POTENTIAL UTILIZATION OF LEGUMINOUS TREES FOR MINIMAL ENERGY INPUT AGRICULTURE AND AMINO ACID ANALYSIS BY GAS-LIQUID CHROMATOGRAPHY

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ABSTRACT

The utilization of leguminous tree orchards with a grass-sod cover is suggested as the ideal minimal energy input agriculture because: 1) This system would not require machinery and fuel for tillage; 2) The perennial soil cover would prevent soil erosion and reduce nutrient leaching; 3) Leguminous trees fix their own nitrogen and often have a high protein content in the seed; 4) Leguminous trees produce nutritious palatable pods with large, or larger yields than conventional annual crops. The uses and yields of leguminous trees of the genera Acacia, Gleditsia, Leucaena, Parkia, Pithecellobium, and Prosopis are documented in North and South America, Africa, and Asia. The protein and amino acid composition of the seeds of 15 species of leguminous trees have been determined and a large genetic diversity found indicating that a breeding and selection program could be successful in further increasing the value of these trees. A leguminous tree seed has been found that contains 69% protein (Prosopis chilensis) as well as one with a chemical nutritional score of 0.79 (Pithecellobium lobatum). This compares favorably with the chemical scores of 0.58 for casein and 0.47 for soy protein. Unfortunately, Pithecellobium lobatum is the seed with the lowest protein content, and the Prosopis chilensis seed has many deficient essential amino acids.

A modification of a quantitative gas-liquid chromatographic assay for the amino acids other than histidine, tryptophan, and cystine as their N(O)-Perfluorobutyryl-O-isoamyl derivatives is reported. An additional

method for quantitative determination of cystine, histidine, and tryptophan as the N-acetyl-n-propyl esters from proteins is also reported. The latter procedure utilizes disulfide reduction and S-alkylation followed by acid hydrolysis in 6 N HCl with dithioethane and metallic tin as a tryptophan protectant. An isotope-dilution method was employed with cystine, histidine, and tryptophan to permit correction for losses during acid hydrolysis and gas-liquid chromatography. The 70 eV fragmentation patterns are presented for N(O)-perfluorobutyryl-O-isoamyl derivatives and for the N-acetyl-o-methyl derivatives.

A colorimetric micro-Kjeldahl method is described which permits nitrogen determinations on 3 to 30 μg of protein.

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BY

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BHT	2,6 di-t-butyl-p-cresol
DTE	dithioerythritol
FAO	Food and Agricultural Organization of the United Nations
FID	flame ionization detector
gc-ms	gas chromatography-mass spectrometry
glc	gas liquid chromatography
IAA	indole-3-acetic acid
IBP	International Biological Program
ir	infrared
NEM	N-ethylmaleimide
nmr	nuclear magnetic resonance
SDS	sodium dodecyl sulfate
TBHP	tertiary butyl hydroperoxide
TCA	trichloroacetic acid
tlc	thin layer chromatography
TMS	trimethylsilane
TVA	Tennessee Valley Authority
uv	ultraviolet

INTRODUCTION

The ultimate objective of this research was the identification of agricultural systems which would require minimal inputs of fossil fuels, capital, and mechanization and still yield large amounts of high quality protein. The bulk of the experimental work of this thesis involved development of glc techniques for amino acid analysis so that amino acid composition data could be used to assess the nutritional quality of plant proteins.

The experimental section consists of 5 papers, the first of which has been published, the second accepted for publication, and the remaining three submitted for publication. Sections I and II deal with glc techniques for amino acid analysis, section III with a micro-kjeldahl method for nitrogen determination, and sections IV and V with the protein and amino composition, and potential for development of tree legume agricultural systems.

I. Gas-Liquid Chromatography of Amino Acids

Leguminous trees have been proposed as the ideal food source for developing nations (Felker and Bandurski, submitted for publication). Thus, it becomes important to survey the seeds from a number of tree legumes for their protein and amino acid content with the hope of determining their protein quantity and quality. Since an amino acid analyzer was not available to me on a regular basis it was decided to assay the amino acids using published methods on glc of amino acids. It soon became apparent that glc of amino acids was not as easily performed as had been reported. Even with conventional amino acid analyzers, problems arise in determining amino acid composition of foods because carbohydrate in the sample and the degree of anaerobicity during hydrolysis markedly affects the yield of threonine, serine, tyrosine, methionine, and cystine. Tryptophan is usually destroyed during acid hydrolysis (Moore and Stein, 1963).

The labile amino acids, especially cystine, methionine, and tryptophan, whose analyses are most difficult, are the most important nutritionally. For example the average amino acid composition of soybeans, beans (Phaseolus vulgaris), wheat, oats, corn, and rice taken from Food and Agricultural Organization (FAO) values (Autret, 1972), as compared to the recommended values for methionine, cystine, and tryptophan in the human diet (Autret, 1972) reveals that all but rice are below the recommended value for methionine; that all but oats are low in cystine; and that all are low in tryptophan.

It is obvious that amino acid composition studies used to determine nutritional quality should include the amino acids cysteine, methionine, and tryptophan. Legume seeds are known to be low in the sulfur amino

acids, and cereals are known to be low in lysine. Practically all grains are deficient in one of the sulfur amino acids and in tryptophan. The fact that these amino acids are present in small quantities further compounds the problem of analysis, as quantitation of these amino acids is made difficult by the presence of other amino acids such as glutamic acid present in concentrations an order of magnitude larger.

Because of the importance of assays for the limiting amino acids, it was decided to see what progress could be made with a glc amino acid analysis method to reduce the cost of analysis, increasing the reliability, and shorten the analysis time for those amino acids. There is a wealth of information on chemical modification of proteins, peptide synthesis, and amino acid chemistry and it was decided to bring this knowledge to bear on the problems of glc analysis of cystine, methionine, histidine, and tryptophan derived from protein.

The review of the glc of amino acids is confined to 1968 to date since literature prior to 1968 is reviewed by Roach and Gehrke (1969) and by Coulter and Hann (1971). The glc of amino acids since 1968 has been almost exclusively devoted to derivatives in which the amino group is acylated with an acid anhydride after the carboxyl group has been blocked by esterification with an aliphatic alcohol. The only exception to this is a paper published by Gehrke and Leimer (1971) on trimethylsilylation (TMS) of amino acids. In spite of the convenient one step synthesis of amino acid derivatives this method is in little use today. The scant use of this technique may be due to the fact that several derivatives are formed for each amino acid. For example three derivatives of glycine are formed each with different retention times. A critique of the TMS-

amino acid procedure has been published by a commercial supplier of glc accessories (Supelco 1971).

The use of the n-propyl-N-acetyl derivatives of amino acids for glc was one of the most successful of the earlier methods. Coulter and Hann (1968), in a very thorough paper, described the preparation of the n-propyl-N-acetyl derivatives in a special apparatus and the conversion of arginine and histidine to a more suitable form for derivatization. Very recently, modifications of this method (the use of a more basic acylation solvent) have been described by Adams (1974) permitting glc of histidine and arginine without modifying these amino acids prior to derivatization. The n-propyl-N-acetyl derivatives are more poorly separated than with other methods.

Most publications of amino derivatization techniques have come from Gehrke's laboratory. Gehrke prepared the amino acid derivatives by two methods which differed in the method of preparation of the alkyl ester. The first method utilized methyl esters prepared in methanolic HCl, followed by evaporation of the methanolic HCl and transesterification to the n-butyl ester. This transesterification method was used as it is difficult to solubilize, and thus derivatize amino acids in n-butanol-HCl. Methyl esterification, followed by n-butyl transesterification, requires about 3.5 hr for completion and this stimulated Roach and Gehrke (1969) to develop a method for direct esterification of the amino acids in butanolic HCl by heating the butanolic-HCl for 1 hour at 110 C. Direct esterification saved approximately 2.5 hr of derivatization time, but did encounter difficulties with the esterification of cystine, as it is the least soluble of all the amino acids. After removal of the butanol-HCl the amino groups were acylated at 150 C for 5 min with a methylene chloride, trifluoroacetic-anhydride mixture. These two esterification methods followed by acylation

at 150 C are the basic methods upon which many modifications and preparations of homologous esters and amides have been used. For example Cancalon and Klingman (1974) studied the effect of sonic oscillation on direct formation of the n-butyl esters.

Gehrke et al. published numerous papers on glc of amino acids using the N-trifluoroacetyl-O-n-butyl ester of amino acids. Gehrke and Takeda (1973) studied the stability and separation of amino acids on ten different liquid phases, the preparation of biological fluids prior to derivatization for glc (Zumwalt et al., 1970), methods for shortening the derivatization time of amino acids by direct esterification (Roach and Gehrke, 1969), and the possibility of using Matsubara and Sasaki's (1968) tryptophan acid hydrolysis protectant, thioglycolic acid, in connection with glc procedures (Gehrke and Takeda, 1973). Gehrke's group is certainly to be lauded for identification of the problems in glc that needed to be solved, and for advancing solutions to the problems of glc of amino acids. While Gehrke's derivatives are not now the derivatives of choice, because the separation of the amino acids by glc has been much better achieved by use of different derivatives, than by different column packings, much of this methodology has been adapted to the new derivatives. For example the use of the N-heptafluorobutyryl-O-n-propyl derivatives of Moss et al., (1971) gives much better separation of the amino acids than Gehrke's methods.

Moss et al., (1971) also found that the use of acetic anhydride as a "chaser" in the syringe markedly improved the recovery of histidine. This has been confirmed by MacKenzie and Tenaschuk (1974), and appears to be superior to the glc method for histidine reported by Gehrke. While the separation achieved by Moss et al. (1971) was much improved over that of Gehrke et al. there remained problems with the separation of methionine

and aspartic acid when seed proteins were analyzed. Methionine and aspartic acid are about 50% resolved as a doublet when using standards, but seed protein hydrolysates may contain 10 times more aspartic acid than methionine and then the resolution is poor.

A solution to the methionine-aspartic acid dilemma was provided by the use of the N-heptafluorobutyryl-O-isoamyl esters of Zanetta and Vincendon (1973) in which the methionine and aspartic acid peaks are completely separated from all other peaks. The preparation of the methyl esters of histidine, arginine, tryptophan, and cystine by the method of Zannetta and Vincendon (1973) was found to be not quantitative by Felker and Bandurski (1975) who reported on a quantitative methyl esterification of these amino acids. Felker and Bandurski (1975) also showed that trans-esterification and preparation of the amides in vacuo greatly increased the yields of methionine, arginine, cystine, and tryptophan. Felker and Bandurski (1975) were unable to obtain a histidine peak following the method of Zanetta and Vincendon (1973), and reported that the derivatization of cystine was too irreproducible to be quantitative. The mass spectra of the N-heptafluorobutyryl-O-isoamyl esters were also reported by Felker and Bandurski demonstrating for the first time that the arginine guanido group was diacylated.

Although the isoamyl esters were superior to the n-propyl esters, there remains a problem with chromatography of tyrosine as the isoamyl ester in hydrolysates of seed proteins because glutamic acid is present in seed proteins in approximately 10 fold excess over tyrosine. This difficulty was overcome by MacKenzie and Tenachuk (1974) who used an ester one methylene shorter than the isoamyl ester. Since glutamic acid has two carboxyl groups esterified in comparison with one of tyrosine, using a shorter

alcohol to esterify the carboxyl shifted glutamic acid to a lower temperature relative to tyrosine. Thus the isobutyl ester gave excellent resolution of all amino acids even from a protein hydrolysate. MacKenzie and Tenaschuk (1974) confirmed the finding of Felker and Bandurski on the inability to chromatograph histidine as reported by Zanetta and Vincendon (1973). However MacKenzie and Tenaschuk did not find that the use of acetic anhydride as a chaser in the syringe would yield a histidine peak. In a later paper MacKenzie and Tenaschuk (1975) reporting on the rapid formation of the isobutyl esters found that the esters could be made satisfactorily at 120 C in 20 minutes. Thus it is now possible to derivatize all the amino acids from a hydrolysate in approximately 0.5 hours, have them all well separated, and have the chromatogram taken only 35 minutes.

Despite the success achieved in glc of amino acids, major problems remain involving stabilization and protection of cysteine-cystine, and tryptophan during acid hydrolysis, and reliable quantitation of the unstable imidazole acetate derivative of histidine during glc. The literature pertaining to analysis of cystine, histidine, and tryptophan will now be discussed in turn.

II. Cystine-Cysteine Analysis

The difficulties associated with glc of cystine-cysteine are numerous. Cysteine is easily derivatized and analyzed by glc but cysteine is spontaneously oxidized to cystine even in acid solution. The resulting cystine is the most water insoluble of all the amino acids (Sober et al., 1970) and in other solvents, making derivatization of this amino acid difficult. Also cystine is the only amino acid which will not methyl esterify

quantitatively under conditions adequate to methyl esterify the other protein amino acids (Felker and Bandurski, 1975). A major problem with amino acid analysis using either an amino acid analyzer or glc of cystine derived from proteins is the instability of cystine and cysteine during acid hydrolysis. If protein hydrolysis is not done at a low oxygen tension in the absence of carbohydrate it is possible to obtain a value of zero cysteine-cystine when indeed cystine is present. The most widely used method for analyzing cysteine involves performic acid oxidation (Hirs, 1967) of the protein to oxidize cystine and methionine to cysteic acid and methionine sulfone since they are more stable to acid hydrolysis. An example, illustrating the necessity of performic acid oxidation is the amino acid composition of the protein associated with the plant photoreceptor, phytochrome done without performic acid oxidation, and with no reported cysteine. Later when performic acid oxidation was performed on the protein 11 cysteines per molecule were found (Briggs and Rice, 1972).

Since performic acid oxidation of proteins was the most widely accepted method for cysteine determination in proteins, and since a method for glc of cysteic acid as the trimethylsilyl (TMS) derivative had been reported (Shahrokhi and Gehrke, 1968) it was decided to attempt to quantitatively determine cysteine by performic acid oxidation followed by glc of the TMS derivative of cysteic acid. In the paper reporting on the derivatization of cysteic acid, the smallest scale on which the derivative was prepared was 10 mg. This synthesis is at least two orders of magnitude higher than would be useful for quantitative glc. With considerable effort it was possible to adapt this derivatization to as low as 25 μg of cysteic acid for glc. Unfortunately, this derivative was not stable in the presence

of other amino acids, and required cleaning of the glc flame ionization detector (FID) after every injection.

Since glc of cysteic acid presented so many difficulties analysis of cysteine by glc of cysteic acid was abandoned. The other major method for stabilization of cystine during hydrolysis involved S-alkylation of the thiol produced after disulfide reduction. The common S-alkylation reagents in use today are iodoacetic acid, N-ethylmaleimide, and acrylonitrile. Use of iodoacetic acid is complicated by iodide released by the light and during storage and thus requires frequent recrystalization (Noltmann et al., 1962). Iodoacetic acid is reported to be more reactive in alkylation of amino groups than is acrylonitrile. N-ethylmaleimide (NEM) does not have to be recrystalized but NEM is not as specific for thiols as acrylonitrile. For example NEM has been shown to react with the peptide N terminal amino group and with the imidazole group of histidine (Smyth et al., 1964). When NEM is reacted with the dipeptide glycyl-L-alanine in 0.2 M phosphate buffer pH 7.4, after 1.2 hours only 50% of the peptide remained (Smyth et al., 1964). In contrast Cavins and Freidman (1968) reported no modification of any of the amino acids in bovine serum albumen at pH 7.0 for 30 min with acrylonitrile except for quantitative S-alkylation of cysteine. Thus acrylonitrile was chosen as the best S-alkylation reagent. Acrylonitrile has the further advantage of yielding a derivative which is easily further derivatized by standard glc procedures.

