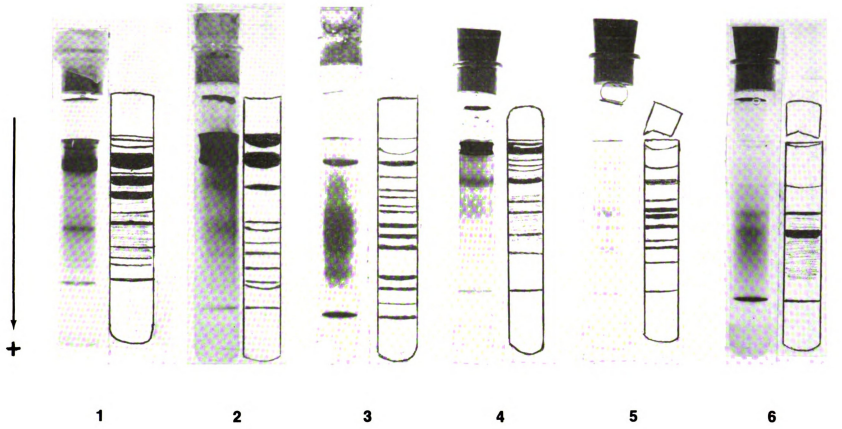


Figure 6.--Polyacrylamide disc gel electropherograms of soluble alfalfa protein fractions as follows: (1) W, (2) G, (3) A, (4) IP, (5) IS, and (6) HS.

components of the G fraction. Diffuse areas of staining were present but not resolved. In general, the G fraction contained most of the protein in one or two zones immediately below the spacer-running gel interface. These may represent carboxydismutase or its aggregate species as described by Romman et al. (1971). Fraction A displayed a broad distribution of protein zones throughout the gel, but a heavy smear is noted in the lower half of the gel. These components may correspond to those present behind the buffer front in the fraction W gel pattern. Kleczokowska (1969b) fractionated an alfalfa extract with ammonium sulfate and found nearly all zones present in the precipitate regardless of the salt concentration employed. In his work the greatest number of zones was present at 0.5 saturated salt, agreeing with the data of this study.

Electropherograms (Figure 6, gel 4) of the IP (isoelectric insoluble) fraction displayed 9 distinct protein zones in addition to the presence of considerable smearing. Most of the material in solution appeared as two zones near the spacer-running gel interface. It is uncertain if these principle zones represent carboxydismutase (or its aggregate) which was apparent in the G fraction. The IS fraction (Figure 6, gel 5) contained several components (11) which were evenly distributed throughout the gel pattern similar to the previously described A fraction.

The electrophoretic pattern of the HS (heat soluble) fraction was characterized by 2 principal bands in the lower

half of the gel (Figure 6, gel 6) which might be similar to zones observed in the IS fraction as indicated by their electropherograms. Immunological analysis (see Appendix, Figure F1) also indicated homology between heat and acid stable fractions. The electropherogram for the HS material showed considerable smearing which was interpreted as an indication of heterogeneity in the fraction. Additional data will be required to determine whether the polypeptides of the HS fraction are indigenously heat stable or are artifacts of the heat treatment of the HP protein mixture.

Electrophoretic patterns of the TCA soluble, non-dialyzable components present in the NPN mixture of fraction IS, A, and HS are presented in Figure 7. Fraction A_s (Figure 7, gel 1) contained at least 10 components whereas Fraction IS_s (Figure 7, gel 2) contained seven. Many of the stained zones originally apparent in the gels for all three TCA-soluble, non-dialyzable fractions either faded quickly or did not reproduce well in the photograph. Fraction HS_s (Figure 7, gel 3) showed six stained zones. From the appearance of the electropherograms, all three fractions appeared to contain common components. Immuno double-diffusion analysis of these fractions (Appendix, Figure F1), confirmed that at least one common, homologous component was present.

The technique of SDS-gel electrophoresis was applied to evaluate the size distribution of the proteins in the

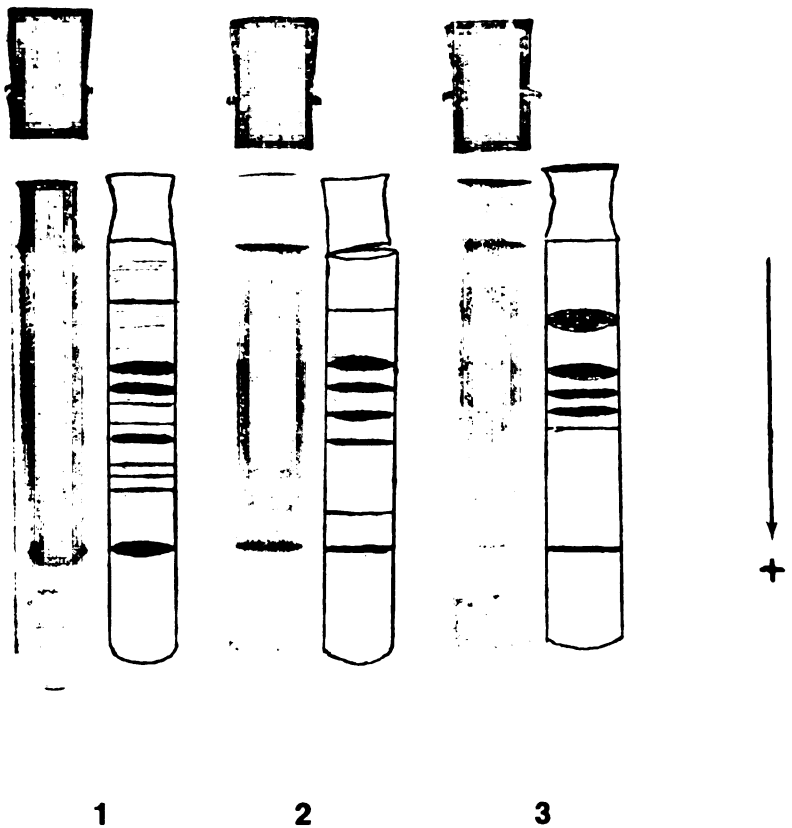


Figure 7.--Polyacrylamide disc gel electropherograms of the non-dialyzable, NPN components of alfalfa protein fractions designated (1) A_s, (2) IS_s and (3) HA_s.

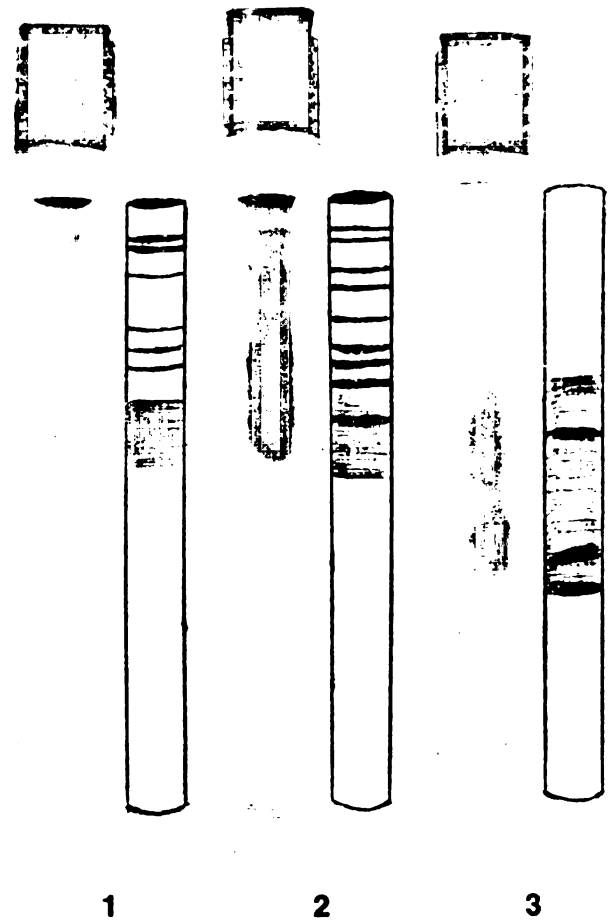


Figure 8.--SDS-gel electropherograms of the non-dialyzable, NPN components of alfalfa protein fractions designated (1) A_s, (2) IS_s, and (3) HS_s.

three TCA soluble, non-dialyzable fractions. The results are presented in Figure 8 (see p. 69).

The electrophoretic patterns indicate that similar proteins exist in fraction IS_s and A_s (Figure 8, gels 1 and 2 respectively). A small amount of material in fraction IS_s (not visible in the photograph) moved behind the ion front in the gel, being smaller than the majority of the other components. Fraction HS_s (Figure 8, gel 3) proteins penetrated farther into the gel than the bulk of the material in the two other fractions, indicating a smaller size distribution.