While acrylonitrile reacts quantitatively with thiols, it doesn't react with disulfides and thus disulfide reduction is necessary prior to S-alkylation. Disulfide reduction is easily accomplished if the disulfides are not buried in the interior of the protein. Proteins are normally denatured in 8M urea or 6 M guanidine prior to disulfide reduction with the

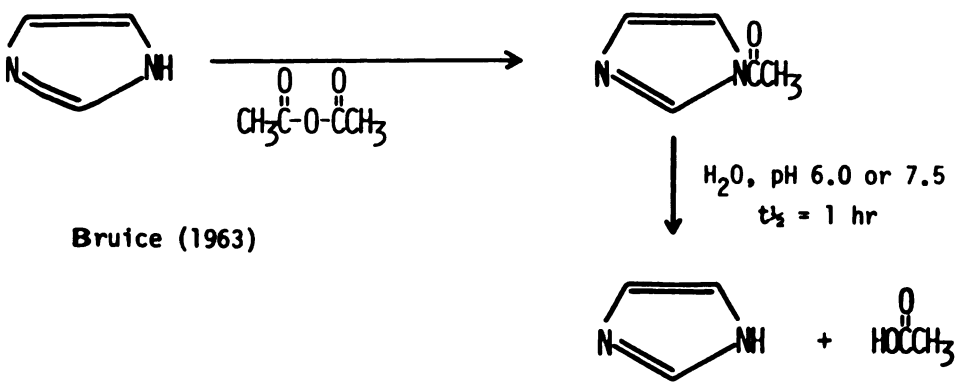
urea or guanidine then being dialyzed away from the protein after S-alkylation. Dialysis was not desirable in our case as quantitative transfer after dialysis is difficult for small, 100 μg, samples and the time required for dialysis is long. Since the proteins from the seeds were being precipitated by trichloroacetic acid (TCA) as a purification procedure, it was felt that the denaturation would be sufficient to expose all disulfides to the reducing agent. Further TCA has the added advantage of being easily removed from the protein by extraction with organic solvents such as diethylether and chloroform. Initially, mercaptoethanol was used as a reducing agent for the disulfide reduction of our model protein, but as it seldom gave complete disulfide reduction, dithioerythritol (DTE) was tried. Because DTE reproducibly gave quantitative disulfide reduction, DTE was adopted as the reducing agent.

In summary, the analysis of cystine finally adopted employed protein TCA precipitation and denaturation, a solvent wash to remove the TCA, disulfide reduction with DTE, and S-alkylation with acrylonitrile.

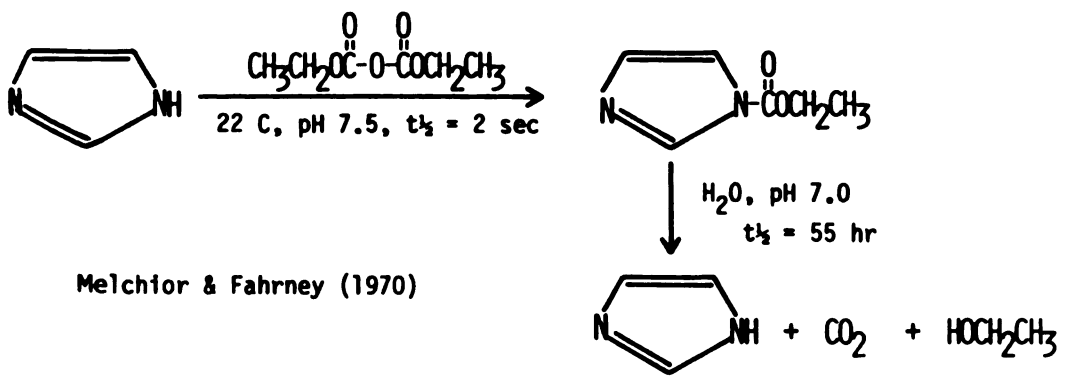
III. Chemistry and Analysis of Histidine

Histidine remains the most difficult amino acid to derivatize for glc. As the carboxy and the amino group are rather easy to derivatize, the difficulty probably arises in derivatization of the imidazole side chain. Difficulties with derivatization of imidazoles have plagued organic chemists for some time. For instance T. C. Bruice (1963) begins a review on acyl-imidazoles with "The great susceptibility of N-acetylimidazoles to hydrolysis delayed its preparation until 1952". As an example the hydrolysis of the acetylimidazole bond in water occurs with a half life of one hour at pH 6 or 7.5. Considering that protein amide bonds are indefi-

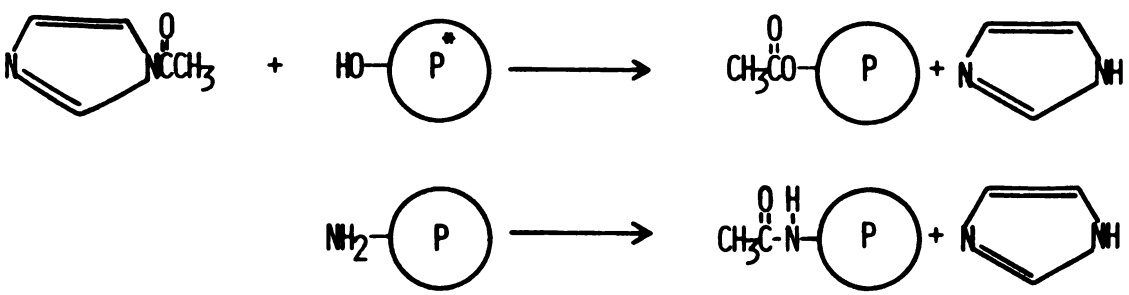
Acylation Reactions of Histidine



Bruice (1963)



Melchior & Fahrney (1970)



P* = protein

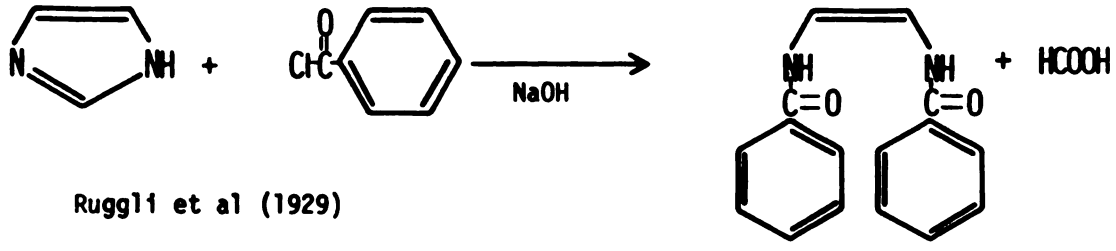
Means & Feeny (1971)

nitely stable at pH 6, the lability of the acetyl imidazole becomes more striking.

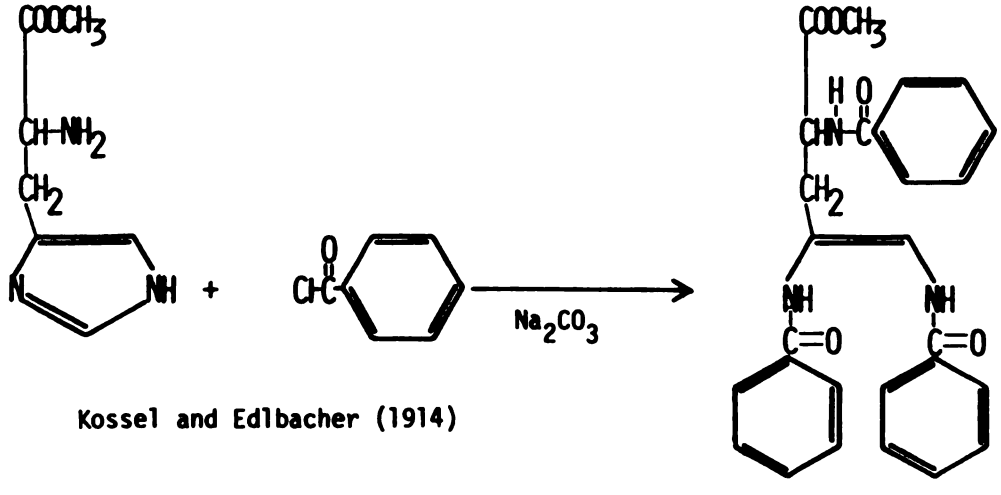
The reaction mechanism for the hydrolytic breakdown of the acyl imidazole has a unimolecular and a bimolecular component (Staab, 1956). Acyl imidazole breakdown was studied with acetyl imidazole replacing the hydrogens one at a time with methyl groups. The Sn2 reaction was studied with amines in anhydrous tetrahydrofuran while the El mechanism was studied with the acylimidazoles in water. The bulky $(CH_3)_3CO$ prevented the amine from attacking the amide bond and thus the order for the rate of hydrolysis was $(CH_3)_3CO<(CH_3)_2CHCO<CH_3CH_2CO<CH_3CO$ as would be expected for an Sn2 reaction. When the derivatives were allowed to hydrolyze in water at neutral pH the order for rate of hydrolysis was exactly the opposite (Staab, 1956). This was as expected as the stability of tertiary carbonium ions are greater than secondary which in turn are more stable than primary. The possibility of acylated histidine reacting with another amino group in the supposedly anhydrous triethylamine acetic anhydride reaction mix, would be reduced by using the t-pentoic anhydride. On the other hand to prevent the unimolecular disociation it would be best to use acetic anhydride. Probably though both reagents should be evaluated.

Not only are acyl imidazoles susceptible to hydrolysis but harsher conditions of acylating the imidazole nitrogen, e.g. Schotten-Baumann conditions with benzoyl chloride and sodium hydroxide, causes ring opening (Ruggli et al., 1929). While the triacylated ring opened product (Kossel and Edlbacher, 1914) of the reaction of histidine methyl ester with sodium carbonate and benzoyl chloride should be quite stable it would have a molecular weight of 448 and consequently would require a high temperature for glc. Even if the volatility was sufficient, there would be the problem of

Imidazole and Histidine Ring Opening Reactions



Ruggli et al (1929)



Kossel and Edlbacher (1914)

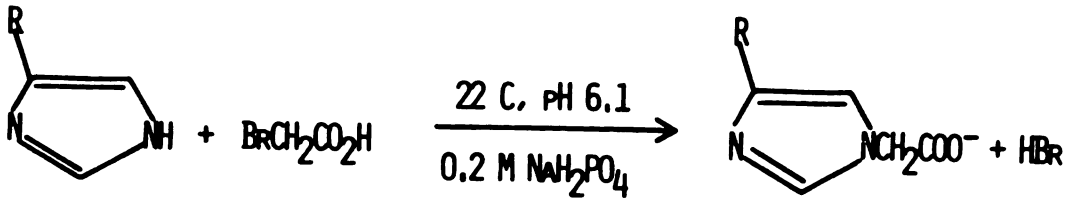
preserving the ester on the carboxyl group under such basic reaction conditions, and of eliminating salts prior to glc.

Acetyl imidazoles not only can be hydrolyzed by water but they can also be destroyed by donating their acetyl group to another amino or hydroxyl group. As an example N-acetylimidazole is used to acetylate tyrosines in carboxypeptidase A (Means and Feeny, 1971). Indeed, trimethylsilyl, perfluorobutyryl, perfluoroacetyl, and acetyl imidazole are commercially available derivatizing reagents for glc. Since these reactions all occur at room temperature, it is little wonder that acylimidazoles are unstable in the injection port of a glc at 230 C in the presence of a derivatized protein hydrolyzate.

Attempts have been made to use acylimidazoles for glc which have longer half lives than acetyl imidazole. For example ethoxyformic anhydride yields an imidazole ethoxyformyl derivative with a half life of 55 hr at pH 7 (Melchior and Fahrney, 1970). The first reported use of ethoxyformic anhydride for glc of histidine was by Moodie (1974). In a study of ethoxyformylation of the methyl esters of amino acids, it was found that the first injection onto a new column packing worked splendidly, but thereafter the peaks became successively smaller (unpublished) unless the injection port was cleaned after every injection. As cleaning the injection port after every injection is clearly an impossibility, this method had been discontinued. The ethoxyformyl derivative has the added disadvantage of being incompatable with strong bases in acylation solvents, since hydroxylamine readily cleaves this linkage (Melchior and Fahrney, 1970).

A little used method for analysis of aliphatic amines by glc without derivatization was also tried (Supelco 1973). This method utilizes the fact that amine free bases are often volatile liquids, whereas the corres-

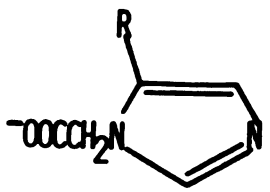
Alkylation Reactions of Histidine

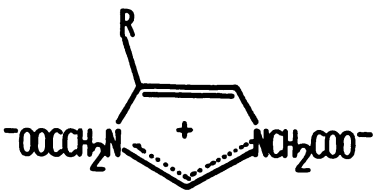


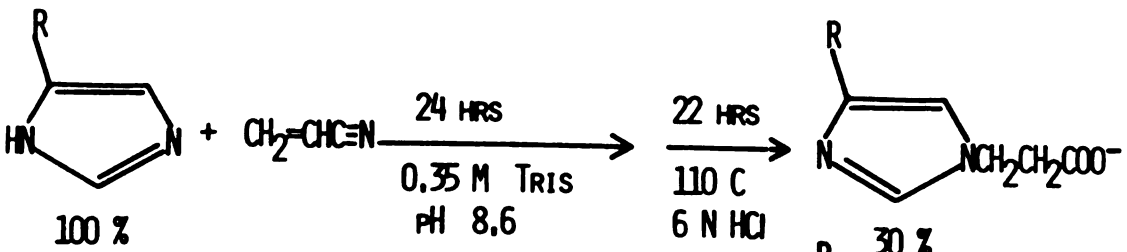
R = $CH_2CH(NH_2)COOH$

His $T_{\frac{1}{2}}$ loss = 74 days

Wieghardt & Goren (1975)

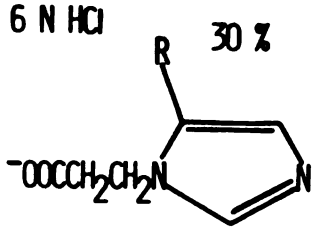






R = Insulin

Bosshard et al (1969)



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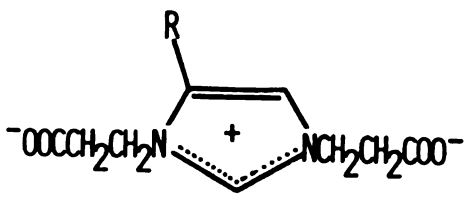


5 %

ponding hydrochloride salts are often solids melting over 200 C. Thus the glc packing had KOH incorporated into it to liberate the free base of the respective amino group. It was found possible to chromatograph imidazole at 90 C, and tryptophol at 160 C but meager success was achieved chromatographing histidinol. At least six combinations of liquid phase and KOH concentration were coated onto several supports, including crushed fire brick and diatomaceous earth before abandoning the method.

A paper by Roach et al. (1969) out of Gehrke's laboratory, reported on three methods for glc of histidine. Two of the methods were based on glc of the N-trifluoroacetyl-n-butyl esters as the monoacyl and diacyl derivatives which were postulated to differ only by the presence of a trifluoroacetyl group on the imidazole nitrogen. No evidence was presented to demonstrate a diacylated histidine. In a later work on gc-ms of the same derivatives the putative diacyl derivative could not be chromatographed (Gelpi et al., 1969). A third method of glc of histidine utilized the trimethylsilyl ethers. The only mention in the literature of this paper by Roach et al. (1969) was by Moodie (1974) who reported "A survey of the literature on the gas chromatography of amino acid derivatives reveals that only Gehrke's group appears to have overcome the (histidine) problem". Moodies (1974) statement while not entirely correct, reflects the feelings of a number of investigators regarding the work of Gehrke's laboratory.

Zanetta and Vincendon (1973) reported on a method for glc of histidine as the N-heptafluorobutyryl-O-isoamyl ester, but two laboratories (MacKenzie and Tenaschuk, 1974, Felker and Bandurski, 1975) have been unable to repeat that extension of Gehrke's work. Following two reports (Moss et al., 1971; MacKenzie and Tenaschuk 1974) that glc of histidine was successful upon injecting acetic anhydride as a chaser in the syringe with alkyl esters

of perfluoroacylamides, the acetyl derivative of histidine was further investigated. As reported in this thesis, it was found that the N-acetyl-O-methyl ester of histidine could be successfully chromatographed after; acetylating the histidine with acetic anhydride in triethylamine, blowing the acylating reagents to near dryness, adding fresh acetic anhydride, flushing the reaction vial with dry nitrogen, and injecting the histidine with very dry acetic anhydride onto a polar (SP2401) column.

CHEMISTRY AND ANALYSIS OF TRYPTOPHAN

The importance of tryptophan as a limiting amino acid in common plant grains was discussed earlier. A review will be made of problems with analytical techniques for tryptophan.

Tryptophan can be estimated in the intact protein by chemical or physical means or the tryptophan can be determined after the protein is hydrolyzed to amino acids with acid or alkali.

The analysis of tryptophan in the intact protein by chemical and physical means will be discussed first. The method in most widespread usage for analysis of tryptophan is the colorimetric method of Spies and Chambers (1948) based on the reaction of p-dimethylaminobenzaldehyde with the indole group to give a purple color absorbing at 600 nm. No other amino acids or carbohydrates appear to interfere with this reaction. The colorimetric reaction may give high results as Noltmann et al. (1962) have shown that myokinase, which contains no tryptophan yielded a value of 0.3 moles of tryptophan per mole of protein using the Spies and Chambers (1948) procedure. Nonetheless because of its simplicity, the Spies and Chambers (1948) method has come into widespread use. As an example, Central Soya uses this method in tryptophan assays on soy protein isolates.

Another colorimetric method is based upon Koshland's reagent 2-hydroxy-5-nitrobenzylbromide. This reagent reacts with the indole ring to give a yellow product having an absorption maximum at 410 nm and an extinction coefficient of 18,000 $M^{-1}cm^{-1}$. With this method it is necessary to remove the excess reagent by a gel filtration step prior to determining the absorptivity.

A widely used method for tryptophan determination in pure proteins is the spectrophotometric method of Goodwin and Morton (1946), employing the fact that tryptophan and tyrosine have different absorption maxima with different molar absorptivities. This method requires that the protein be pure, and best results are achieved if the tyrosine composition is known. Thus, this method is completely inapplicable to crude plant proteins which always have present at least small amounts of ultraviolet absorbing non-protein contaminants. It is also possible to measure the fluorescence from proteins and to attempt to relate this to the tryptophan content but because of large effects on fluorescence emission of salts, solvent polarity, viscosity, temperature, and concentration dependent bimolecular quenching, this author doubts the quantitative reliability of any fluorescence assay. Certainly for crude materials derived from plants a spectrofluorometric assay is out of the question.

The literature on hydrolysis of proteins to recover tryptophan began with the isolation of tryptophan from casein by Hopkins and Cole (1901). Since then, both acid and base protein hydrolysis procedures have been developed. Methods for recovery of tryptophan from acid hydrolysis are of more recent origin (Matsubara and Sasaki, 1969) but cannot be used in the presence of large amounts of carbohydrates.

Hydrolysis of proteins in base gives the most reliable results from a wide variety of substances, but the method is not without its drawbacks. For instance base hydrolysis causes extensive destruction of arginine, histidine, serine and threonine. Base hydrolyses require special vessels as glass is soluble in hot alkali and requires special handling procedures to remove the base if column chromatography is to be employed following the base hydrolysis. For example, in the procedure of Noltmann et al. (1962) the calculated volume of water and amount of solid barium hydroxide to achieve a 4 N concentration has to be added to the hydrolysis tubes separately because the base will not dissolve until it reaches the hydrolysis temperature of 110 C. In addition expensive vycor tubes were used as normal glass dissolves under these conditions. After evacuation of the air with an oil pump the tube was sealed off and the protein hydrolyzed. The removal of barium from the hydrolysate was affected by directing a stream of CO_2 over the surface which caused barium carbonate to precipitate. The precipitated $BaCO_3$ occluded some of the amino acids during the precipitation so that a recovery coefficient of amino acids from the $BaCO_3$ had to be determined. Noltmann et al. (1962) determined this figure to be 85±4% for an average of nine experiments. It is easy to see why barium hydroxide hydrolyses are so infrequently carried out. In the discussion of Noltmann et al. (1962) they compare their value of 6.7± .2 moles of tryptophan per mole of enzyme with a value of: 9.6 determined by the method of Spies and Chambers Chambers (1948) employing p-dimethylaminobenzaldehyde on the intact protein; and of 11.6 moles of tryptophan per mole of enzyme by Friedberg using p-dimethylaminobenzaldehyde; and of 10 calculate from the uv absorption spectrum; and of 13-14 by reaction with N-bromosuccinimide

and notes that all the indirect methods give high values. The discrepancy among tryptophan values is striking and it is unfortunate that sequence data are not yet available to resolve this issue. Since Spies and Chambers (1949) have shown that a 4 fold excess of cystine over tryptophan can produce a 25% loss of tryptophan during a 20 hr -100 C- 5 N NaOH hydrolysis and as there were 8 moles of cysteine per mole of enzyme, this could partially account for Noltmann's low value. As Noltmann's work is explicitly described and appears to be carefully done it is difficult to see how his value could be so much in error.

A valuable study on the effects of different amino acids on the recovery of tryptophan from base hydrolysis was done by Spies and Chambers (1949). While they found that cystine, cysteine, lanthionine, serine, and threonine all caused destruction of free tryptophan during base hydrolysis, only cystine caused degradation of tryptophan during base hydrolysis of the 4 proteins tested. Evidently proteins retard destruction of tryptophan by serine during base hydrolysis. Spies and Chambers (1949) also proved that glucose and fructose in a molar ratio of 4 carbohydrate to one tryptophan had a negligible destructive effect on tryptophan. This fact has made base hydrolysis of high carbohydrate foodstuffs the method of choice for tryptophan hydrolysis.

Improvements in the method for base hydrolysis have been made recently which makes the procedure more amenable to routine analysis (Oelshegel et al., 1970). Those changes consist of carrying out the base hydrolysis in plastic centrifuge tubes placed inside of a glass vessel equipped with an O-ring joint and a Teflon stopcock. This apparatus makes it possible to evacuate air through the stopcock and yet retain the chemical resistance

of plastics to hot alkali. These workers also reported that thiodiglycol significantly improved their recoveries (Oelshlegel et al., 1970).

A thorough examination of parameters relating to alkaline hydrolysis has been done by Hugli and Moore (1972). These authors modified the techniques of Oelshlegel et al. (1970) by the use of starch, which they found to be a superior antioxidant to thiodiglycol, and by sealing the liners (plastic centrifuge tubes) in glass because of the tendency of the O-ring joint to leak. In order to achieve the low pressures necessary for good recovery of tryptophan (less than 50 μ Hg) it was necessary to add octanol to prevent foaming. The recovery of tryptophan from 8 proteins or peptides of known composition was 100 ± 3%. It appears as if a quantitative procedure for tryptophan determination by base hydrolysis is now available.

Since only tryptophan cannot be determined after acid hydrolysis, while with base hydrolysis serine, threonine, arginine, histidine, and cysteine are completely destroyed, efforts have been intensified in the last 10 years to preserve tryptophan during acid hydrolysis. Matsubara and Sasaki (1969) reported a tryptophan recovery of 85% by adding 2% thioglycolic acid to the 6 N HCl. Shortly afterwards Liu and Chang (1971) reported the use of p-toluenesulfonic acid and methanesulfonic acid for use as protein hydrolysing acids. The toluenesulfonic acid gave improved recoveries over thioglycolic acid, but the tryptophan recoveries were still dependent on the carbohydrate content in the hydrolysate. For example addition of carbohydrate to give 30% by weight reduced the tryptophan recovery by over 20%. Penke et al. (1974) further improved the methanesulfonic acid method of Liu and Chang (1971) by the introduction of β-mercaptoethanesulfonic acid. This acid gives superior yields of

tryptophan, in the presence and absence of carbohydrate, than does p-toluenesulfonic acid. However, even β-mercaptoethanesulfonic acid in the presence of a 2 fold excess of glucose yields only 55% of the theoretical tryptophan value.

While recent workers have made progress with acid hydrolysis techniques that preserve tryptophan, they have made little attempt to determine the mechanism for tryptophan destruction as did the workers in the 20's and 30's. For example Mitchell and Hamilton (1929) tried to account for the nitrogen in humin (a dark brown precipitate formed during acid hydrolysis) as coming from tryptophan. If zein, which contains no tryptophan or carbohydrates, was heated in acid no humin was formed. If tryptophan and carbohydrate were added to zein hydrolysis, 87% of the nitrogen from added tryptophan could be recovered from the humin. If histidine and carbohydrate were added to the humin only 0.5% of the added histidine nitrogen could be recovered in the humin. Gortner and Norris (1923) reported that "artificial humins can be produced by condensing tryptophan with aldehydes by means of 20% hydrochloric acid, and that when a protein is hydrolyzed in the presence of an appropriate aldehyde, it is possible to recover the tryptophan nitrogen practically quantitatively as the acid-insoluble humin nitrogen". Mitchell and Hamilton (1929) reported in a discussion of protein hydrolysis, that "humin is formed by a condensation of the indole nucleus of tryptophan with an unknown aldehydic constituent of the protein molecule" and furthermore "artificial humin formed during acid hydrolysis is not a simple condensation product but is due to a condensation followed by a rearrangement or oxidation of both with the ultimate formation of an extremely resistant molecule or molecules". These observations are interesting in the light

of what is now known about indole chemistry. For example three methyl indole (Remers and Brown, 1972) is known to condense with benzaldehyde in ethanolic HCl on carbon 2 of the indole ring to form a 1:1 adduct. This 1:1 adduct may condense with itself to yield an indole dimer, bridged by a methylene from the benzaldehyde. p-Quinone may also react with carbon three substituted alkyl indoles in acetic acid to give a cycloadduct which may also dimerize (Remers and Brown, 1972). By analogy it is easy to see how carbohydrates with reducing sugars under acidic conditions could form adducts with tryptophan.

Similar kinds of products have been found upon reacting the plant hormone, indole-3-acetic acid (IAA) with p-dimethylaminobenzaldehyde under oxidizing strongly acidic conditions (Dr. Axel Ehmann personal communication). When the colored product product, which is very stable in 10 N H_2SO_4, is subject to tlc, four spots appear. After preparative tlc and mass spectrometry the chromophores have been identified as a dimer, trimer, and tetramer of the product of IAA condensation with the reacting aldehyde. Thus, there is good chemical evidence to lend support to the theories of the early workers on tryptophan degradation, and it is unfortunate that research directed towards a mechanistic understanding of tryptophan acid degradation is not being carried out today.

As the indole ring of tryptophan is reactive there are other possibilities for mechanisms of tryptophan destruction during acid hydrolysis. The tryptophan degradation product arising from either photooxidation of tryptophan in formic acid or from performic acid oxidation of proteins is kynurenine (Means and Feeney, 1971). Whether kynurenine, which has the pyrrole of the indole ring opened, ever normally occurs as an acid degradation product is not known.

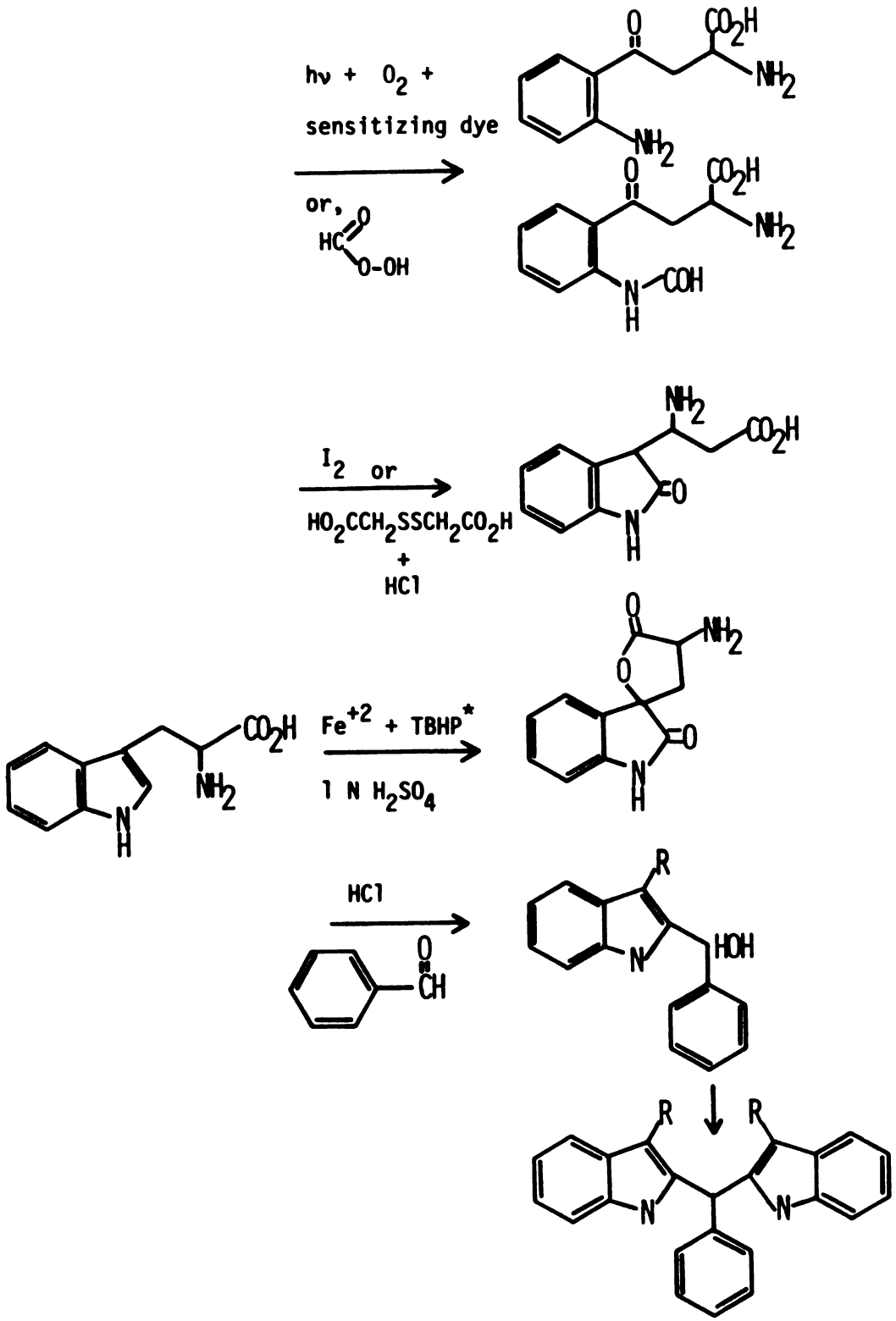
The Nakai and Ohta (1976) report of tryptophan degradation to β-3-oxindolylalanine could be classified as a third kind of degradative mechanism for tryptophan destruction during acid hydrolysis. The β-3-oxindolylalanine formation in protein hydrolysates of lysozyme was attributed to the presence of cystine and disulfides. The authors show a large peak on the short column of the amino acid analyzer after addition of dithiodiglycolic acid which they suggest is the primary acid degradation product of tryptophan. Since the collected unkown peak matched the ir spectra of synthetic β-3-oxindolylalanine quite well there can be little doubt that the unknown was β-3-oxindolylalanine. While the β-3-oxindolylalanine may be the primary tryptophan degradation product in the presence of large amounts of cystine, cystine cannot be the major cause of tryptophan degradation since fully S-alkylated lysozyme also shows tryptophan degradation(unpublished). There is a further discrepancy in Nakai and Ohta's (1976) hypothesis that β-3-oxindolylalanine is the main intermediate in tryptophan degradation. This discrepancy appears on their published amino acid chromatogram of a 24 hr lysozyme hydrolyzate showing tryptophan and the proposed acid degradation product. Their chromatogram shows tryptophan, β-3-oxindolylalanine, and histidine from lysozyme all with the approximate same peak area. Since there are 6 moles of tryptophan in lysozyme to 1 mole of histidine, the combined peak areas of β-3-oxindolylalanine and tryptophan should be 6 times the histidine peak area. The fact that the combined areas of tryptophan and β-3-oxindolylalanine are only twice the histidine peak area, in combination with the fact that 85 % of the tryptophan is hydrolyzed under these conditions(Matsubara and Sasaki, 1969) implies that they are missing at least 50 % of the tryptophan acid degradation products.