Assuming that the proteins constituting these fractions are ideal, i.e., non conjugated, etc., molecular weights can be estimated by reference to standard proteins (see Figure 17). By referring to this calibration curve it was concluded that (1) fraction A_s contains components of 30,000 Daltons and higher, (2) fraction IS_s contains components of 25,000 Daltons and higher with traces of material between 18,500 to 21,000 and (3) fraction HS_s proteins range between 19,500 to 43,000 Daltons. With proper consideration for the assumptions involved, there appears to be a definite size correspondence between the broad range of proteins found in all three fractions. This supports the previous conclusion that there is one or more protein species common to all TCA-soluble fractions.

Major Protein Component

Preparative Procedure

Previously presented electropherograms of the W and G fractions support the conclusion that most of the soluble protein of alfalfa extracts exists in one principal component (Romman et al., 1971). Additionally, sedimentation velocity analysis on the G fraction (Appendix, Figure F2) revealed that about 60% of the refractive material sediments as a single boundary of about 18S. Because the nature of the alfalfa protein fractions reflect the presence of the major component, this protein was isolated and its properties investigated.

Freshly prepared G fraction was equilibrated with 0.1M TRIS-HCl buffer, pH 8.0 and applied to a DEAE cellulose column. Elution was conducted as described previously. Gel electropherograms (not shown) of fractions from the cellulose column showed extensive heterogeniety with some of the principal protein present in all fractions. However, the major component was recovered in relatively high purity near the break-through of the NaCl-phosphate buffer elution. A typical DEAE elution pattern is shown in Figure 9A. The elution volume indicated by the horizontal bracket identifies the portion of the elution retained for further purification on Sepahdex G-200. Gel filtration was conducted on the DEAE-derived fraction following dialysis and concentration as previously described. The Sephadex elution

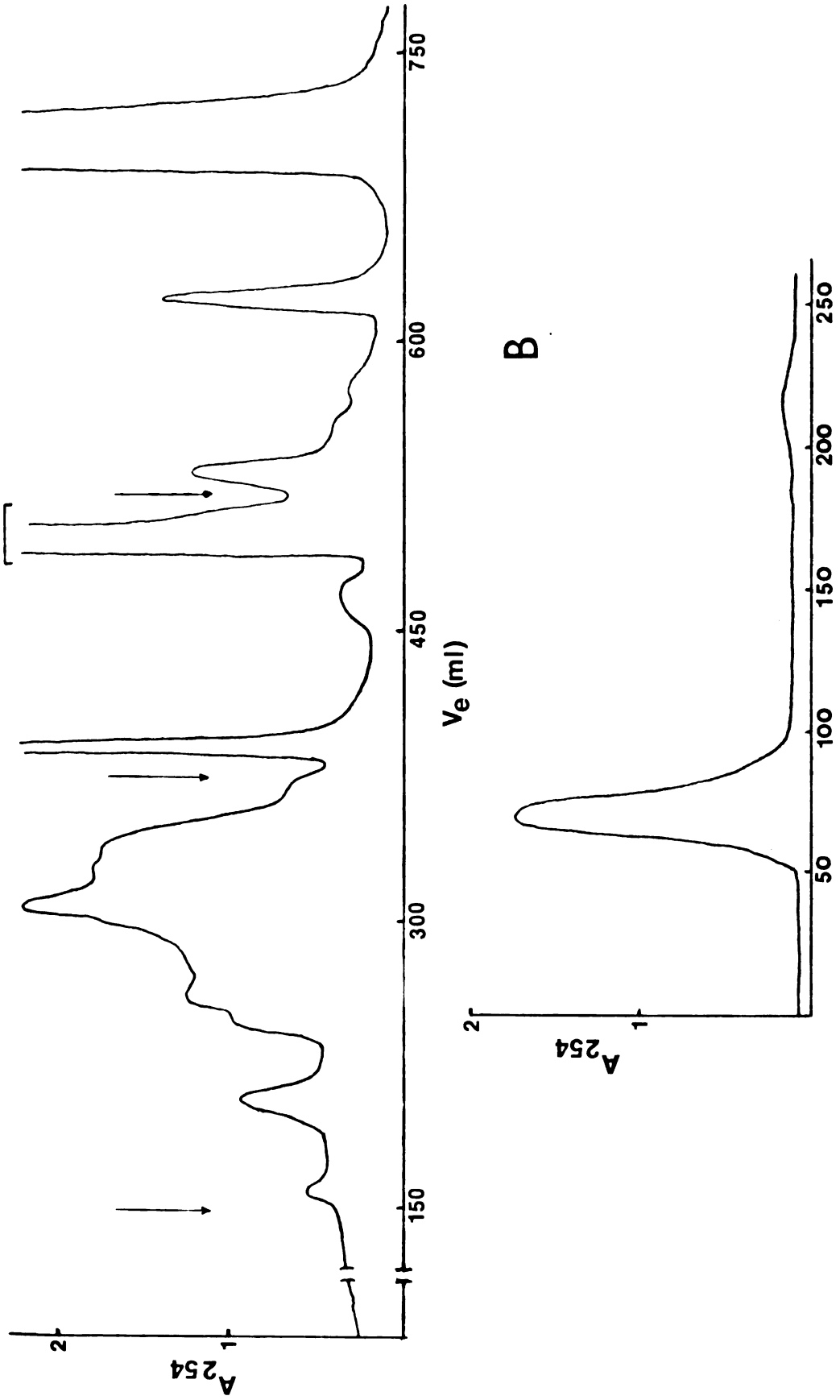


Figure 9.--Elution chromatograms obtained during purification of the major protein component from alfalfa as follows: (A) DEAE cellulose column. Arrows indicate buffer changes during elution of fraction G. (B) Sephadex G-200 chromatography of major protein fraction obtained from DEAE cellulose chromatography.

pattern is presented in Figure 9B and indicates that most of the material absorbing at 245nm appears in a single peak near the void volume of the column. Generally, the middle portion of the large peak was collected for analysis. Gel electrophoretic and sedimentation velocity analyses on this material support the conclusion that the protein exists in a state of purity suitable for detailed study. Figure 10 shows the gel electrophoretic and sedimentation patterns of the purified component. Sedimentation analysis (Figure 10A) revealed that most of the refractive material was present as a single boundary of about 18.5S and that less than 5% of the area constituted a faster sedimentating species. Figure 10B represents the electropherogram obtained with the purified component and indicates that most of the material migrated as one zone which was followed by a small "eyebrow" located directly below the spacer-running gel interface. Additional gel filtration did not remove this minor component.

Experimental observations indicated that studies on the physical nature of the major alfalfa protein must be conducted immediately after isolation because the purified protein was adversely affected if held at 4°C for more than about 2-3 weeks. For example, following a period of storage the protein displayed an atypical sedimentation pattern, i.e., two boundaries of equal area appeared in place of the previously observed single boundary, and the solution

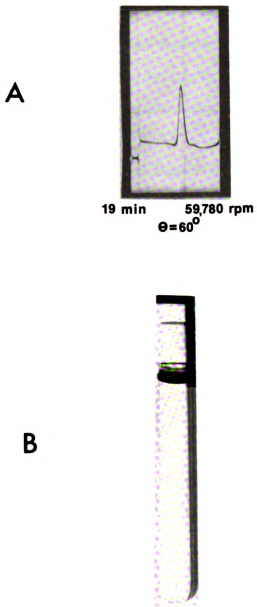


Figure 10.--A) Sedimentation velocity pattern of the major protein component in pH 8.6 veronal buffer containing 12.5 mM $MgCl_2$ ($\Gamma/2 = 0.1$). Protein concentration was 6.9 mg/ml. B) Electropherogram of final preparation of major protein component. Sample consisted of 25 µl of a solution containing approximately 5 mg/ml of protein.

appeared to be hazy or have a fine precipitate. Apparently, these phenomenon are generally encountered frequently (Stahman, 1963). Physical transfer of the major protein while in solution, i.e., by pipetting or pouring, require extreme care to avoid the development of a fine precipitate. Throughout the course of this study, extraneous precipitates were removed from small volumes of protein solution by filtration through Whatman No. 4 paper discs cut to fit a millipore filter adapter for small glass syringes. Unless otherwise indicated, chemical and physical analyses on the major protein were conducted with non-lyophilized samples in which the protein concentration was determined by the biuret method.