The oxidation products of tryptophan in proteins have been studied in connection with determination of the active sites of enzyme catalysis. For example tryptophan residue 108 in lysozyme has been shown to be specifically oxidized with I_2 to β-3-oxindolylalanine (Strickland et al., 1973) which is the main tryptophan acid degradation product reported by Nakai and Ohta (1976). Strickland et al. (1973) postulated a change in configuration of carbon 3 of tryptophan residue 108 of lysozyme after specific oxidation of residue 108 because of the appearance of a strong new center of optical activity in the circular dichroism spectra. Strickland et al. (1973) also discussed how the indole keto-enol tautomerism might relate to oxidation of tryptophan. A slightly different oxidation form of tryptophan has been reported by Stohrer (1976) as a result of an attempted alkylation of tryptophan in acid solution. This oxidation product is the spirolactone of β-3-oxindolylalanine in which the α-carboxy group cyclizes with carbon 3 of the indole to form an ester.

Since most of the postulated degradation products of tryptophan in acid hydrolysis involve either aldehydic or keto attack on the indole ring or oxidation of the indole ring to form the oxindole, Felker (in press) added metallic tin to 6 N HCl with the thought of lowering the redox potential to prevent the formation of aldehydes and ketones. Addition of tin has been shown to improve recoveries of tryptophan by 10-12% over the use of the protecting agent 1,2 dithioethane alone.

To summarize, the most widely used method for tryptophan assay in proteins is the colorimetric procedure of Spies and Chambers (1948) which is applicable to crude non-colored materials. This colorimetric method has been reported to give higher values than are correct. Spectrophotometric and spectrofluorometric procedures are also available but must be

Summary of Tryptophan Degradation Reactions



* TBHP = $(CH_3)_3COOH$

limited to pure samples. Base hydrolysis gives the most reliable tryptophan value in the presence of carbohydrate but base hydrolysis drastically destroys other amino acids. Acid hydrolysis of proteins yields accurate values for tryptophan and all other amino acids, but only in the absence of carbohydrate. The mechanism of tryptophan degradation is unknown but is catalyzed by metal salts, aldehydes, and ketones. The structures of tryptophan acid degradation products are not well characterized but include ring opened kynurenine structures, condensation products with aldehydes and ketones, and structures with the indole oxidized to the oxindole.

V. BIOCHEMISTRY AND CHEMISTRY OF LEGUME SEED PROTEINS

Since a large part of the experimental section of this thesis deals with protein and amino acid composition of tree legume proteins, it is appropriate to review the literature on chemical and biochemical aspects of seeds. A historical review of seed proteins will not be given, but instead a number of current reviews will be cited which provide an entry to the older literature.

A review of the chemical composition of the soybean seed has recently appeared (Smith and Circle, 1972) which covers subjects such as: the protein and non-protein nitrogen of a soybean seed; nitrogen distribution in meal fractionation; amino acid distribution in meal fractions; ash mineral content; phytin; inorganic phosphorus; phospholipids; nucleic acids; phenolic acids; and soluble and insoluble sugars of the seed. A sequel to the review on chemical composition of the seeds is the excellent review by Wolf (1972) "Purification and Properties of the (Soybean) Proteins." The review by Wolf covers subjects such as: subcellular localization of seed proteins; protein extraction and fractionation methods;

column chromatography of the proteins; amino acid composition of purified proteins; solubility, molecular size, and molecular structure of the soy proteins; electrochemical properties of the proteins; and denaturation of the proteins.

A review on "The Biochemistry of Legume Seed Proteins" has recently appeared by Millerd (1975) of Australia, which places heavy emphasis on the developmental biochemical aspects during seed maturation. Millerd discusses topics such as nucleic acid profiles during cotyledon development and transcription and translation in developing seeds. She also points out that the sulfate nutrition of the plant can greatly alter the ratio of methionine and cysteine containing storage proteins to total storage proteins. Since methionine and cysteine are the limiting amino acids in legume seed proteins for human nutrition, the regulation of sulfur rich storage protins by sulfate levels in the soil is an important observation.

A very thorough review by Derbyshire et al. (1976) on the extraction, purification, and biochemical and physical properties of the major storage proteins in the subfamily Papilonoideae of the leguminosae has recently appeared. The review by Derbyshire et al. (1976) lists amino acid composition data, sedimentation coefficients, N-terminal amino acids, and postulated subunit structure for the two major storage proteins, vicilin-7s, and legumin-11s, of common legumes. The biochemistry described in these reviews is carried out on one subfamily of the Leguminosae, the Papilonoideae, and a phylogenetic understanding of these proteins by study of the more primitive subfamilies, the Caesalpinoideae and Mimosoideae might also prove interesting.

Reviews of an applied nature have recently appeared on the "Biochemical basis for differences in Plant Protein Utilization" by Kakade (1974) and "Genetic Improvement of Plant Protein" by Johnson and Lay (1974). Kakade discusses the effects that the primary and tertiary structure may have on enzymatic digestion of plant proteins, as well as the inhibitory effects on digestion of saponins, phytates, tannins, hemagglutinins, and trypsin inhibitors. Johnson and Lay (1974) discuss the protein, amino acid, and nutritional variations that exist in world plant seed collections, and the heritability of these traits.

While discussing reviews on seed proteins it would be amiss to omit work on the systematic screening of the plant kingdom for variations in protein and amino acid composition. Van Etten et al. (1967) reported on the amino acid composition of the seeds of 379 species of plants and found that the mean amino acid content of some essential amino acids differed remarkably from one family to another. In a similar paper Jones and Earle (1966) reported on the protein and oil content of 759 species of plants in a systematic survey of the plant kingdom and found some seeds with a protein content of 70%. Jones and Earle (1966) established an inverse correlation between protein content and seed size by noting that seeds having 30% or more protein (423 samples) weighed 47 grams per thousand seeds, while seeds having less than 30% protein averaged 210 g per thousand seeds.

Having discussed the major reviews on the subject of seed proteins, the literature pertinent to the experimental protocol in this thesis will now be discussed. It was necessary to prepare a protein fraction from the tree legume seeds that would be as free from carbohydrates and fatty acids as possible, and the protein extraction procedure of Wolf and Briggs

(1956) as modified by Hill and Breidenbach (1974) was initially used. This procedure consists of extraction of the proteins in 0.4 M NaCl pH 7.6 phosphate buffer with mercaptoethanol. The sodium chloride is used to solubilize the globulins, and the mercaptoethanol is used to prevent disulfide interchange. Wolf (1972) in a review pointed out that the solubility of soy proteins increases with pH from 4 to 10, so pyrophosphate buffer at pH 8.2 (pKa=8.2) was used instead of phosphate buffer at pH 7.6 (pKa=6.8). Since dithioerythritol is a much better reducing agent than mercaptoethanol, (Cleland,1964) dithioerythritol was used to prevent disulfide interchange. As a trichloroacetic (TCA) precipitation was used to separate proteins from carbohydrates it is instructive to remember the work of Jansen et al., (1952) showing that glycoproteins may not be precipitated by TCA, and that glycoproteins may prevent non-glycosylated proteins from being TCA precipitated.

The literature on the protein and amino acid composition of leguminous trees is indeed meager. By far the most active worker in the field is Busson who has measured the protein and amino acid content of many African foods including several tree legumes and lists these values in his book (Busson et al.,1965). In addition Busson has published papers on the protein and amino acid composition of the pulp (Lanza et al.,1962) and seed (Busson et al.,1958) of the African locust bean. More recently Fetuga et al (1974) have made similar analytical measurements, but have also done feeding trials with rats which determined the African locust bean to be nutritionally very deficient in methionine and tryptophan. Since the addition of methionine and tryptophan changed the rats from a net nitrogen loss to a net nitrogen gain status, the selection of African locust beans with higher methionine and tryptophan is essential if it is to be used as a large part of the diet.

The first amino acid composition data for a New World Prosopis has recently been reported in a Ph.D. thesis from Brazil (Figueirdo, 1975) and agrees well with that reported in this thesis with the exception of cystine in which Figueirdo (1975) reported a ten fold higher value than is reported here. The Brazilian work on the protein composition is in good agreement with Walton (1923) who reported 69% protein in the true seed.

There is currently some exciting work in the field of seed protein biochemistry which is not yet covered in reviews. Romero et al. (1975) have demonstrated that the heritable difference in a high methionine level Phaseolus strain can be partially attributed to the presence of an extra band on an SDS polyacrylamide gel. Furthermore, Hall's group (Sun et al., 1975) has achieved cell-free synthesis of the major storage protein of a Phaseolus after preparation of polysomes from Phaseolus and translation of the message using a wheat germ extract. Shortly after Hall's report of in vitro seed protein synthesis two laboratories (Burr and Burr, 1976; Larkins and Dalby, 1975) reported on zein synthesis by polyribosomes. This is most significant as zein is the protein in corn seeds whose synthesis is suppressed in the production of high lysine corn. The control of lysine biosynthesis in maize has recently been studied (Cheshire and Miflin, 1975).

A new method for separation of the major legume seed proteins in 10 minutes by electrophoresis on cellulose acetate membranes has been reported (Blagrove and Gillespie, 1975) and should be valuable in screening legume seeds for their nutritional quality by establishing the ratio of high methionine, cystine, and tryptophan 11s storage protein, to the low methionine, cystine, and tryptophan 7s protein.

VI. Uses and Potential of Tree Legumes

The literature on the uses of leguminous trees spans 100 years and originates from four continents. For this reason, the review will be divided into the following sections;

1. Uses of leguminous trees in North America and Hawaii
    a. Prosopis (Mesquite or Keawe)
    b. Gleditsia (Honey locust)
    c. Ceratonia (Carob) and Leucaena (Koa haole)
2. Uses of Prosopis in South America
3. Uses of leguminous trees in Africa
    a. South Africa
    b. Subsaharan region
4. Uses of leguminous trees in Asia

1. USES OF LEGUMINOUS TREES IN NORTH AMERICA AND HAWAII

a. Prosopis

The use of Prosopis in N. America is the subject of a thorough, annotated bibliography by Schuster (1969). The first section of this work is devoted to the phytogeography and taxonomy of Prosopis in N. America and provides a key to and descriptions of 35 species. A second section reviews the uses of mesquite in N. America from the late 1800's, detailing the use of mesquite by 13 tribes of Indians in the American Southwest and uses by poor Mexicans and white pioneers. Mesquite pods were a staple in the Indian diet while mesquite wood was used for weapons, tools, and building materials. Mesquite as a source of food and shelter for many types of wildlife were cited. A third section deals with natural enemies of the mesquite, primarily the insects. The last section of the work is the annotated bibliography of over 50 pages of abstracts.

Other references on the uses of Prosopis are in Smith's (1952) book entitled "Tree Crops-A Permanent Agriculture". Smith's hypothesis was that

many tree crops could be developed to replace annual cultivated crops which, over a period of years, cause soil erosion and loss of fertility. Smith (1952) discusses the use of Prosopis in two sections, one on Keawe, a Hawaiian vernacular for Prosopis, and another section on mesquite. In the section of uses of Prosopis in Hawaii, Smith states the yield of pods may vary from 2 to 10 tons per acre with a yield of a good forest being 8,000 lbs per acre. "Large areas of trees stand for the most part in rock soil where cultivation would be practically impossible". Citing a letter from 1926, Smith notes that the Hawaiian Commercial and Sugar Company on the island of Maui, hired laborers to pick 1,000 to 1,400 tons of beans from 9,000 acres of Keawe (a common name for Prosopis). The beans were then ground for animal feed. Many of Smith's citations (1952) on the uses and yields of Keawe are letters from USDA officials in the period ca 1910 to 1930 and are virtually impossible to verify. Smith also points out a controversy on the taxonomy of Keawe, with some workers suggesting that Keawe is not a true algaroba (Prosopis). It is this authors opinion that Smith's photo of Keawe (fig.38 Smith 1952) looks more like a Carob than a mesquite.

In a chapter on mesquite Smith (1952) details yields and uses by Indians and farmers in Texas, Arizona, and New Mexico in the early 20th Century. The references are mostly letters from USDA personnel, Agricultural Experiment Station Directors and a few Agricultural Experiment Station Bulletins. There were no numerical data on yields or utilization of mesquite, but the consensus of opinion from observations on wild mesquite was the mesquite yielded an appreciable quantity of pods in an area where very little else would grow and with the same feeding value as barley.

Smith (1952) also notes that mesquite, or algaroba as it is called in South America, grows wild in Argentina and is native to regions as far as 40° S latitude.

While the work of Smith (1952) and Schuster (1969) cover most of the N. American literature, it is instructive to look at a few other references in detail. Forbes (1895), later to become the Director of the Arizona Agriculture Experiment Station, published a bulletin on mesquite and stressed the importance of examining indigenous plants for development by man and suggesting that mesquite might be such a plant. This bulletin (Forbes 1895) gives protein values for the pods and seeds of mesquite which are in agreement with those found today (6 % and 53 % protein in pods and seeds respectively). Considering the fact that this report was published before Hopkins and Cole's (1901) discovery of tryptophan and at the time of development of the Kjeldahl procedure (1883) the values are remarkable.

Essentially the same report by Bentley (1898) and Smith (1900) appeared in USDA publications on mesquite. They noted that pods are eaten by Mexicans and Indians and that an acre of mesquite produces not less than 100 bushels of mesquite beans which at 21 lbs per bushel, is 2,100 lbs per acre. It was their experience that the feed value of mesquite beans is approximately equivalent to barley (Bentley, 1898; and Smith, 1900).

Because of the large crops of mesquite beans and the high prices of corn in New Mexico in 1916, Garcia (1916) of the New Mexico Agricultural Experiment Station undertook a nutritional study of the value of mesquite beans as pig feed. Garcia (1916) ground the beans "to secure the full

nutritive value of the beans", and mixed the ground beans with one part ground corn. One lot of pigs were fed ground corn and another lot were fed the 1 to 1 mixture of ground corn and mesquite. During the first four weeks the mesquite beans were 4 % less efficient than corn in sustaining weight gain but after the first four weeks the beans were only 53 % as valuable pound for pound as corn. Garcia (1916) stated that the beans tended to promote growth rather than rapid fattening.

The importance of the feeding trial becomes apparent considering that the mesquite beans were of a wild variety growing on the grounds of the New Mexico College of Agriculture at State College, New Mexico from a bush receiving no irrigation, had 6.2 inches of precipitation the previous year, was 60 feet from the water table and had a mean diameter of 18 feet 9 inches. The air dry weight of the pods was 38.1 pounds and would contain 7 1/2 lbs of sugar, 5 1/4 lbs of protein, and one pound of fat (Garcia, 1916). Approximately 69% of the protein and 60% of the fat would be in the seed while 87% of the carbohydrates would be in the husk. While the feeding value of the wild mesquite pod was less than corn, which was undoubtedly shipped from the midwest, it is certain that the yield of corn in New Mexico with no irrigation and 6 inches of rain would have been zero.

At approximately the time the above trials were conducted Wilcox (1910) wrote that "Keawe is the most valuable tree thus far introduced into the territory of Hawaii". Wilcox discussed, but did not resolve, the controversy of whether Prosopis was introduced from Paris, Chile, or California. Wilcox (1910) stated that Keawe grew all over the island to elevations of 800 to 1,000 ft and estimated that 500,000 bags were

picked annually and stored with, at least, 15,000 bags stored annually on two or three estates. Grinding the pods proved difficult because the sugar tended to "gum up" the grinder, but it was found that by placing a minute spray of water on the rollers, the seeds could be crushed and the rollers would stay clean. Wilcox (1910) also reported that when the pods were fed after milking, instead of before, no flavor would be imparted to the milk by the mesquite beans.

It was found (Wilcox, 1910) that thinning of Keawe forests would increase bean production, and that the wood gained from thinning almost paid for the thinning operation.

In 1923 in an effort to promote utilization of mesquite, carob, and honey locust, Walton (1923) did chemical and structural studies of these tree legumes. An interesting introduction is given prior to Walton's analysis on the use and geographical distribution of mesquite which cites papers showing that mesquite beans were shipped by the carload in Texas, and where the yearly estimated feeding value of mesquite in Hawaii was $225,000.

Walton (1923) separated the mesquite seeds from the pods, both manually and mechanically, analyzed the isolated parts, and reported a milling procedure for removing the mesquite seed coat. On an air dry basis he found 6.7 % protein in the pods free of seeds and 65 % protein in the true seed after removal of the seed coat. These values are in good agreement with those of Forbes (1895) and those reported in this thesis.

Walton's (1923) report was the last major USDA or southwestern experiment station study sympathetic to the idea of using mesquite as a

food plant. The remainder of USDA and agricultural experiment station publications are concerned with eradication of mesquite on the southwestern ranges. The reason for the attempted eradication is that mesquite competes with grass for water, causing the death of grass, which could support more grazing livestock than unground, unselected mesquite beans.

Cattle nutritional problems do result from a diet consisting solely of unground mesquite pods (Hendershot,1946;and Adler,1949). A conservative estimate of the animals showing signs of sickness was about 1 % when cattle in Hawaii were pastured on the leeward size of the island when all other forage was exhausted and the beans were abundant (Hendershot,1946). The illness resembled ketosis and was fatal in some animals. At autopsy the rumen was found impacted with Kiawe pods and seeds with the fibrous part of the bean and seed coat undigested. In summary Adler (1949) commented "A high sugar diet low in good forage factors depresses bacterial multiplication. Digestion of cellulose to available sugar is not accomplished. Protein synthesis induced by normal bacteria is sharply reduced".

Management of the disease produced by ingestion of an unbalanced diet of mesquite beans was reported from Texas by Dollahite (1964) who stated that although many cattle men depend on mesquite beans, especially in period of drought, malnutrition due to ingestion of beans probably occurs in all areas where large quantities of beans are produced. The author theorized that the high concentration of sucrose in the pods (30%) altered the rumen microflora to such an extent that the animals could no longer digest cellulose or synthesize the "B vitamins". The treatment

shown to be effective by Dollahite (1964) consisted of the use of rumen flora transplants from healthy cattle and the feeding of a balanced ration that contains cellulose and available carbohydrates.

Felger, a botanist, and Moser, a linguist (1971), studied the use of mesquite by the Seri Indians in the Mexican-Sonoran desert as a food source for making bread, and in basketry, dyes, face paint, firewood, and for games. In a second paper on Seri Indian use of desert plants Felger and Moser (1976) list Prosopis grandulosa as the most important of over 70 plants used for food in Sonoran Mexico. They further state that mesquite could be relied upon even during years of extreme drought.

A popular account of mesquite (Bush, 1972) entitled "Symbol of the Southwest" describes earlier use by the Indians as well as its use today in making jelly, and as charcoal for grilling steaks. Bush (1972) also reports on the use of young chopped mesquite trees as a cattle food and as a substrate for single cell protein production.

1. USES OF LEGUMINOUS TREES IN NORTH AMERICA

b. Gleditsia triacanthos

Perhaps the first report on the potential uses of Gleditsia triacanthos (honey locust) was given by Walton (1923) who reported on protein and carbohydrate composition of the pods of honey locust. Walton reported that "the pods and young trees are relished by livestock. The ripe pods, with their sirupy pulp, are popular with human beings, especially children." Analytical data were then presented showing the entire fruit to be 13% protein, and 23% sucrose, while the entire seed (seed plus seed coat) was 26% protein.

A contest was sponsored by the American Genetic Association for the

best honey locust trees (Anon, 1926) with the purpose of identifying superior tasting honey locust pods for breeding purposes. The awards were based on size and thickness of the beans, weight of beans, sweetness, and palatability. The results of the contest were reported in the Journal of Heredity (Anon, 1928) with the winning entry having 24 % sucrose and 5 % other sugars. A second contest (Anon, 1929) produced beans with a much better flavor than the first contest and it was noted that none of the entries from northern states were comparable to the entries from southern states.

In 1934 spurred by the impetus of the honey locust contest, the Tennessee Valley Authority (TVA) began a program to develop thornless honey locusts with high yielding, nutritious, palatable pods with a high sugar content to be used for livestock food. The successful propagation of thornless high bearing varieties of honey locust, Millwood, and Calhoun, was reported by Chase (1947). Propagation relied on the principle that thornless trees could be propagated by grafting scions from branches which had ceased thorn production.

Yield and sugar content data for 5 year old trees of the thornless honey locusts developed by the TVA were reported by Atkins (1942) of the Alabama Experiment Station. The four highest yielding trees of the Calhoun variety yielded pods of 32 % sucrose equivalent to 1,500 lbs per acre, while the 4 highest yielding Millwood varieties yielded pods of 29% sucrose equivalent to 3,150 pounds per acre. Feeding tests using dairy cows over a two year period and using honey locust pods showed that the ground pods may be satisfactorily substituted, on a weight for weight basis, for oats. The yield of some of these trees at 8 years of age was

250 lbs of pods per tree which would be 8,750 lbs per acre or the equivalent of 275 bushels of oats per acre (Smith, 1952). In comparison the yield of oats in 1976 in Michigan was only 56 bushels per acre (Hines and Pscodna, 1976). To my knowledge, there has been no further work on development of honey locust pods for fodder since letters to the experiment stations and institutions previously cited as being involved in honey locust studies have revealed no further work. Zarger (1957) did note that 12 varieties of honey locust were being maintained by the TVA at Norris, Tennessee.

A report on Gleditsia by the U.S. Forest Service (Anon, 1965) outlines honey locust cultural conditions such as climate, soil and topography, reproduction and growth and common diseases. It was noted that honey locust is drought hardy, tolerant of salinity and alkalinity, and grows best between pH 6.0 and 8.0.

1. USES OF LEGUMINOUS TREES IN NORTH AMERICA

c. Ceratonia siliqua and Leucaena leucocephala

Unlike both the honey locust and the mesquite, the carob is not native to North America but was introduced from the Mediterranean. The carob is not-nodulated and has less protein in its pods and seeds than either the honey locust or mesquite and thus the literature on carob is not reviewed as thoroughly as for the former trees. Most of the references dealing with carob in N. America can be found in Smith's book (1952). Most carob trees in N. America are grown in California, where a number of individuals and groups have promoted the development of carob and where it is one of the few trees that can survive the southern California climate without irrigation (Smith, 1952). A chemical analysis of the pods and seeds of carob has been cited by Walton (1923) listing

the protein content of pods as varying between 5 to 8 %. It takes 20 years for a carob tree to produce 150 lbs of pods and this may explain why its use is not widespread (Smith, 1952).

Unlike the three leguminous trees mentioned so far the Leucaena pods are not used as much for food as are the young leaves and twigs. Brewbaker et al. (1972) of Hawaii has established a collection of over 104 Leucaena strains and has examined some of them for their potential yield. He found that the yearly average of the top three varieties in replicated plots was 42.2 tons freshweight per acre or 13.4 tons dry weight per acre with 12 % moisture after being cut and harvested for forage. Unfortunately all of the Leucaena varieties have the alkaloid mimosine present which is a depilatory when eaten in excess (Brewbaker and Hylin, 1965), although Malynicz (1974) has shown that the depilatory effect of mimosine can be overcome with ferrous sulphate. From these yield data there can be no doubt that even though the tree legumes are presumably C-3 plants, having photorespiration, their yield potential is as great as any C-4 plant.

2. USES OF PROSOPIS IN SOUTH AMERICA

The three major groups or individuals who have worked on Prosopis from South America are: 1, Burkart (1943) who monographed the genus Prosopis and wrote a book on the legumes of Argentina in which uses of Prosopis are discussed; 2, the International Biological Program (IBP) under Solbrig of Harvard made an ecological and taxonomic study of Prosopis; and 3, a Ph.D. thesis by Figueirdo (1975) on the chemical composition of the pods of Algaroba.

Burkart (1943) in the book "Las Leguminosas Argentina" notes that there are 40 species of Prosopis in Asia, Africa, and North and South

America. Argentina is the center of polymorphism of the group, having the great majority of the species. It is Burkart's contention that the Prosopis subshrubs, shrubs and trees are more adapted to dry conditions than any other group of legumes.

Burkart (1943) describes Prosopis as growing most profusely in the northern part of Argentina where there is a semiarid region extending into Chile and Peru. In this semiarid region, known as the Chaco, the Matacos Indians depend on the fruiting Prosopis and have special words to denote Prosopis flowering and maturation periods. There are many Indian names for Prosopis, but the Spanish called it algarobo because of its similarity to the European carob.

Seven species of Prosopis are commonly used for wood to make wagons, posts, barrels, firewood, and charcoal. In Buenos Aires in 1943 the best streets were paved with blocks of P. nigra or P. alba. (Burkart, 1943)

The composition of the pods differs from species to species. Burkart (1943) reports that some species are bitter, acidic, and astringent while P. alba, P. nigra, and P. alba var. panta have sweet pulp. Importance is attached to the annual harvest of Prosopis in the interior dry regions of Argentina where there is a scarcity of pasture, and where the Prosopis pods are fed to livestock. Arata, cited by Burkart (1943), noted that ranchers found that Prosopis fattened the cattle a great deal, and suggested cultivating Prosopis for cattle food. "Prosopis dulcis an argentine tree par excellence is the most outstanding tree" (Burkart, 1943).

Prosopis is used as food only by the poorer people, and in earlier times by Indians who prepared a number of kinds of food from Prosopis.

The pods of the mature and dry Prosopis were ground in mortars into a floury paste known as patay and then pressed into vessels, dried in an oven and stored for later use. A concentrated infusion of the fruits sometimes translated as honey has a rather agreeable taste (Burkart, 1943). A sweet and refreshing drink called Anapa is prepared from Prosopis by crushing the pods in a mortar and pestle and adding water. Burkart (1943) describes in detail the preparation of the alcoholic drink, Aloja, from a yeast fermentation of the pods. Due to the high content of monosaccharides, Medina cited in Burkart (1943), suggested that the fermentation and distillation of ethanol from the pods should be possible. Other uses reported by Burkart included use of the bark to tan leather, use of the gum as a substitute for gum arabic, and use of various parts of Prosopis as a herb for medicinal purposes.

Prosopis played such an important part in the lives of the people in arid areas that the Province of Santiago del Estero forbade the cutting of Prosopis for forestry purposes (Burkart, 1943). Burkart (1943) advised that mountain areas be reforested with Prosopis. He states, "The belief that the growth of the Prosopis is slow is erroneous, as there are great differences between species and varieties, the best of which are of sufficiently rapid growth to make cultivation economically possible". Furthermore, Prosopis hybrids are possible as they hybridize naturally (Burkart, 1943).

Two IBP sponsored papers on Prosopis have appeared comparing the North American Sonoran desert and the semidesert regions of Argentina. Solbrig and Bawa (1975) attempted to determine the level of genetic diversity among and within species of Prosopis by looking at leaf isozymes with starch gel electrophoresis and concluded that species of Prosopis

are characterized by a high degree of genetic variability. A second paper by Solbrig and Cantino (1975) presents quantitative data concerning: phenology; production of flowers, fruits, and seeds; destruction of seeds by bruchid beetles, and the percent germination of three Prosopis species from North and South America. In the South American region of study the rainfall ranged from 75 to 300 mm with nearly all of the rain coming in the summer months.

Much of Solbrig and Cantino's (1975) data have immediate application in seeking better varieties of Prosopis for food. For example, since the pods fall to the ground the first month after maturity, it would not be necessary to shake the trees mechanically, but merely to find a way of picking the pods off of the ground. The selection of bruchid resistant Prosopis varieties is possible since damaged seeds varied from 2 to 25 % for different species. Since the number of seeds per fruit varies from 3 to 19 and since the seeds contain most of the protein of the fruit (Garcia, 1916), it should be possible to select higher protein pods by selecting for a pod with a large number of seeds (Solbrig and Cantino, 1975). Simpson's work (cited by Solbrig and Cantino, 1975) showed that fruit production was increased two to four fold by artificial pollination. Other cited work on the effect of planting soil depth and temperature on seed germination and emergence would be of obvious horticultural importance in a mesquite breeding program. As an interesting sidelight, one site of 10 P. chilensis trees had to be abandoned since a herd of domesticated goats ate all the fruits which had fallen from the tree before they could be weighed.

The latest work on Prosopis from South America is a Ph.D. thesis by

Figueirdo (1975) on the chemical composition of the pods of algaroba. Figueirdo was born in Portugal, raised in Brazil, and obtained his Ph.D. in Germany. This thesis is an excellent study on the purification and chemical characterizations of components from Prosopis pods. Analysis of the intact pod showed 13 % protein and 29 % sucrose, while the isolated seed and pod minus seed had protein contents of 32 % and 9 % respectively. SDS gels of the seed proteins showed molecular weights of 77,000 and 96,500 respectively. Amino acid composition of proteins, following hydrolysis under milder conditions than normally employed i.e. 8 hours with 3 N HCl versus the standard 20 hours with 6 N HCl, yielded lower levels of hydrophobic amino acids than reported here. Molecular weight analysis, nmr data, and gc-ms permethylation analysis of carbohydrate fractions from Prosopis are also given.

Figueirdo (1975), also lists references on the uses of Prosopis showing that Algaroba was widely used by the Indians in South Ecuador, Chile, and Argentina as food during the conquest of South America in the sixteenth century. He cites Oveido, a chronalist in colonial times as reporting that Algaroba was a major nutrient for the Indians and when the rain was not sufficient and the rivers did not carry enough water to irrigate maize, the Indians lived almost exclusively on Algaroba.

Algaroba is not indigenous to Brazil being first imported in 1942 by the former director of the Agronomy School in Viscosa. In 1947 large quantities of a good Prosopis variety from Northern Peru were imported to Brazil and planted by farmers as food for livestock. By the 1950's algaroba was much favored and was proposed as a good plant for the dry infertile regions of northeastern Brazil (Figueirdo, 1975).