Enzymatic Nature of the Major Component

The assay procedure of Paulsen and Lane (1966) revealed that ribulose 1,5 diphosphate carboxylase (E.C. 4.1.1. 39) activity corresponded closely to the elution of the major protein from the G-200 column (Figure 11). The enzymatic nature and physical properties discussed below indicate that the major protein component from alfalfa tissue extracts is probably the much investigated Fraction 1 protein described by Kawishima and Wildman (1970). It exceeded the scope of this thesis to study the kinetic properties of this protein in detail. However, the enzymatic activity served as a useful monitor for assessing the native state of the protein submitted to physical analyses.

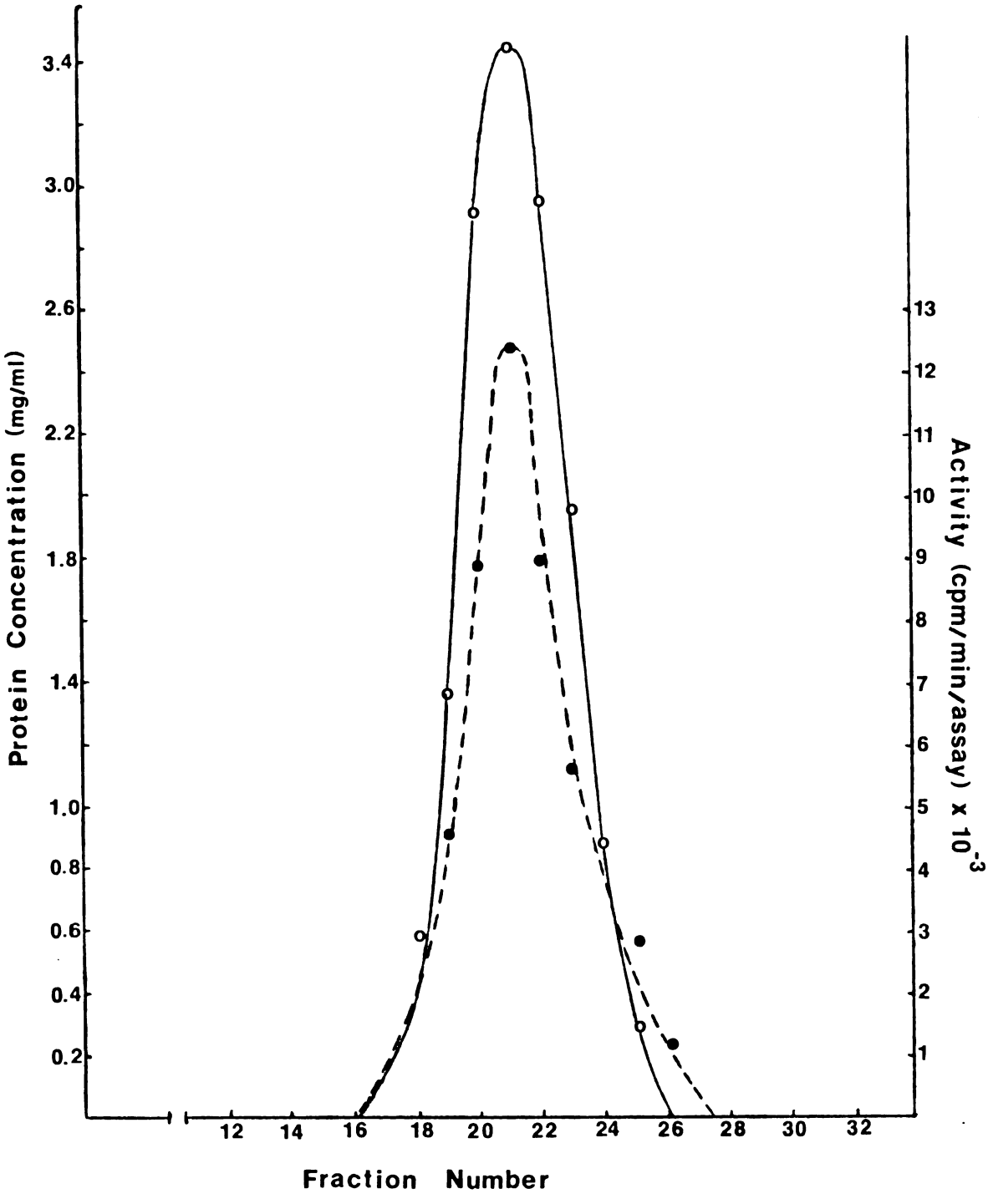


Figure 11.--Sephadex G-200 chromatography of major protein fraction obtained from DEAE cellulose chromatography. Column eluant was monitored for protein concentration (•———•) and 3.5 ml fractions collected and assayed for ribulose 1,5 diphosphate carboxylase activity. Carboxylase activity (•-----•) is expressed as counts per min of $^{14}C\text{-}HCO_3$-fixed x 10^{-3}.

Fractions from the Sephadex column were periodically checked for the 280/260 nm absorbancy ratio. For several preparations the value ranged from 1.69 to 2.0 indicating an absence of nucleic acids associated with the protein. Although earlier publications indicated that the protein contained associated nucleic acids, more recent reports suggest the absence of these materials (Paulsen and Lane, 1966; Ridley et al., 1967; Kawishima and Wildman, 1970).

Moving-boundary Electrophoresis

Few investigators utilize the technique of Tiselius electrophoresis, thus, it is not surprising that mobility determinations for carboxydismuates are rarely reported. Pon (1967) performed extensive physical characterization on the enzyme from spinach and reported mobilities of -3.21 to -6.08×10^{-5} $cm^2 volt^{-1} sec^{-1}$ (Tiselius units) for pH values from 6.3 to 9.45. An earlier publication by Mertz and Matsumoto (1955) reported the isolation of the major 19S protein from alfalfa (possibly carboxydismutase) by centrifugal precipitation for 2-2.5 hr at 40,000 rpm on a Spinco Model L preparative centrifuge, utilizing a No. 40 rotor. The protein contained nucleic acids and, when examined in pH 7 cacodylate buffer, possessed a mobility of -5.37 T.U. measured from the descending channel.

The major component of alfalfa which was isolated in this study possessed carboxydismutase activity and had a mobility (at infinite dilution) of -4.4 T.U. in pH 8.0

veronal buffer as shown in Figure 12. This value is lower than that reported by Mertz and Matsumoto (1955) possibly because of the absence of nucleic acids. However, it agrees quite closely with the value reported by Pon (1967), i.e., -4.57 T.U. as observed at pH 8.07.

Ultraviolet Absorption Spectrum

The ultraviolet absorption spectrum of the major protein component shown in Figure 13 is typical for a protein, having an A_{max} at 280nm. The 280/260 nm absorbancy ratio for the preparation (0.32 mg protein/ml) was 1.71, indicating an absence of associated nucleic acids. This is in agreement with the reports of previous investigators (Kawishima and Wildman, 1970).

Sedimentation Analysis

The apparent sedimentation coefficients of the major protein of the major protein component in pH 8.6 veronal buffer at various protein concentrations are summarized in Table 4.

The apparent sedimentation coefficient of the major boundary displayed concentration dependency, increasing with decreasing concentration of protein. Information in the literature on carboxydismutase appears to be inconclusive on this issue. Kawishima and Wildman (1970) state in their review article that the sedimentation coefficient of Fraction 1 protein was not appreciably concentration dependent. This is in contrast to the data reported by

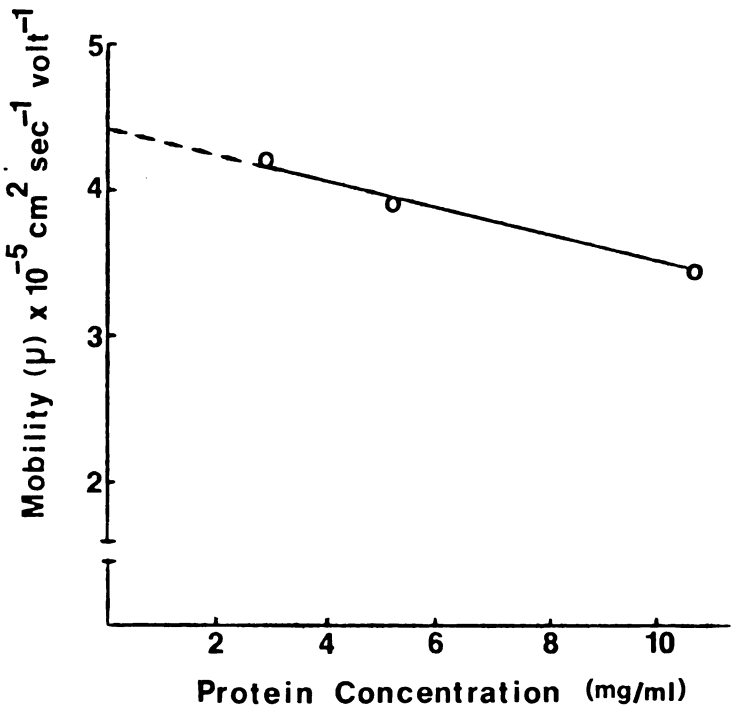


Figure 12.--Electrophoretic mobility of the major protein at different concentrations in pH 8.6 veronal buffer containing 12.5 mM $MgCl_s$ ($\Gamma/2 = 0.1$).