A list of preparative methods for Algaroba pods in human diets was reported by Azevedo (1966) where an attempt was made to adapt the recipes and names of algaroba products from Chile, Argentina, and Peru to Brazilian customs. Recipes were described for production of: mel, a boiled water extract of pods used like sirup or honey; Farinha or flour; coffee; a cold drink; of alua-a fermented alcoholic drink; and aguardente, a stronger alcoholic drink (Azevedo, 1966). It is unfortunate that in the preparation of the farinha or flour, the seeds are not ground with the pods resulting in a flour of much lower protein than need be. Azevedo (1966) also mentions that there are several houses in Buenos Aires which sell algaroba products such as "Casa Da Catamarca" and "Casa De San Jose De Estero", and that in restaurants in Lima one finds the famous cocktail algarobina on the menu. A study showing the high contents of calcium, thiamin, vitamin B_1, riboflavin, and vitamin B_2 in algaroba pods is cited by Azevedo (1966).

3. USES OF LEGUMINOUS TREES IN AFRICA

a. South Africa

The three leguminous trees which have been recommended as fodder for the farmers of South Africa are Prosopis, Gleditsia, and Ceratonia, and have been introduced either from the U.S.A. or the Mediterranean region. There appear to be only three papers relating to cultivation of leguminous trees in South Africa. The first paper by Loock (1947) described methods of propagation, climatic and soil requirements, and yield potential for these trees. Loock (1947) notes that Prosopis grows best on deep and sandy soil, and since it has a taproot sometimes as long as 60 feet, it can withstand any drought. From the recommended 20

ft spacing and suggested yield of 200 lbs of pods from a 10 yr old tree (Loock, 1947) one can calculate a density of over 200 trees/ha with approximately 18,000 kg/ha of mesquite pods-truly a phenomenal figure considering a good corn crop yields 6,000 kg/ha. The ripe pods are reported to have the same nutritional value as maize when crushed and used as a livestock food (Loock, 1947). This claim is not substantiated and is in disagreement with the Hawaiian and Texas work noting nutritional deficiencies in cattle. A significant difference betweeen the American and S. African study is that the South African workers appear to have crushed the pods which would have released the protein in the seed.

Loock (1947) points out that Gleditsia is one of the hardiest fodder trees known and that it is resistant to frost, cold and severe droughts. A honey locust tree from Middleburg C.P. South Africa is reported to yield an average of 500 lbs of dried pods per year between the 7th and 12th year of age. The carob is not used as extensively in South Africa as Prosopis or Gleditsia because it is not as frost resistant, does not have as high protein in the pods, and does not yield as well at an early age.

A Republic of South Africa Forestry Bulletin (Jurriaanse, 1973) examined the possibility of planting Prosopis, Gleditsia, and Ceratonia as fodder trees in South Africa. The original research on fodder trees had stopped in 1956 and, probably, advances could have been made if the work had been continued.

Much of what Jurriaanse (1973) reports has been discussed above so only new information will be mentioned here. Jurriaanse describes, scarification treatments, seed bed and nursery care, suggested orchard planting densities, pollination requirements, and useful vegetative propogation techniques for Gleditsia. Since the honey locust is shade intolerant 12 m

x 12 m spacings are recommended to give room for crown development. It was suggested that adult trees would bear 180 kg of pods, corresponding to 11,500 kg/ha. The pods should be thoroughly ground and mixed with other types of feed when used for livestock food.

Jurriaanse (1973) notes that Prosopis is already hybridized to such an extent that it is difficult to distinguish species and since some wild Prosopis are thornless it should be possible to cultivate thornless types. Unlike honey locust, Prosopis does not do well in high rainfall areas and seems to do best when receiving between 250-500 mm rain per year (Jurriaanse, 1973). Once established, the trees can withstand severe frost although the young trees must be protected from frost. An interesting feature of Porsopis is their adaptability to unfavorable conditions. If ample rain comes, after a dry spell, when not many pods were produced, the trees will flower and produce a second crop of pods. An orchard spacing of 9 x 9 m was suggested for Prosopis with a conservatively expected pod yield of between 90-140 kg/tree thus yielding 11,000 to 17,000 kg/ha (Jurriaanse, 1973). The suggested use of Prosopis pods is for livestock food. It is noted that Prosopis could become a weed on land used for other crops. Livestock feed willingly on carob beans but Jurriaanse recommended against carob plantations for fodder until further research is done.

An interesting but very sketchy report of plantings of algaroba (Prosopis) and carob (Ceratonia) in South Africa, Rhodesia, and Tanzania was prepared by Douglas (1967). High yielding, thornless varieties of algaroba were obtained from Hawaii while high yielding varieties of carob were obtained from E. Coit of California. Douglas reported that

"good algaroba varieties yield up to 10 tons of edible beans per acre annually", but it is not clear if Douglas measured that or if he is repeating data from Smith's (1952) book. Douglas reports that algaroba seeds were sown in small grass baskets and set out 25 feet apart when the seedlings were 18 inches tall. At 16 months the trees were in flower and at 18 to 20 months the first pods were harvested. This is rapid and means that selection for pod and seed protein quantity and quality could be accomplished in under two years - not much longer than for annual crops. Yields were not reported for the carob but it was stated that the carob begins to bear at 4 to 6 years.

Although they do not yield food, the Wattle (Acacia meansii) of South Africa is grown in many plantations for tannin extraction from the bark. The importance of wattle in South Africa is indicated by the presence of the "Wattle Research Institute", and a Ph.D. thesis on "A Site evaluation study in Black Wattle" (Schonau, 1969). Wattle is reported to fix nitrogen at rates of 200 $\pm$ 45 kg N/ha/year (Orchard and Darb, 1956) and thus comparable to the highest reported nitrogen fixation rates for alfalfa.

3. USES OF LEGUMINOUS TREES IN AFRICA

B. Subsaharan region

The earliest, comprehensive, review of the useful plants of West Africa including tree legumes was by Dalziel (1937). Considerably later Irvine, (1961) published a book expanding on the work of Dalziel. A book concerning food plants in, French speaking Africa (Busson et al., 1965) gives proximate analysis, fatty acid and amino acid composition data and is the last major work on tree legumes. While these three

works discuss all three subfamilies in the Leguminosae, only the material from the subfamily Mimosoideae will be discussed here since it contains the great majority of the tree legumes.

Dalziel (1937) lists 16 Acacia species used in W. Africa with four having importance as a livestock food, and Irvine (1961) expands the list of uses of Acacia, presents several color prints, and gives tables of proximate composition of various parts of Acacias. The Fulani tribesmen, in Western Sudan, drive their cattle during dry season to where Acacia seyal grows, and there the leafy branches and pods of A. seyal may supply the cattle with food for several months (Dalziel, 1937). Acacia sieberiana and A. tortilis are used in the same manner as A. seyal (Dalziel, 1937).

Acacia albida is the largest Acacia in the savannah with the pods and leaves being used as camel and cattle food (Dalziel, 1937). The pods may also be mixed with maize, groundnut meal, and hay as a livestock food (Irvine, 1961). More recently Charreau and Vidal (1967) found that millet grown directly under A. albida yielded 2.5 times more than millet not grown under the treetree and that the soil nitrogen content was at least twice as high under the A. albida cover than away from it.

Perhaps the most important genus used as a food for humans and livestock in the subsaharan region of Africa is Parkia where there are four species Parkia bicolor, P. biglobosa, P. filidoidea, and P. clappertoniana (Busson et al., 1965). Parkia clappertoniana, P. biglobosa, and P. filicoidea are differentiated from each other by the size and shape of their leaflets, and from P. bicolor by virtue of the fact that P. clappertoniana, P. biglobosa, and P.filicoidea have a yellow pulp surrounding the seeds while P. bicolor does not (Busson et al.,1965).

Nomenclature problems do exist as this simple taxonomic treatment is not in agreement with that of Hagos (1961). Also Dalziel (1937), Irvine (1961), and Busson et al. (1965) describe P. filicoidea, P. clappertoniana , and P. biglobsa respectively as being the most important Parkia food source in West Africa.

Dalziel (1937) reports that in inhabited districts every P. filicoidea is sold as a meal and is a valuable food when used with rice, cereals, meat or soup. The yellow meal can be pressed into cakes for storage (Dalziel, 1937). As the P. filicoidea seeds have a hard seed coat, the seeds are boiled for 24 hr to loosen the seed coat, and then pounded and washed to remove the seed coat. The naked seeds are then boiled to form a paste and set aside for several days to ferment. After fermentation, the paste is made into balls sometimes called dawa-dawa and are sold and used as Europeans use cheese. The somewhat unpleasant smell from dawa-dawa is destroyed when the fermented product is fried or roasted (Dalziel, 1937).

Irvine (1961) reported much the same uses of Parkia but for P. clappertoniana and P. biglobsa instead of P. filicoidea and gave proximate analyses for the seeds and pods. Busson et al. (1965), gave the most complete chemical analyses of seeds and pods giving proximate analysis, and composition values for 9 fatty acids, and the amino acids. The 4 Parkia's range in protein from 21 to 45% protein with methionine and cystine being the limiting amino acids. No poisonous substances were present in the pulp or seed of P. filicoidea (Dalziel, 1937) although Irvine (1961) reported that the young P. clappertoniana pod contains an alkaloid and a cyanogenic glucoside.

The whole Parkia fruit is a good fodder for domestic stock and the percentages of husk, yellow powdery meal, and seeds are 41, 33, and 25 % respectively (Dalziel, 1937). Busson et al. (1965) notes that the average yield of pods per tree is 25 kg but an exceptional tree may yield 100 kg of pods. After strong winds, women and children collect the pods of Parkia and store them in graneries. Parkia was highly esteemed as a food by the early voyagers to West Africa and the Muslim people believe that the African locust bean (Parkia), the Baobab tree, and the Cola tree are gifts from heaven being introduced into Africa by the prophet (Busson et al., 1965).

A recent study of the protein quality and amino acid composition of Parkia has been completed (Fetuga et al., 1974) indicating that the Parkia protein was of poor quality. The poor quality of this protein could be largely overcome with supplementation by methionine and tryptophan.

A tree legume much less widely used for food is Pentaclethra macrophylla (Dalziel, 1937). The seeds contain 30-36 % oil and are rich in protein but because of the strong odor, the beans are primarily suited for use as a manure. The beans can be eaten after roasting, but are used more as a condiment than as a staple food. Presumably the heating destroys the poisonous alkaloid paucine which is found in the seed (Dalziel, 1937). P. macrophylla has 40 x 10 cm pods and 7 x 3 cm seeds (Busson et al., 1965). The seed has a protein content of 29 % and a low methionine value (Busson et al., 1965).

Prosopis africana is found in West Africa but it is used much less than the North American derived Prosopis. The seeds of P. africana

contain a tannin which deters cattle from eating them (Dalziel, 1937). Busson (1965) lists the protein content of Prosopis africana as being 19 % and a methionine content of 350 mg/gm N. This high methionine value is of importance as it is almost 3 fold higher than that recommended by the FAO for adequacy in human nutrition and since other legumes are below FAO recommendations for methionine. Unfortunately, there seems to be a probability of a typing error transposing the lysine value in the table of Busson et al. (1965). Correspondence with Busson has not clarified the issue since the raw data had been discarded.

Leucaena glauca is reported by Dalziel (1937), Irvine (1961), and Busson (1965) to be used in the same manner in Africa as it is in Hawaii. Tetrapleura tetraptera, of minor food use, has as unusual pod shape in that it has four longitudinal ridges, two of which are soft, filled with a sugary pulp and edible (Dalziel, 1937). Neither the pods or seeds of T. tetraptera contain alkaloids or cyanogenic glucosides. The two tree legumes, Pithecellobium dulce and Samanea saman which are used as fodder trees in West Africa, have been introduced from their native tropical America (Irvine, 1961).

4. USES OF LEGUMINOUS TREES IN ASIA

Leguminous trees are perhaps used in all parts of tropical Asia, but literature on the uses of leguminous trees arises from two major areas, the Indian subcontinent, and the Malaysian-Indonesian region.

This review of tree legume uses in Asia will begin with India where the most prominently used tree legumes are Acacia, Pithecellobium, and Prosopis. One of the most general reviews on these trees can be found in Wealth of India (1969). The three species of Prosopis chilensis, P.

cineraria, and P. stephaniana are found to be useful in India, with Prosopis chilensis probably the most valuable (Wealth of India, 1969). Prosopis chilensis syn. P. juliflora was introduced to the new world while P. cineraria is native to India (Wealth of India, 1969; and Gupta and Balera, 1972).

In India, the mesquites are reported to be fast growing, hardy, drought resistant and suitable for reforestation of saline arid and semiarid areas. Prosopis starts fruiting at 3-4 years and a 10 year old tree may produce 90 kg of pods annually (Wealth of India, 1969). In India dried and crushed Prosopis pods did not induce deleterious effect when fed to cattle and feeding trials have shown that dry pods can be used to make up for the protein deficiency of roughages such as wheat and rice straw (Wealth of India, 1969).

As a prelude to a physiological study on mesquite (P. juliflora) Gupta and Balera (1972) reported that P. juliflora was declared the "Royal plant" and placed under government protection in the Jodhpur state. This protection caused its widespread distribution where it provides the bulk of fuel for the local population. Douglas (1967), without citation, reports that an algaroba (Prosopis) forest 400 miles long and 2 miles wide is being established to prevent the eastward advance of the Rajputan desert. In Arabia, Afghanistan, Persia, India, and West Pakistan the Prosopis cineraria foliage and pods are used as a livestock fodder, the wood is used as a fuel, and the sweetish pulp around the seeds is eaten green or dry, raw or cooked (Ali, 1973).

The encyclopedic reference Wealth of India (1969) lists 5 species of Pithecellobium which exist in India with the most important being a

Mexican introduction, Pithecellobium dulce, used as a fodder for cattle sheep, goats and other livestock. The pulpy aril of the ripe Pithecellobium dulce seeds is reported to be used in preparation of a beverage similar to lemonade in Mexico (Wealth of India, 1969). The two Parkia species P. biglandulosa and P. roxburghii have only minor importance in India as a foodstuff (Wealth of India, 1969).

The most thorough coverage of the presence and uses of tree legumes in Malaysia and Indonesia was given by Ochse (1931). An older taxonomic study of "The Flora of the Malay Peninsula" by Ridley (1922) gives brief references to the uses of the leguminous trees Acacia, Leucaena, Parkia, and Pithecellobium. A more recent but less detailed review of these leguminous trees is given by Burkill (1966).

Leucaena glauca, the tropical American tree introduced to many tropical areas of the world is found in Indonesia where the immature and mature, but not dry cooked pods are eaten as a side dish with rice (Ochse, 1931). Varieties of Leucaena with both large and small pods exist (Ochse, 1931).

Malynicz (1974) from Papua, New Guinea studied the effect of adding ground Leucaena leaves to a commercial ration in pig feeding trials. Since Leucaena contains the depilatory mimosine, 0.2 % of ferrous sulphate was added to prevent the absorption of mimosine. In the trials it was found that the addition of 10 or 20 % Leucaena leaf meal significantly improved the growth rate of pigs over the commercial ration alone.

The genus Parkia contains some of the most important tree legumes used for food in Indonesia and has three common species P. intermedia, P. javanica, and P. speciosa (Ochse, 1931). As the name implies P. intermedia is intermediate between the species P. javanica and P.

speciosa. Although P. intermedia is not cultivated its seeds are eaten raw or roasted. P. javanica is a very large tree having a height of 20-50 m. The seeds of P. javanica are often roasted since they are not as well liked as other species. P. speciosa is much shorter than P. javanica having a height of 5-25 m. and is frequently cultivated. Often P. javanica does not live long being killed by borers at about 8-10 years of age. A large and small seeded variety of P. speciosa exists. P. speciosa is enjoyed as a food and Ochse (1931) notes that P. speciosa "is one of the most relished native vegetables. However large the supply on the market may be it is always readily sold. The most important part are the seeds which young or ripe, cooked or roasted are eaten in many ways with rice". The P. speciosa pods are often dried in the sun prior to storage or for shipping. The seeds are 2-2.5 cm long, 1.4-1.9 cm wide and have a very thin testa (Ochse, 1931). It should be noted that P. roxburghii found in Africa is believed to be P. javanica introduced from Indonesia (Hagos, 1961).

Pithecellobium lobatum (Djenkol bean) is a leguminous tree used as widely as the Parkia spp. and was the source for the first isolation of djenkolic acid. Ochse (1931) reports that the fruits "young as well as old, raw as well as cooked, form a side dish with the rice table. In the native household these fruits are very important and of great commercial value. Therefore, they should be cultivated in every native kitchen garden". It was also stated (Ochse, 1931) that eating of the djenkol bean can cause inflammation of the urethra, and that several common remedies for this condition are to drink water containing a special straw ash, to drink a large glass of milk or to drink whipped eggs. Ochse (1931) advised that in order to prevent the occurrence of the inflammation, one

should drink lots of water when eating the seeds. If the seeds are buried for about 7 days and allowed to germinate the danger of getting urethritis is almost entirely gone. The P. lobatum tree is of moderate size ranging from 10-26 m tall.

In dry regions of Indonesia the tamarind (Tamarindus indicus) thrives well. The pods of the tamarind are collected at harvest time, and the pulp removed and sold to Chinese buyers (Ochse, 1931).

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Quantitative Gas–Liquid Chromatography and Mass Spectrometry of the *N*(*O*)-Perfluorobutyryl-*O*-Isoamyl Derivatives of Amino Acids[1,2]

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A modification of a gas–liquid chromatographic method for quantitative analysis of amino acids as the *N*(*O*)-perfluorobutyryl-*O*-isoamyl derivatives is described. The modifications include changes in time and temperature for esterification, improved preparation of the esterification reagents and conducting the derivatizations *in vacuo* to obtain reproducible values for amino acids such as methionine and arginine. Mass spectral data are presented for all the derivatized amino acids.

Quantitative analysis of amino acids in protein hydrolysates by gas–liquid chromatography (glc) is potentially faster and inherently less expensive than chromatography with conventional amino acid analyzers. Several methods using gas–liquid chromatography have been described (1–3) but, for a variety of reasons, have not gained wide acceptance. We wish to describe a modification of the method of Vincendon and Zanetta (3) which permits the quantitative determination of the isoamyl heptafluorobutyryl derivatives of 17 amino acids. The method requires less than 0.050 mg of protein, and a complete analysis can be accomplished in less than 1 hr excluding derivatization. The mass spectral (ms) fragmentation patterns of the amino acids are presented to permit confirmation of their elution order. Typical analyses for standard mixtures and for protein hydrolysates are presented.

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MATERIALS AND METHODS

Methyl esters of methionine, lysine, tyrosine, glutamic acid, arginine, histidine, and tryptophan were from Nutritional Biochemicals (Cleveland, OH), cystine dimethyl ester from Sigma Chemical Co. (St. Louis, MO) and solvents from Mallinckrodt. Methanol and isoamyl alcohol were anhydrous reagent grade and were further purified (1). Equally satisfactorily, they were redistilled and stored in glass-stoppered bottles over Linde 4A at room temperature and, transferred as needed, to a separatory funnel fitted with a drying tube. Acetyl chloride and methylene chloride were redistilled from reagent grade material.

Methanolic HCl and isoamyl alcoholic HCl were prepared by bubbling dry HCl gas into the respective alcohol (3). Upon storage, such preparations yield the respective ether and alkyl chloride. Thus, more satisfactorily, alcoholic HCl was prepared daily by slowly pipetting 0.2 ml of redistilled acetyl chloride into 1 ml of the respective alcohol at 0°C.

Ethyl acetate and acetonitrile were reagent grade and, after further purification (4,5), they were stored in glass stoppered bottles over Linde 4A.

Heptafluorobutyric anhydride was obtained from Pierce Chemical Co. (Rockford, IL), dispensed in 0.2-ml aliquots in ignition tubes, flushed with nitrogen and sealed *in vacuo*. Screw-capped, thin-walled reaction vials of 1–2-ml capacity were purchased from Regis or drawn in a flame from 13 × 100-mm culture tubes. Other, thicker-walled vials, did not sonicate as well. A 3-mm hole was drilled through the screw caps and the liner was replaced with a resealable, Teflon-faced disk (Tuf-Bond) from Pierce Chemical Co.

Transesterification and acylation were in a nitrogen atmosphere obtained by repeated evacuation and nitrogen flushing of the reaction vials through a stainless-steel 25-gauge needle piercing the Teflon-coated disc. By using a vacuum of 0.2 Torr (on the vacuum-pump side of the needle), the reaction vials were evacuated for 5–10 sec, and nitrogen was admitted through a two-way stopcock. This was repeated three times, with the vial finally being left evacuated. As the needle is easily plugged, it is important to check for disturbance of the reaction mixture surface by the incoming nitrogen.

Sand baths used to heat the reaction vials were prepared with temperatures 1 cm below the surface of 50, 70, 100 and 150°C by placing sand to depths of 12, 7, 5, and 1.5 cm, respectively, in tin cans on a temperature-regulated hot plate. Prepurified nitrogen was further dried by passage through a $CaSO_4$ drying tube and used to reduce derivatization mixtures to dryness.

The column packing was 3% SP 2100 on 80/100 mesh Supelcon

AW-DMCS and was obtained from Supelco Inc. (Bellefonte, PA) or prepared in this laboratory with the aid of a "fluidizer" (6).

Glc was on a Varian Model 2740 equipped with a flame ionization detector using a 3 m long × 3-mm i.d. glass column. A dual-channel recorder was utilized to permit recording peak size at a 1 and 0.1 × sensitivity, thus extending the range of concentrations assayable. The glass insert for the injection port was filled with silanized glass-wool and changed daily. Due to the extremes of temperature, programming Hi-Temp Vespel ferrules from Anspec were used. Quantitation of the amounts of amino acids was obtained by photocopying the chart paper and cutting out and weighing the peaks.

Combined glc–ms was on an LKB-9000 mass spectrometer interfaced with a 2.0 m long × 3.0-mm i.d. glass column packed with 1% SP-2100 on 100/120 mesh (Supelcoport). The helium flow rate was 25 ml/min, the ionizing energy was 70 eV, the flash heater was at 160°C, the molecular separator at 240°C, and the ion source temperature at 290°C. The mass spectra were recorded with an on-line data acquisition and processing program. The oven temperature was held at 90°C for 10 min and then programmed at 4°C/min to 250°C. Since complete resolution of all the amino acids was not obtained on this column, mixtures of a few well-separated amino acids were used to obtain the mass spectral data.

Exact mass measurement of the $m/e = 70$ ion was performed with a Varian MAT CH5/DF instrument using a direct probe at 90°C and an ionization voltage of 70 eV. Perfluorokerosene was used as a standard.

For preparation of the protein from soybeans (*Glycine max*, var. Hark), the defatted, 40-mesh meal, was homogenized three times with 10% NaCl in 30 mM phosphate buffer, pH 7.2, for 5 min in a micro-omnimixer at 45,000 rpm. After each extraction the homogenate was centrifuged in a clinical centrifuge, the supernatant fluids were combined and solid trichloroacetic acid was added to give a 10% (w/v) solution. The suspension was heated in a boiling-water bath for 10 min, cooled and centrifuged at 6,000*g* for 5 min. The supernatant fluid was discarded, and the pellet was washed with diethyl ether to remove the trichloroacetic acid. The precipitated protein was then dried *in vacuo*. Hydrolysis was in sealed, evacuated ignition tube at 110 ± 4°C for 18 hr as previously described (10).

RESULTS

Derivatization of Amino Acids

The procedure for derivatization of amino acids was as follows: A protein hydrolysate or amino acid solution containing up to 50 μg of amino acids was dried with a stream of nitrogen at 70°C in a reaction

vial. Redrying with methylene chloride was unnecessary. Then 0.4 ml of freshly prepared 20% (v/v) acetyl chloride in methanol solution was added, the reaction vial capped, sonicated for 15–20 sec after cavitation occurred, and then heated at 70°C for 30 min. After drying at 50°C under a stream of nitrogen, 0.4 ml of 20% (v/v) acetyl chloride in isoamyl alcohol was added, the vials flushed with nitrogen and evacuated. The vials were sonicated as above, heated at 100°C for 2.5 hr, then dried at 70°C under a stream of nitrogen. Next, 100 μl of ethyl acetate and 20 μl of perfluorobutyric anhydride were added and the vials were flushed with nitrogen and evacuated. The vials were again sonicated and held at 150°C for 5 min. After cooling to room temperature, the reaction mixture was reduced in volume in a stream of nitrogen until the sides of the vial were barely wet. This step is critical and requires close attention. Excessive drying leads to a loss of arginine while inadequate drying results in a tailing glc solvent-front. A suitable volume of ethyl acetate was added and the solution was sonicated prior to glc.

Esterification

Incomplete derivatization was encountered in the methyl esterification of arginine, histidine, tryptophan and cystine using previously described procedures (1,3). Transesterification of methionine must be done *in vacuo*. A 20-min methyl esterification at 70°C followed by a 150-min isoamyl transesterification gave a quantitative yield of the esters as judged by a single ninhydrin-reactive spot on thin layer chromatograms (tlc). Cystine did not chromatograph as a single spot on tlc after 45 min of the methyl esterification treatment. The R_f values on tlc in a 95% ethanol solvent for the free amino acid, methyl ester, and isoamyl ester, respectively, were: 0.08, 0.66, 0.81, for glutamic acid; 0.03, 0.22, 0.54 for arginine; 0.01, 0.21, 0.56 for histidine; 0.41, 0.71, 0.80 for tryptophan; and 0.11, 0.33, and 0.77 for cystine.

Acylation

As is shown in Fig. 1, authentic, commercial methyl esters can be acylated and examined by glc, thus providing a convenient check of the acylation procedures. The two tryptophan peaks probably correspond to the monoacyl and diacyl derivative as discussed later in the text. Arginine is the most difficult of the amino acids to acylate, although, within limits, the time and temperature of acylation are not critical. A time of 3–10 min at 150°C, or 5 min at 130–150°C yields similar results. If the ethyl acetate is not anhydrous, if the perfluorobutyric anhydride is impure (owing to storage in air for a month), or if the acylation mixture is improperly reduced in volume, the arginine peak will be diminished or lost. Other acylating reagents which donate the perfluorobutyryl group

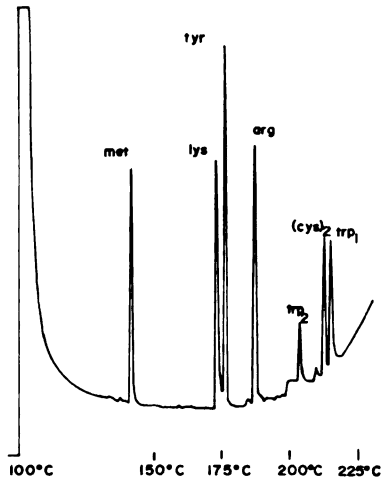

FIG. 1. Gas-liquid chromatogram of the perfluorobutyryl derivatives of the methyl esters of methionine, lysine, tyrosine, arginine, monoacylated (trp_1) and diacylated (trp_2) tryptophan, and cystine. Gas chromatography was done on a 4 m long × 3-mm i.d. glass column. The temperature was 100°C initially and was programmed at 4°C/min. The flash heater was 230° and the detector was 250°. Approximately 1 μg of each of the amino acids was injected.

such as perfluorobutyryl imidazole and *N*-methyl-*bis*(perfluorobutyramide) yield unsatisfactory results. In addition tertiary amines, such as triethylamine and pyridine, cannot be used since they are quaternized by the perfluoro anhydrides.

Tryptophan, Histidine and Cysteic acid

Tryptophan, cysteic acid and histidine similarly present problems. The diacyl derivative of histidine has been reported (7), but there has been no mass spectral identification of diacyl histidine (8). We do not obtain a histidine peak under the above conditions, and this is in keeping with the known instability of acyl imidazoles (9). Similarly we were unable to derivatize cysteic acid. The diacyl derivative of tryptophan can be obtained particularly by using acetonitrile as the acylation solvent and a somewhat longer heating time. Since, however, tryptophan, histidine, cysteic acid, or cysteine and cystine, require alternative methods of protein hydrolysis or derivatization, these amino acids are not assayed in the procedure here described.

Gas–Liquid Chromatography

A photoreduction of a glc tracing showing the retention times of the isoamyl-perfluorobutyryl derivatives of the common protein amino acids

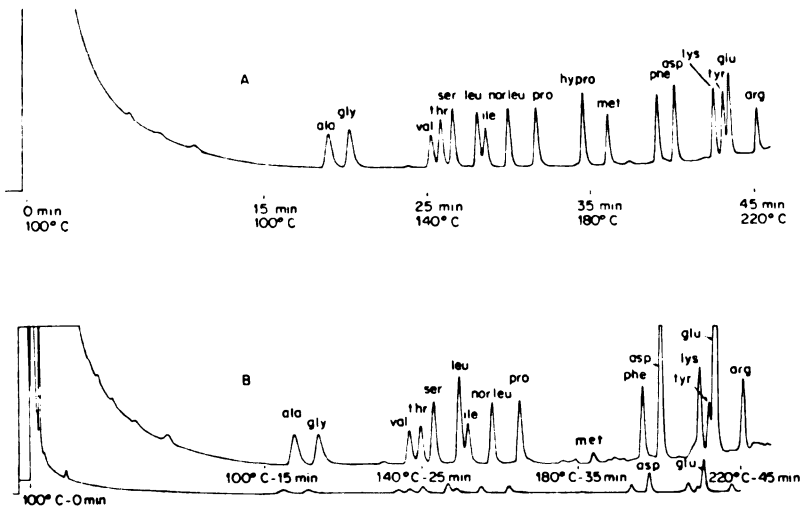

FIG. 2. (A) Gas–liquid chromatography of 0.7 μg of each amino acid from a standard mixture. A 3.3 m long × 3-mm i.d. glass column with 3% SP2100 on 100/120 mesh Gas Chrom Q was used, with a carrier gas flow rate of 22 ml/min. The oven was initially held 15 min at 100°C followed by a 4°C/min program. The flash heater and detector were both 250°C. (B) Gas–liquid chromatogram of an aliquot of a soybean hydrolysate. A total of approximately 14 μg was injected. Evacuation for hydrolysis was performed as previously described (10). The lower trace was recorded at tenfold less sensitivity to permit a more accurate determination of methionine and glutamic acid in one run. The use of a two-pen recorder created the horizontal offset of the time scales. All other conditions were as in (A) except that the carrier gas flow rate was 35 ml/min. The flow rate in (A) was later found to achieve somewhat better resolution of tyrosine and glutamic acid.

is shown in Fig. 2A. As discussed above, tryptophan, histidine, cysteine–cystine, and cysteic acid are not included. As can be seen, the components are sufficiently well resolved to permit quantitative assay of all of the amino acids present. Where necessary, a somewhat lower carrier gas flow gives improved resolution of tyrosine and glutamic acid.

Quantitative Analysis

By photocopying the glc recorder chart and weighing the cut-out peaks it is possible to determine the relative weight response (RWR) for each amino acid (1). These values, relative to norleucine as an internal standard, are shown in Table 1. Retention times for each of the derivatized amino acids are also shown and range from 19 min for alanine to 46 min for arginine.