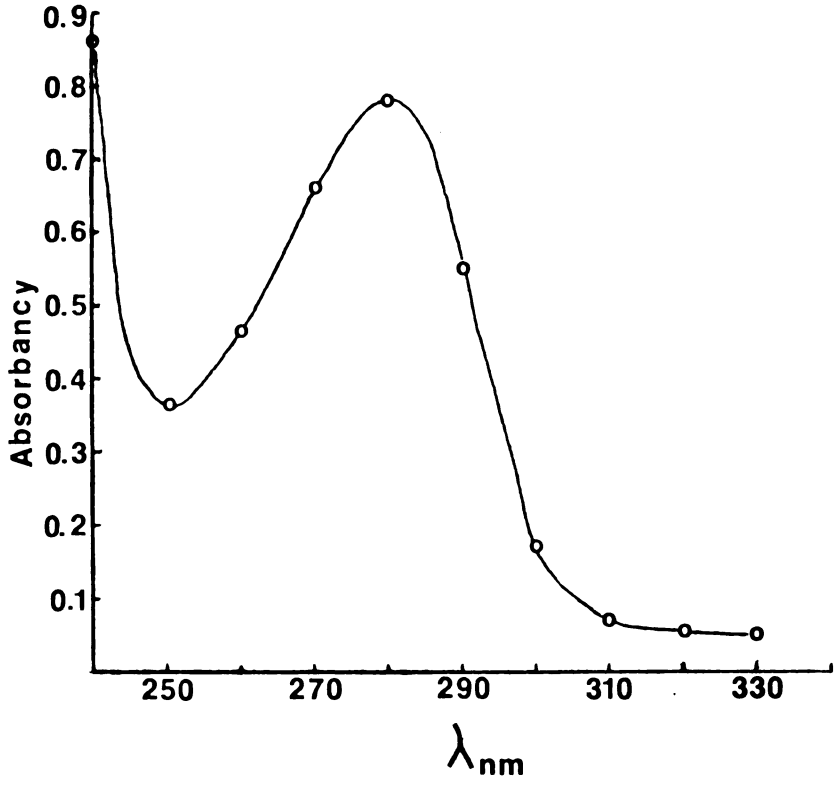


Figure 13.--Ultraviolet absorption spectrum of the purified major protein component. Protein concentration was 0.32 mg/ml of pH 8.0 TRIS-HCl buffer (0.1M) containing 12.5 mM $MgCl_2$ and 0.3mM sodium azide.

TABLE 4.--The sedimentation coefficients of the major protein component in pH 8.6 veronal buffer containing 12.5mM $MgCl_2$ ($\Gamma/2 = 0.1$).

Protein concentration	Sedimentation coefficient
10.35 mg/ml	16.12
6.90	18.46
5.18	20.64

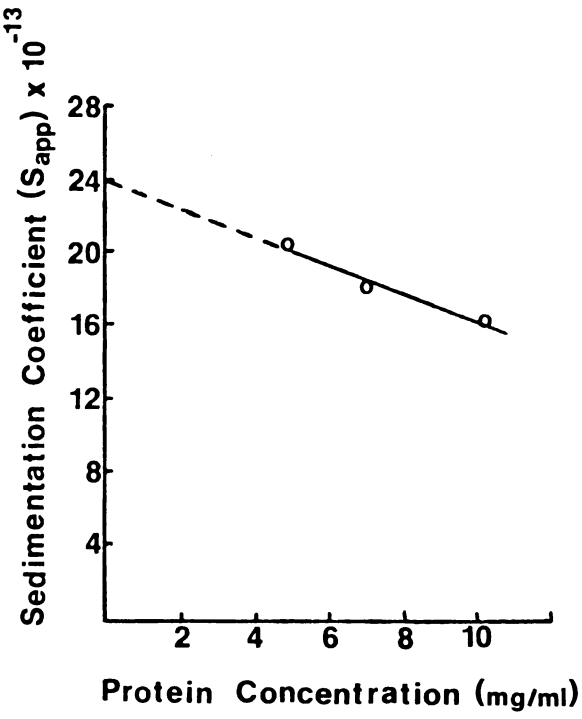


Figure 14.--Sedimentation behavior of the major protein component dissolved at several concentrations in pH 8.6 veronal buffer containing 12.5 mM $MgCl_2$ ($\Gamma/2 = 0.1$).

Ridley et al. (1967), Paulsen and Lane (1967), Pon (1967) and Trown (1965). Even among these authors, the type of dependency was unclear. Pon (1967) found that the sedimentation coefficient of Fraction 1 protein from spinach increased with decreasing protein to about 0.34 mg/ml. At this concentration, he found the trend reversed itself and the sedimentation coefficient decreased with lower protein levels. Trown (1965) reported that the sedimentation coefficient for Fraction 1 protein was proportional to concentration (decreased with decreasing concentration). Ridley et al. (1967) found that the sedimentation coefficient of carboxydismutase (Fraction 1) increased with decreasing protein concentration.

Figure 14 shows that the major protein possess an S_{obs} of approximately 24.0 when extrapolated to zero concentration and an $S^{\circ}_{20,w}$ of 25.3 when corrected for buffer. This value is higher than that reported for the same protein isolated from other sources (Kawishima and Wildman, 1970) but is reasonably close to the $S^{\circ}_{20,w}$ of 21.0 for spinach Fraction 1 protein as reported by Paulsen and Lane (1966).

Diffusion Coefficient

The apparent diffusion coefficients for the major protein component was also determined at several concentrations in pH 8.6 veronal buffer. The results are recorded in Table 5. The concentration dependency of the apparent

TABLE 5.--Diffusion coefficients of the major protein component in pH 8.6 veronal buffer containing 12.5mM $MgCl_2$ ($\Gamma/2 = 0.1$).

Protein concentration	Diffusion Coefficient
6.9 mg/ml	2.25
5.18	2.38
3.45	2.59

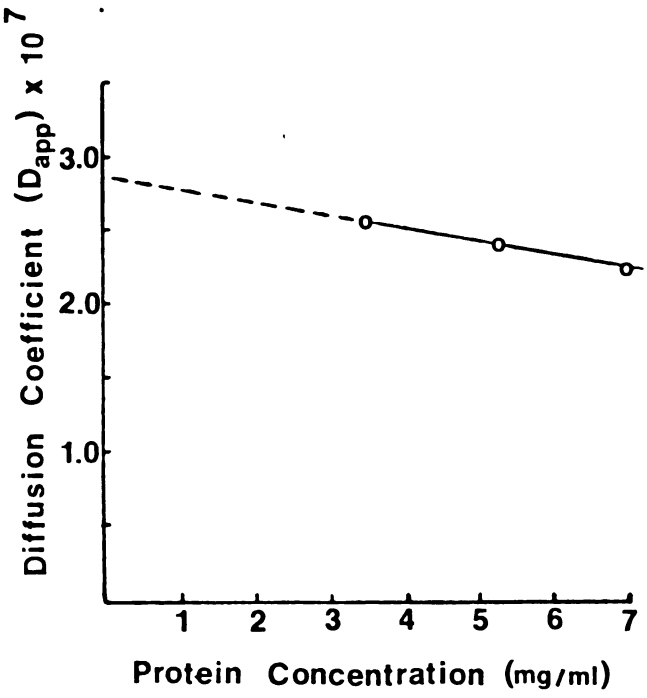


Figure 15.--Diffusion properties of the major protein component at several concentrations in pH 8.6 veronal buffer containing 12.5 mM $MgCl_2$ ($\Gamma/2 = 0.1$).

diffusion coefficient (D_{app}) is illustrated by the plot in Figure 15 which indicates that D_{app} increases slightly with decreasing protein concentration. This kind of data could indicate the protein is dissociating into smaller species or monomer units at low concentration. Extrapolation of the data yielded a D_{obs} of 2.84 Ficks Units and, after corrections for the buffer, a value of $D^{\circ}_{20,w} = 2.98 \times 10^{-7}$. This value falls within the range for diffusion coefficients -2.75 to 3.3 F.U.--previously reported (Kawishima and Wildman, 1970; Pon, 1967; Trown, 1967; Lyttleton, 1956).

Estimation of Molecular Weight

The molecular weight of the major protein was estimated from the Svedberg equation--$M = RTs/D(1-\bar{v}\rho)$--where R is the gas constant, T the absolute temperature, $\bar{v}$ the partial specific volume and ρ the density of the protein solution (assumed to be the same as the buffer). A value for $\bar{v}$ of 0.73, reported by Trown (1965) and a value of $\rho = 1.0039$ for the buffer as reported by Rhee (1969), were used in the calculation.

The molecular weight of the major alfalfa protein estimated with the above equation was approximately 786,800 Daltons. The value is slightly greater than 500,000-600,000 Daltons reported for this enzyme (Trown, 1965; Pon, 1967; Ridley et al., 1967; Kawishima and Wildman, 1970). These workers obtained molecular weights by employing equilibrium methods which are usually considered more reliable than the sedimentation velocity-diffusion technique

used in this study. Attempts to study the molecular weight of the Fraction 1 protein from alfalfa by equilibrium analysis were thwarted by technical difficulties.

The high value of the sedimentation coefficient undoubtedly contributes to the high molecular weight obtained in the present study. Other researchers incorporated reducing agents such as cysteine or mercaptoethanol in the buffer used for centrifugal analysis. Apparently, they were concerned with disulfide interchange leading to aggregation of the protein during molecular weight determinations. Similar reagents were not included in the present studies, perhaps accounting for the high sedimentation coefficient. Evidence will be presented to demonstrate that the carboxydismutase molecule isolated from alfalfa contains several free sulfhydryl groups, thus, it is conceivable that aggregation could occur through these moieties.

Effect of Dissociating Agents

The effect of six dissociating conditions on the major protein was studied by the techniques of gel electrophoresis: (1) mercaptoethanol (ME), (2) urea, (3) urea + ME, (4) pH 11 buffer, (5) pH 11 buffer + ME, and (6) sodium dodecyl sulfate (SDS). The results are presented in Figure 16. Gel 1 represents the undissociated major component following electrophoresis according to the method of Davis (1964). Gel 2 is the major component following

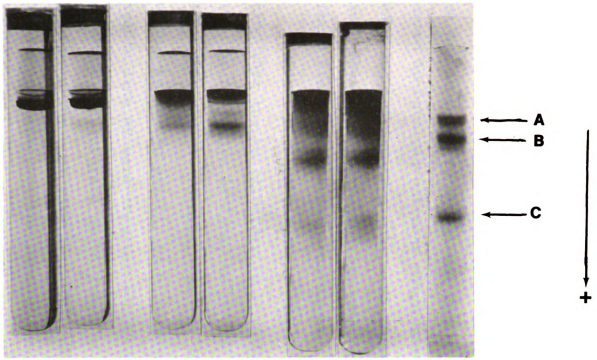


Figure 16.--Electropherograms of the major protein component as influenced by various dissociating agents. Gel (1) no dissociating agent, (2) after 30 min treatment with 10 mM mercaptoethanol (ME), (3) after equilibration in 5M urea, (4) after equilibration in 5M urea with 10mM ME, (5) after equilibration with pH 11 phosphate buffer, (6) after equilibration with pH 11 phosphate buffer with 10mM ME, and (7) after equilibration with 0.1% SDS according to Weber and Osborn (1969).

electrophoresis after a 30 min exposure to 10mM ME. Apparently this agent causes the release of a small amount of protein as indicated by the presence of a zone migrating ahead of the principal zone. Mercaptoethanol appears to have little affect on the major isolate. Gel 3 shows the purified protein previously equilibrated with 5M urea and electrophoresed in a 5M urea gel system. Gel 4 shows the 5M urea gel system plus ME added to the sample. The presence of ME in the urea gel resulted in a sharpening of zones but no dramatic change was noticed. Diffuse zones appeared in both gel patterns which were absent in gels 1 and 2 and may indicate that urea causes unfolding of the protein molecule, increasing its axial ratio and physically limiting its migration into the support.

Gels 5 and 6 show nearly identical patterns suggesting that the pH 11 buffer is an effective dissociating system for the major isolate, as is the combination of pH 11 buffer and ME. Similar results equating the disulfide bond-splitting ability of high alkaline pH conditions to agents like ME were reported by Donovan et al. (1972). Approximately 7 separate zones occur in both gel patterns derived with the high alkaline system which indicates that this treatment resolved the protein into more components or subunits than either 5M urea or the urea-ME system.

Gel 7 shows the results of SDS gel electrophoresis on the major protein (see Weber and Osborn, 1969). Three principal subunits, presumably differing in size, were

observed and were designated A, B, and C. In addition, 3 minor species were noted. A comparison of the relative mobilities for the three principal zones with those in the standard curve in Figure 17 yielded approximate molecular weights of A-56,000 Daltons, B-49,000 Daltons, and C-29,000 Daltons. Specimens of carboxydismutase isolated from several different sources have been extensively studied and most authors contend that the molecule is composed of various combinations of two distinct sizes of polypeptides, one of approximately 50,000 Daltons and the other of about 12,000 to 16,000 Daltons (Rutner and Lane, 1967; Akazawa et al., 1972; Rutner, 1970; Sugiyama et al., 1971; Moon and Thompson, 1967). The size distribution for the large subunits of alfalfa carboxydismutase (represented by zones A and B, Figure 16, gel 7) was in reasonable agreement with these values. However, the smallest subunit (zone C) was considerably larger than previously reported.

The application of SDS-gel electrophoresis to carboxydismutase isolated from several plant species has produced variable results. Rutner and Lane (1967) reported two subunits for the enzyme from spinach as did Sugiyama et al. (1971) who studied the Chlorella ellipsodia protein. However, Sugiyama and Akazawa (1967) found that carboxydismutase from wheat was dissociated into several subunits depending upon the concentration of SDS used. Sugiyama et al. (1968) observed a concentration dependent

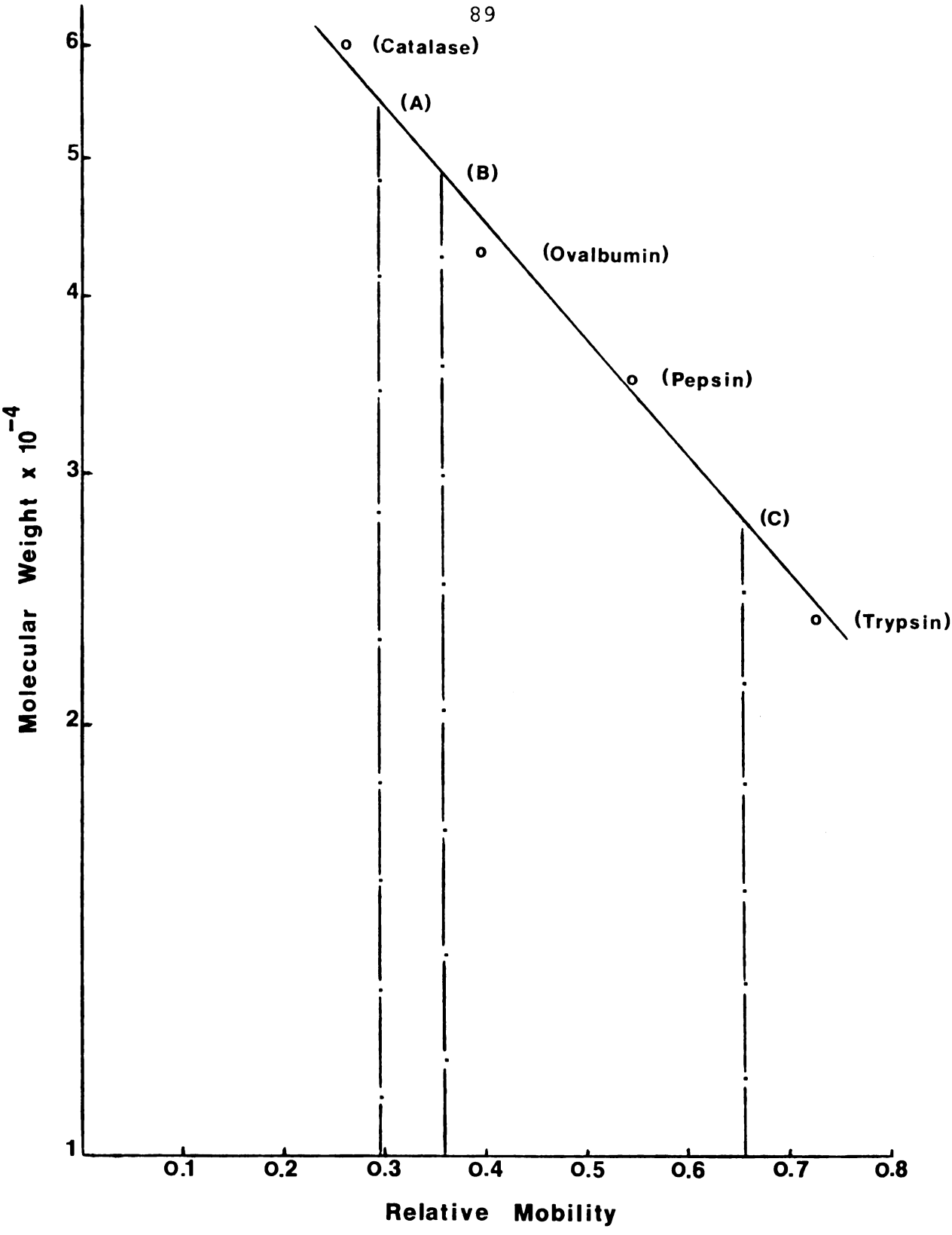


Figure 17.--Standard curve of molecular weight versus relative mobility in SDS gel. Standards used were trypsin (23,300), pepsin (35,000), ovalbumin (43,000) and catalase (60,000).

affect of SDS on the number of subunits obtained for the enzyme from spinach. This latter work appears to contradict the finding of Rutner and Lane (1967) who observed only two subunits for the spinach enzyme. Because the data regarding the subunit structure of alfalfa carboxydismutase doesn't conform to the commonly accepted model for the enzyme, additional data should be gathered on this question. It is possible that SDS is dissociating the protein in a non-specific manner as suggested by Sugiyama and Akazawa (1968) or other non-specific interactions are occurring during electrophoresis. Weber and Osborn (1969) cite the need to be cognizant of the possibility of disulfide bond formation between protein subunits during the application of their method but point out that this was no problem in the majority of the proteins studied. Possibly the method of SDS-gel electrophoresis should be reapplied to the major alfalfa isolate after dissociation of the protein, followed by reduction and alkylation of free sulfhydryl groups.

Data previously presented relative to the effect of urea and high alkaline pH conditions also suggest the existence of greater heterogeneity in the substructure of alfalfa carboxydismutase than previously noted (Ruter and Lane, 1967; Akazawa et al., 1972; Kawishima and Wildman, 1971). Alternatively, these results might reflect a small amount of contaminant in the preparation or non-specific splitting of the molecule under the influence of high

alkalinity or urea (Akazawa et al., 1972). However, the results of the electrophoresis experiments in the presence of all dissociating agents studied herein, were reproduced on several trials.

Results presented and discussed below indicate that the major protein from alfalfa contains several disulfide bonds. Because ME treatment of the protein did not release significant amounts of electrophoretically distinct polypeptides compared to urea or pH 11 treatment, the disulfide bonds were considered to be of the intrachain type.

Chemical Analyses

Table 6 summarizes the chemical analyses performed on the major protein component.

The isolated material was found to contain 16.6% nitrogen, 1.1% hexose and no hexosamine or sialic acid. Ridley et al. (1967) reported that carboxydismutase isolated from spinach contained 16.75% nitrogen as determined from amino acid analysis. Their final preparation however, contained some ash, lipid and carbohydrate material. They were also able to calculate a conversion factor for nitrogen to protein content of 5.97 and, accordingly, the protein content in the final preparation isolated from alfalfa would be 99.1%.

The protein isolated in this study contained about 1.1% hexose but lacked other carbohydrates normally occurring

TABLE 6.--Summary of chemical analyses on the major protein component.

Item	Value
%N	16.6
%Protein[a]	99.1
%Carbohydrate	
Hexose	1.1
Hexosamine	none
Sialic acid	none
Exposed -SH[b]	1.4
Unexposed -SH[b]	46
Unexposed -SH and S-S[b]	110
Unexposed -SH and S-S[b,c] (alkaline treated)	95

[a]Computed as %N x 5.97 according to Ridley et al., 1967.

[b]Expressed as number of SH (or S-S reduced to SH) per molecule.

[c]Determined following 24 hr exposure to pH 11 buffer at 4°C.

in glycoproteins. In their review, Kawishima and Wildman (1970) cited the confusion surrounding the question of whether this protein contains carbohydrate moieties. For example, Ridley et al. (1967) and Akazawa et al (1965) described the protein as containing carbohydrate while Paulsen and Lane (1966) and Pon (1967) have pronounced its absence. Plant material must be thought of as an excellent source of phenols which could contribute to a positive hexose analysis with the method used in this study (Dische, 1955). Therefore, it is premature to designate carboxydismutase isolated from alfalfa as a glycoprotein based

upon the small amount of hexose detected. The absence of other commonly occurring carbohydrates found in glycoproteins suggests that the hexose detected in the protein preparation was fortuitously bound during isolation. Trown (1965) points out the tendency of Fraction 1 protein to bind other materials (including proteins) during isolation.

The data in Table 6 also include the results of analyses for free (exposed) sulfhydryl, unexposed sulfhydryl and reduction-induced sulfhydryl groups ostensibly involved in disulfide bond formation.

If one assumes a molecular weight for this protein of 786,000 Daltons, there exist approximately 1.4 free sulfhydryl groups that can be detected without unfolding the molecule. Unfolding the protein with 4.3M urea produced a total of 46 measurable sulfhydryl groups whereas unfolding of the molecule and associated reduction of disulfide bonds produced a total of approximately 110 -SH groups. This suggests the presence of about 32 disulfide bonds throughout the interior of the molecule and/or its subunits.

It is interesting to note that Kawishima and Wildman (1970) describe the enzyme as lacking disulfide bonds. This conclusion was supported by the evidence of Sugiyama and Akazawa (1967) who applied PCMB titration to carboxydismutase and found 96 free sulfhydryl groups. In the latter publication the authors interpreted the data of

Ridley et al. (1967) to indicate that they would have found 96 -SH groups (by cysteic acid determination) if the protein was composed of 24 identical subunits. Sugiyama and Akazawa also detected similar numbers of free sulfhydryl groups which would leave none for disulfide bond formation. The literature relating to the presence of disulfide bonds is further complicated by the findings that the molecule was actually comprised of non-identical subunits (Moon and Thompson, 1966; Rutner and Lane, 1967) and the presence of 30-40 sulfhydryl groups on the enzyme as detected by other techniques (Sugiyama et al., 1968).

The 46 free -SH groups found in this study on alfalfa Fraction 1 protein is in reasonable agreement with the 30-40 per molecule detected by Sugiyama et al. (1967). Additionally, other data obtained suggest that 32 disulfide bonds exist in the molecule, although the technique applied does not distinguish between intra and intermolecular bonds. The higher molecular weight observed here than normally reported could be the result of aggregation through disulfide bonds. Additional work to determine the molecular weight in the presence of reducing agents (i.e., mercaptoethanol) will be required to test this hypothesis.

Finally, the data in Table 6 indicate that a 24 hr exposure of the major protein to pH 11 buffer caused a drop of approximately 8.7% in the number of detectable -SH groups (i.e., 95 per molecule). This probably represents

the degradation of S-S bonds by the high alkalinity of the media as observed by earlier workers with other proteins (Young and Potts, 1963; Swaisgood et al., 1964; Donovan et al., 1972). These observations suggest that alkaline dissociation of alfalfa Fraction 1 protein is degradative in nature, and caution should be applied when interpreting the subunit structure indicated by high pH gel electropherograms.

Amino Acid Analysis

The amino acid composition of the major alfalfa protein is presented in Table 7 expressed in two ways: (1) as moles of each amino acid per 1000 moles of recovered amino acids, and (2) as relative molar ratios of each amino acid compared to Phe.

The more descriptive data-moles/1000 moles amino acids-indicates that the Fraction 1 protein (carboxydismutase) from alfalfa is a mildly acidic protein, i.e., the ratio of the acidic Asp and Glu residues to the basic Arg, His and Lys residues is approximately 1.1. For comparison, a similar ratio for the milk caseins is 2.0-2.5. Additionally, the alfalfa isolate possesses a hydrophilic nature because the ratio of the hydrophilic residues Asp, Glu, Tyr, Lys, His to the hydrophobic residues Phe, Pro, Met, Val, Leu and Ileu is about 1.3. This relationship confirms the theory of Trown (1965) who suggested that the enzyme has a hydrophilic character because of its large

TABLE 7.--Amino acid composition of carboxydesmutase from different species of plants.

Amino acid	Alfalfa[a] (Moles/1000) moles	Molar ratio relative to phenylalanine			
		Alfalfa[a]	Spinach beet[b]	Spinach[c]	Spinach[d]
Phenylalanine	51.45	1.00	1.00	1.00	1.00
Aspartic acid	82.80	1.60	1.76	2.01	2.37
Serine	31.14	0.61	1.08	0.68	0.87
Threonine	55.48	1.08	1.41	1.37	1.54
Glutamic acid	98.89	1.92	2.08	2.18	2.55
Proline	56.09	1.09	1.17	1.24	1.29
Glycine	87.56	1.70	1.95	2.05	2.22
Alanine	84.57	1.64	1.80	1.89	2.06
Half-cystine	12.42	0.24	0.40	0.46	--
Valine	67.24	1.31	1.80	--	1.39
Methionine	3.91	0.08	0.40	0.45	0.50
Isoleucine	45.24	0.88	1.00	0.84	0.65
Leucine	81.41	1.58	1.89	2.00	2.12
Tyrosine	44.45	0.86	0.89	1.07	1.10
Lysine	54.21	1.05	1.19	1.15	1.18
Histidine	42.07	0.82	0.89	0.67	0.67
Arginine	69.26	1.35	1.00	1.34	1.42
Tryptophan	17.43	0.34	0.30	0.64	--

[a]This study

[b]Ridley, et al.

[c]Rutner and Lane (1966)

[d]Kawishima (1969)

charge to mass ratio as well as its tendency to bind ionic substances during isolation.

The amino acid composition of carboxydismutase is conventionally reported as molar ratios relative to Phe (Kawishima and Wildman, 1970). The analysis of the major alfalfa protein was therefore computed for comparison to three typical preparations of carboxydismutase from other species. Differences between species are readily apparent. The alfalfa protein possesses a lower ratio for all residues determined. This indicates a relatively higher amount of Phe in the alfalfa protein than for the other species indicated in Table 7. Also ½CyS and Met are partially degraded during hydrolysis, yielding lower values than that reported by Rutner and Lane (1967) and Ridley et al. (1967) who determined these residues by separate analysis. A general agreement between the computation of the alfalfa protein and the three reference proteins was found. Specifically, the ratio value for Asp, Thr, Glu, Pro, Gly, Ala, Val, Leu and Arg exceed one, whereas the values for ½CyS, Met, His and Try are less than one. The remaining residues were observed to vary in a random manner for all four analyses presented.

SUMMARY

Soluble alfalfa proteins can be extracted with a variety of different comminution media. However, superior yields and solubility for the two fractions studied were achieved with an extraction medium consisting of 0.1M TRIS-HCl buffer, pH 8.0 and containing 10mM $K_2S_2O_5$ and 5.8mM (0.1%) ascorbic acid plus an associated dialysis against 0.02M TRIS-HCl buffer, pH 8.0. The presence of reducing substances in the comminution media appears to ensure maximum solubility of protein components, but the exposure time and amount of such reagents must be carefully regulated to avoid protein denaturation.

Six alfalfa protein fractions were prepared by subfractionation of a whole cellular extract (fraction W), using: (1) 0.5 saturated ammonium sulfate, (2) adjustment to pH 4.4-4.5, or (3) application of heat to 80°C. With these conditions, precipitated material (Fractions G, IP, and HP) was isolated and subsequent chemical analysis revealed that the precipitates contained more protein and lipid, less carbohydrate and ash than the associated soluble fractions (A, IS and HS). The heat precipitated fraction (HP) was rendered permanently insoluble and was

not studied by solution techniques. All other fractions were completely soluble above pH 8.5 when initially isolated.

Gel electrophoresis of all fractions revealed considerable heterogeniety, demonstrated as well as unresolved, with most of the protein in one or two zones for the G, IP and W fractions. The A, IS, and HS fractions were characterized by numerous zones distributed throughout the gel. The results of these studies suggested considerable correspondence between fractions G and IP as well as between A and IS. Fractions IS, A, and HS contained protein soluble in 15% trichloroacetic acid and were designated IS_s, A_s, HS_s, respectively. Gel electrophoresis in the presence of SDS revealed that comparable molecular species occurred in the IS_s and A_s fractions ($2.5\text{-}7.0 \times 10^4$ Daltons), whereas the HS_s proteins were different and smaller in size ($1.9\text{-}4.3 \times 10^4$ Daltons).

The IP fraction (isoelectric precipitate) represented the soluble protein mixture most easily attainable by commercial methods and some of its functional properties were investigated. This fraction was highly soluble (80%) below pH 2.5 and above pH 7.0. Minimum solubility was observed between pH 4.0-5.0. The fraction displayed heat stability in highly acid or alkaline buffers but was heat sensitive when in solution at neutrality. The observed stability in acid or alkaline buffers may be the result of a protein-protein repulsion due to a high charge: mass ratio.

The IP fraction was calcium sensitive in pH 8.9 buffer with maximum precipitation occurring at 50mM Ca++. The same fraction was calcium stable at pH 2.3 and the onset of precipitation did not occur below 125mM and was maximum at 250mM Ca++. Calcium-induced precipitation of the IP fraction solubilized in alkaline buffer may have resulted from the neutralization of charge-charge repulsion leading to chelation or salt bridging and thence aggregation of molecules. Coagulation by calcium ion in acid buffer may have resulted from the slow formation of inter-molecular chelates as the concentration of the calcium became overwhelming.

Most of the protein present in the soluble G fraction was of one species which was isolated by a combination of DEAE cellulose and Sephadex G-200 chromatography. This component possessed ribulose 1, 5 diphosphate carboxylase (E.C. 4.1.1. 39) activity. The protein possessed an $S^{\circ}_{20,w}$ of 25.3 and an estimated molecular weight of 786,800 Daltons in veronal buffer, pH 8.6 ($\Gamma/2 = 0.1$) containing 12.5mM $MgCl_2$. The electrophoretic mobility in the same buffer was -4.4 Tiselius Units at infinite dilution. Gel electrophoresis of the major protein in the presence of 5M urea revealed 5 separate subunits with associated smearing indicating extreme molecular unfolding or unresolved subunits. Electrophoresis in the presence of 10mM mercaptoethanol did not affect the characteristics of the protein patterns, indicating that disulfide bonds are present as

intrachain moieties. Electrophoresis in the presence of 0.1% SDS revealed three major protein zones with molecular weights of 5.6, 4.95 and 2.8 x 10^4 Daltons, as well as three minor species of undetermined size.

Chemical analyses of the alfalfa Fraction 1 protein revealed 16.1% nitrogen, 1.1% hexose and the absence of nucleic acid constituents. Other carbohydrate moieties commonly associated with glycoproteins were absent.

The protein possessed 32 disulfide bonds and a total of 110 reduced sulfhydryl groups, some of which were found to be sensitive to high alkaline conditions. Amino acid analysis revealed that the major protein possessed a slightly acidic and hydrophilic nature and contained Glu, Asp, Gly and Ala in highest concentrations. The relative distribution of all residues compared favorably with previously published reports for the protein.

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APPENDIX

TABLE A1.--Some important chemicals used in this study and their sources.

Chemical	Source
Diethylbarbituric acid Sodium diethylbarbituric acid Mannose Acetlyacetone 2-mercapotethanol Urea Sodium dodecyl sulfate	Fisher
2-thiobarbituric acid N,N,N',N',-titramethylethylenediamine (TEMED) Acrylamide N,N'-methylenebisacrylamide Sodium azide	Eastman Organic Chemicals
TRIS-hydroxymethyl aminomethane (Sigma 121, Trisma base and Sigma 7-9)	Sigma
Galactosamine	Nutritional Biochemicals
Tryptophan N-acetylneuramic acid	Calbiochem
Special-Noble Agar Plate count agar	Difco
Galactose	Phanstiehl Laboratories, Inc.

TABLE A2.--Method for determination of total lipid.

a. weigh 50-100 mg sample into conical centrifuge tube*
b. add 1.5 ml 2% KCl and agitate tube
c. add 1.0 ml 95% ethanol; seal tube with stopper wrapped in saran wrap; shake tube 30 sec.
d. release pressure and add 2.5 ml ethyl ether, making certain to rinse stopper
e. seal tube and shake 30 sec
f. release pressure and add 2.5 ml petroleum ether, making certain to rinse stopper
g. seal tube and shake 30 sec
h. centrifuge tube 1 min on clinical centrifuge
i. carefully remove upper layer with syringe and place fluid in previously tared dish held on electrical plate
j. wash tube with 1.0 ml of ethyl, petroleum ether mixture (1:1)
k. repeat steps c-j twice
l. evaporate pooled washing to dryness and place dish in 110°C vacuum oven for 30 min
m. cool dish in desicator and reweigh

*Samples and solvent blanks were done in duplicate and results averaged.

TABLE A3.--Timetable for immunization procedure using fraction W as sensitizing material.

Injection No.	Interval	Route of Injection	Total Volume	Adjuvant
1.	1st day	Subcutaneous-over ear and groin nodes	4 x 1.0 ml[a] (10mg/ml)	Freund's Complete
2.	7th day	Subcutaneous-top of neck	1 x 0.5 ml (10mg/ml)	None[b]
3.	21st day	Subcutaneous-top of neck	1 x 0.5 ml (10mg/ml)	None
	26th day	Test for antibody titer with small amount of blood		
4.	32nd day	Subcutaneous-top of neck	2 x 0.4 ml (10mg/ml)	None
	37th day	Collect whole blood for immunological analyses		

[a]Fraction W dissolved in 0.1M TRIS-HCl buffer, pH 8.0 (20 mg/ml) and diluted 1:1 with complete adjuvant before injection.

[b]Fraction W dissolved in 0.1M TRIS-HCl buffer, pH 8.0 (10 mg/ml) before injection.

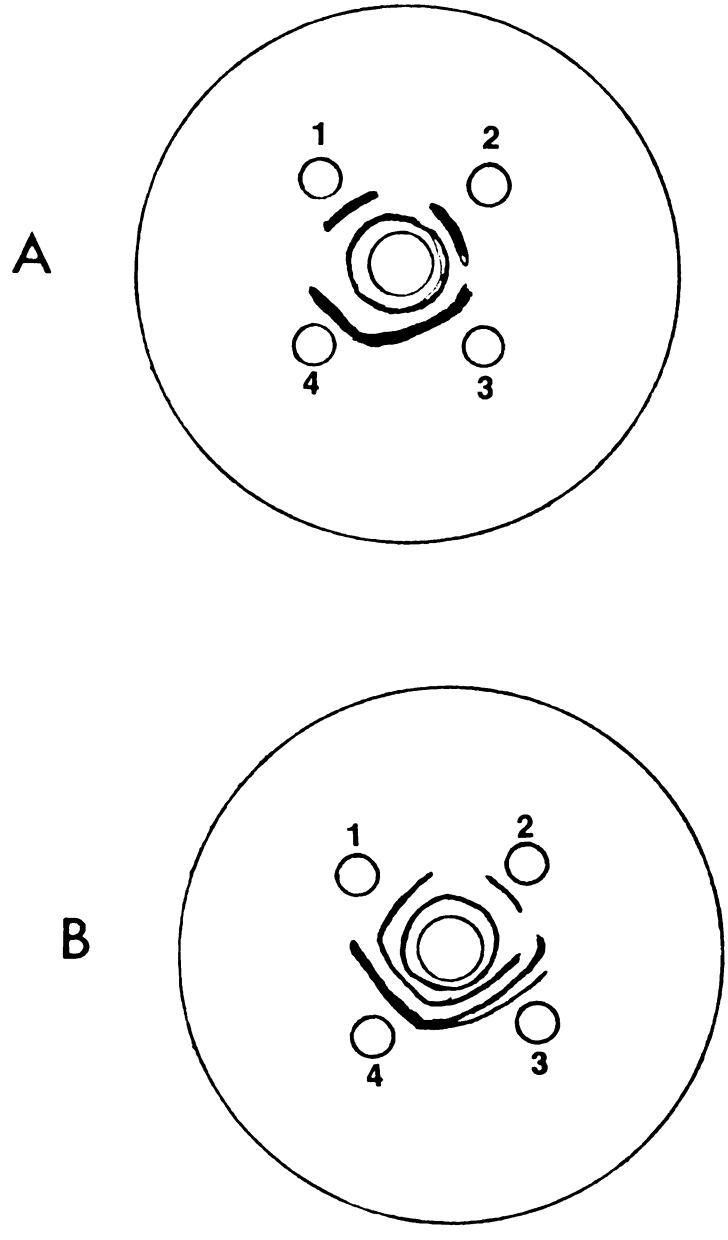

Figure F1.--Immuno-double diffusion patterns of selected alfalfa protein fractions (2%) in pH 8.6 veronal buffer. Center wells contained whole serum derived from a rabbit previously sensitized to alfalfa protein fraction W. Other wells contain antigen solutions as follows: Pattern A--(1) fraction A, (2) fraction HS_s, (3) fraction IS_s and (4) fraction IS. Pattern B--(1) fraction IS_s, (2) fraction IS_s, (3) fraction IS, and (4) fraction IP.

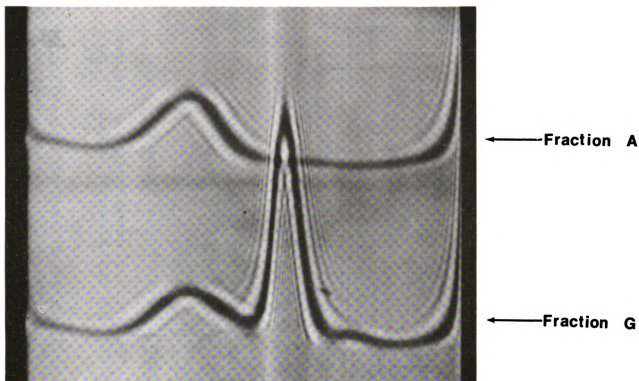

Figure F2.--Sedimentation velocity pattern of alfalfa protein fractions after 26 min at 39,470 rpm in pH 8.6 veronal buffer ($\Gamma/2 = 0.1$), 20°C and 0 = 60°. Concentrations of fraction A and G were 15 mg/ml and 6 mg/ml respectively.

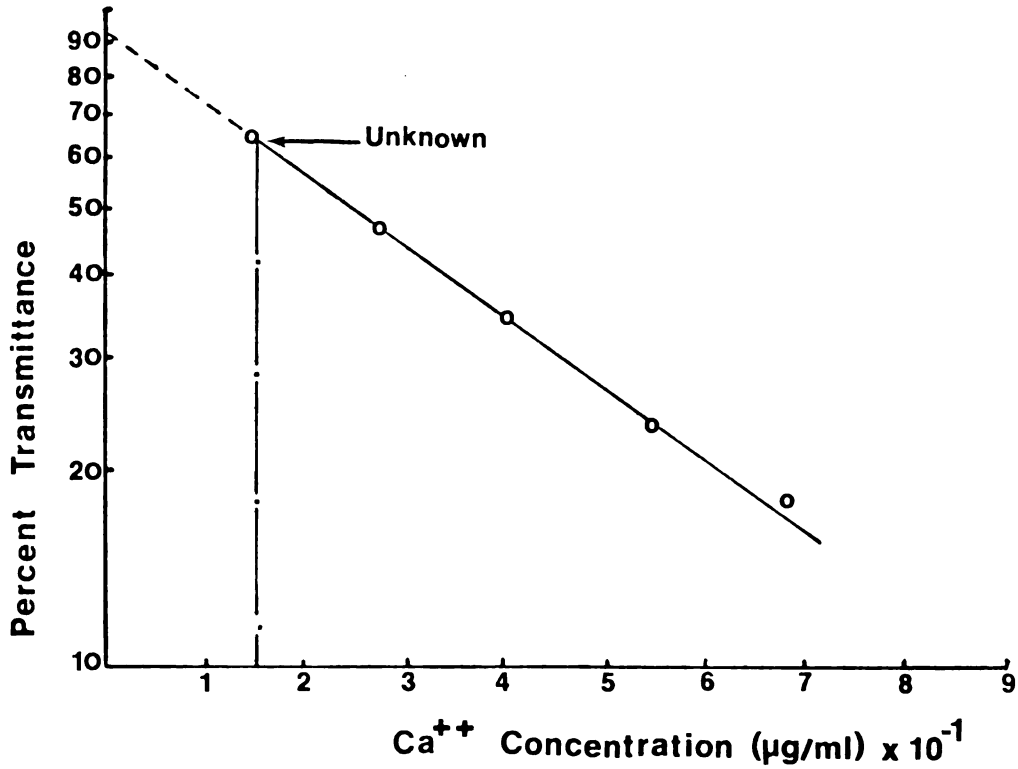

Figure F3.--Standard curve for determination of Ca++ by atomic absorption spectroscopy.

Calculation of Ca++ in Unknown

Sample: Fraction A ash
wt. 0.000095 mg in 100 ml 0.1N HCl (95 µg/ml)
%T. 65.0

Concentration of Ca++ is 14 µg/ml and sample contains 14 µg/95µg sample or 15% Ca++