During the course of a single day, RWR values differed by less than 10%. Over a period of several days variations as large as 10% were observed for valine, proline, hydroxyproline, and phenylalanine, with

TABLE 1
RELATIVE WEIGHT RESPONSE (RWR) COEFFICIENTS AND RETENTION TIMES FOR THE *N*-HEPTAFLUOROBUTYRYL-*O*-ISOAMYL AMINO ACID DERIVATIVES[a]

Amino acid	Retention time (min)	RWR
Alanine	18.8	0.99
Glycine	20.1	1.05
Valine	25.2	0.63
Threonine	25.8	0.92
Serine	26.6	1.10
Leucine	28.2	0.94
Isoleucine	28.8	0.73
Norleucine	30.1	1.00
Proline	31.9	1.11
Hydroxyproline	34.8	1.22
Methionine	36.4	0.82
Phenylalanine	39.5	1.09
Aspartic acid	40.7	1.24
Lysine	43.1	1.06
Tyrosine	43.7	0.98
Glutamic acid	44.1	1.22
Arginine	45.8	0.73

[a] The retention times are measured from the start of the solvent front. The RWR values are calculated from Fig. 2A and are relative to norleucine. The flash heater was at 245°C, and the detector was at 250°C. The oven was at 100°C with a 15-min delay followed by a program of 4°C/min to 250°C. Chromatography was on a 3.3 m long × 3-mm i.d. glass column on 3% SP2100.

smaller variations for the remaining amino acids. However, as the time required for derivatization and analysis of standards is small, daily calibrations to check RWR coefficients are made.

Assays of Protein Hydrolysates

Figure 2B illustrates the retention profile obtained upon hydrolysis of a crude protein prepared from soybean meal. Evacuation prior to hydrolysis was done as previously described (10). As can be seen, there are no major extraneous peaks, and resolution is comparable to that obtained for pure amino acids. Table 2 compares the quantitative values for soybean protein as determined by this method and as previously measured (1,11).

Table 3 compares the number of residues, relative to alanine, for lysozyme as determined by this method and as previously determined (12). Agreement is good except for valine and isoleucine and this discrepancy may be due to the lower hydrolysis temperature employed in the present study.

TABLE 2
COMPARISON OF LITERATURE VALUES AND VALUES DETERMINED IN THIS STUDY FOR AMINO ACIDS DERIVED FROM SALT-EXTRACTABLE, TCA-PRECIPITABLE SOYBEAN PROTEIN[a]

Amino acid	This method	FAO(11)	Gehrke *et al.* (1)
Alanine	1.06	1.02	1.01
Glycine[a]	1.00	1.00	1.00
Valine	0.99	1.15	1.27
Threonine	0.88	0.92	0.76
Serine	1.14	1.22	1.26
Leucine	1.53	1.86	2.08
Isoleucine	1.03	1.08	1.23
Proline	1.09	1.31	1.34
Methionine	0.31	0.30	0.22
Phenylalanine	1.11	1.18	1.31
Aspartic acid	2.46	2.80	3.26
Lysine	1.38	1.53	1.60
Tyrosine	0.90	0.75	1.02
Glutamic acid	3.80	4.48	5.68
Arginine	1.69	1.73	1.86

[a] Values normalized to glycine.

TABLE 3
COMPARISON OF LITERATURE VALUES AND VALUES[a] DETERMINED IN THIS STUDY FOR A 22-HR LYSOZYME PROTEIN HYDROLYSATE

Amino acid	This method	Literature (12)
Alanine	12.0[b]	12.0[b]
Glycine	11.6	12.1
Valine	4.43	5.59
Threonine	6.19	6.76
Serine	9.74	8.87
Leucine	7.52	7.84
Isoleucine	4.57	5.78
Proline	2.07	2.06
Methionine	2.21	1.89
Phenylalanine	2.91	2.91
Aspartic acid	19.60	21.40
Lysine	5.96	6.00
Tyrosine	2.93	2.94
Glutamic acid	4.86	4.84
Arginine	10.20	9.01

[a] Average of three derivatizations from the same hydrolysate.
[b] Values normalized to alanine.

TABLE 4
COMMON 70-eV FRAGMENTS OF THE ISOAMYL-HEPTAFLUOROBUTYRYL AMINO ACID DERIVATIVES

Ion	*m/e*	Ion	*m/e*
CH_3	15	CF_3	69
$(CH_3)_2CH$	43	CF_3C	81
$(CH_3)_2CHCH_2$	57	CF_3CF	100
$(CH_3)_2CH(CH_2)_2$	71	CF_3CF_2	119
$(CH_3)_2CH(CH_2)_2O$	87	CF_3CF_2C	131
$(CH_3)_2CH(CH_2)_2OCO$	115	CF_3CF_2CF	150
		$CF_3CF_2CF_2$	169
CH_3S	47	$CF_3CF_2CF_2CO$	197
CH_3SCH_2	61	$CF_3CF_2CF_2CO_2$	213
		$CF_3CF_2CF_2CONH$	212
		$CF_3CF_2CF_2CONHCNH$	239

Mass Spectrometry

The 70-eV mass spectral fragmentation patterns for the *N*-perfluorobutyryl-*O*-isoamyl amino acid derivatives have not previously been described. They are somewhat analogous to the 20-eV patterns described for the *N*-trifluoroacetyl-*O*-butyl esters (8) although relative intensities are different as would be expected since the spectra reported here were obtained with a higher ionizing potential (70 eV). In addition, the bulkier substituents lead to fragments such as isopropyl ($m/e = 43$) from the isoamyl substituent and CF_3 through C_3F_7 from the heptafluorobutyryl group. The data of Table 4 show the most common ion fragments observed in this study.

The mass spectra of the isoamyl heptafluorobutyryl derivatives studied here and the trifluoroacetyl butyl derivatives of proline, hydroxyproline, alanine, glycine, serine and threonine previously described (8) are in good agreement (Tables 5 and 6). For alanine and glycine the major differences from the trifluoroacetyl butyl derivatives are an intense $m/e = 43$ peak, which was the base peak for glycine, and the presence of the perfluoroalkane series. While Gelphi *et al.* (8) found the base peak to be the $CF_3CONH{=}CHR$ peak for serine and threonine, we have found an analogous peak, but one mass unit smaller, to be twice as intense for both serine and threonine. Proline and hydroxyproline fragmentations are quite similar in both kinds of derivatives.

Intensities of identical fragment ions for isoleucine, leucine and norleucine differ sufficiently to identify the amino acid (Table 7). The differences arise from varying stabilities of the side chains to yield primary and secondary carbonium ions. Since fragmentation yields tertiary > secondary > primary carbonium ions (13), isoleucine cleaves

TABLE 5
ALIPHATIC AND SIMPLE HYDROXY-AMINO ACID 70-eV MASS FRAGMENTATION PATTERNS

Ion	Ala *m/e* (%)	Gly *m/e* (%)	Val *m/e* (%)	Thr *m/e* (%)	Ser *m/e* (%)
M	355 (0.0)	341 (0.1)	383 (0.0)	581 (0.0)	567 (0.0)
$M-CH_3$			368 (0.1)		
$M-(CH_3)_2CH$	312 (0.2)	298 (0.7)	340 (0.1)		
$M-(CH_3)_2CH(CH_2)_2O_2C$	240 (100.0)	226 (31.5)	268 (100.0)	466 (0.9)	452 (0.8)
$M-CF_3$	286 (1.9)	272 (4.9)	314 (0.6)	512 (0.4)	498 (0.6)
$M-C_2F_5$			214 (8.4)		
$M-C_3F_7CO_2$				368 (1.1)	354 (0.3)
$(CH_3)_2CH$	43 (79.8)	43 (100.0)	43 (67.5)	43 (100.0)	43 (100.0)
$(CH_3)_2CH(CH_2)_2$	71 (35.6)	71 (49.1)	71 (37.1)	71 (84.9)	71 (68.5)
$(CH_3)_2CCH$			55 (64.1)		
CF_3	69 (16.6)	69 (13.9)	69 (9.8)	69 (14.9)	69 (23.0)
CF_3CF_2	119 (3.7)	119 (3.2)	119 (2.2)	119 (1.3)	119 (1.7)
$CF_3CF_2CF_2$	169 (10.2)	169 10.4)	169 (3.5)	169 (11.9)	169 (9.3)
$CF_3CF_2CF_2CO$				197 (1.1)	197 (0.2)
$CF_3CF_2CF_2CO_2$				213 (2.1)	
$C_3F_7CONHCCHCH_3$				252 (21.7)	
$C_3F_7CONHCCH_2$					238 (13.5)
$CH_2CHCO_2C_3F_7$				241 (2.9)	
$C_3F_7CO_2CH$					226 (0.9)
$M-(CH_3)_2CH(CH_2)_2O-(CH_3)_2CH$			253 (6.7)		
$M-C_3F_7CO_2-(CH_3)_2CH(CH_2)_2$					283 (2.2)

between the α and β carbons, while leucine will cleave between the β and γ carbons. These fragmentation patterns occur in conjunction with loss of another ion. Loss of the pentoxy and loss of the CF_3 group, in conjunction with cleavage between α and β carbons, corresponding to loss of a $m/e = 57$ fragment, yield higher intensities of $m/e = 271$ and

TABLE 6
PROLINE AND HYDROXYPROLINE 70-eV MASS FRAGMENTATION PATTERNS

Ion	Pro *m/e* (%)	Hyp *m/e* (%)
M^+	381 (1.6)	593 (0.0)
$M-(CH_3)_2CH(CH_2)_2O$	294 (0.3)	
$M-(CH_3)_2CH(CH_2)_2O_2C$	266 (100.0)	478 (5.7)
$M-CF_3$	312 (0.7)	524 (0.5)
$M-C_3F_7COO$		380 (0.9)
$(CH_3)_2CH$	43 (13.8)	43 (34.7)
$(CH_3)_2CH(CH_2)_2$	71 (6.3)	71 (18.8)
CF_3	69 (10.2)	69 (12.9)
C_2F_5	119 (0.9)	119 (1.5)
C_3F_7	169 (6.8)	169 (7.6)
C_3F_7CO	197 (0.2)	197 (0.1)
$M-C_3F_7CO_2-(CH_3)_2CH(CH_2)_2O_2CH$		264 (100.0)

TABLE 7
ALIPHATIC AMINO ACID 70-eV MASS FRAGMENTATION PATTERNS

Ion	Leu *m/e* (%)	Ile *m/e* (%)	Nle *m/e* (%)
M^+			397 (0.1)
$M-CH_3$		382 (0.2)	382 (0.1)
$M-(CH_3)_2CH$	354 (0.1)	354 (0.1)	354 (0.2)
$M-(CH_3)_2CH(CH_2)_2$	326 (0.1)		
$M-C_4H_8$	341 (5.1)	341 (11.0)	341 (3.2)
$M-(CH_3)_2CH(CH_2)_2O$	310 (0.2)	310 (0.3)	310 (0.5)
$M-(CH_3)_2CH(CH_2)_2O_2C$	282 (54.5)	282 (100.0)	282 (100.0)
$M-CF_3$	328 (0.6)	328 (0.7)	328 (1.0)
$(CH_3)_2CH$	43 (100.0)	43 (74.4)	[a]
$(CH_3)_2CH(CH_2)_2$	71 (34.5)	71 (74.9)	71 (29.9)
$C_6H_{11}O_2$	115 (0.8)	115 (1.2)	115 (0.7)
$C_6H_{10}O_2$	114 (1.1)	114 (3.1)	114 (1.8)
CF_3	69 (67.9)	69 (96.4)	69 (54.3)
C_2F_5	119 (0.5)		
C_3F_7	169 (4.9)	169 (6.4)	169 (5.3)
$M-CF_3-C_4H_9$	271 (0.9)	271 (7.2)	271 (0.8)
$M-(CH_3)_2CH(CH_2)_2O-C_4H_9$	253 (2.4)	253 (14.8)	253 (2.2)
$M-(CH_3)_2CH(CH_2)_2OH-(CH_3)_2CH$	266 (2.9)	266 (0.7)	266 (0.4)
$M-C_6H_{10}O_2-(CH_3)_2CH$	240 (38.4)	240 (1.5)	240 (6.5)
$M-C_6H_{11}O_2-C_4H_8$	226 (5.0)	226 (10.3)	226 (22.9)

[a] Data lacking.

$m/e = 253$ for isoleucine than for leucine and norleucine. Similarly, loss of the pentoxy ($m/e = 88$ and $C_6H_{10}O_2$ group ($m/e = 114$) shows that the loss of the secondary carbon ($m/e = 43$) is preferred in leucine to yield $m/e = 266$ and $m/e = 240$ ions.

A molecular ion is not observed for aspartic acid and the intensity of M^+ is only 0.3% of the base peak for glutamic acid (Table 8). In both cases the base peak is $m/e = 71$ assignable to the isoamyl alcohol. The $M-(CH_3)_2CH(CH_2)$ peak is much less than previously reported (8). This is to be expected from a 70-eV spectrum.

While the fragmentations of the aromatic amino acids are very similar in this study and that of Gelphi *et al.* (8), the relative intensities are different. This is exemplified by phenylalanine (Table 9) in which the derivative reported here has *m/e* (intensity) values of 91 (100), 148 (51.5), 218 (17.4), while the analogous relative intensities for the derivatives of Gelphi *et al.* (8) are 91 (39), 148 (100), and 204 (95). Both phenylalanine and tryosine have an anomalously high intensity $m/e = 70$ peak. Exact mass measurement of the $m/e = 70$ peak showed it to be 70.07809 ± 0.00016 amu, corresponding to the composition of C_5H_{10} with no nitrogen or oxygen. This peak may arise from preferred elimina-

TABLE 8
DICARBOXYLIC AMINO ACID 70-eV MASS FRAGMENTATION PATTERNS

Ion	Asp m/e (%)		Glu m/e (%)	
M^+	469	(0.0)	483	(0.3)
$M-CH_3$			468	(0.3)
$M-(CH_3)_2CH$	426	(0.1)	440	(0.4)
$M-(CH_3)_2CH(CH_2)O$	382	(0.1)	396	(2.7)
$M-(CH_3)_2CH(CH_2)_2O_2C$	354	(14.4)	368	(15.8)
$(CH_3)_2CH$	43	(90.5)	[a]	
$(CH_3)_2CH(CH_2)$	71	(100.0)	71	(100.0)
$C_6H_{10}O_2$	114	(0.2)	114	(0.4)
$C_6H_{11}O_2$	115	(0.3)	115	(0.5)
$M-CF_3$	400	(0.5)	414	(0.8)
$M-C_3F_7CONH$			271	(0.3)
$(CH_3)_2CH(CH_2)_2O_2C(CH_2)_2$			143	(0.5)
$(CH_3)_2CH(CH_2)_2O_2C(CH_2)_2CH$			156	(0.1)
CF_3	69	(7.0)	69	(5.3)
C_2F_5	119	(0.6)		
C_3F_7	169	(1.8)	169	(1.0)
$M-(CH_3)_2CH(CH_2)_2O-(CH_3)_2CH$	339	(0.1)	353	(0.1)
$M-(CH_3)_2CH(CH_2)_2-C_6H_{10}O_2$	284	(5.4)	298	(23.8)
$M-(CH_3)_2CH-C_6H_{10}O_2$	312	(3.5)	326	(3.6)
$M-2(C_6H_{11}O_2)$	239	(11.0)	253	(3.4)
$M-C_6H_{11}O_2-C_6H_{12}O_2$	238	(0.4)	252	(19.4)
$M-C_6H_{11}O_2-(CH_3)_2CH(CH_2)_2O$	267	(2.5)	281	(2.7)
$M-C_6H_{12}O_2-(CH_3)_2CH(CH_2)_2O$	266	(3.1)	280	(15.6)
$M-2(CH_3)_2CH(CH_2)$			341	(1.1)

[a] Data lacking.

tion of the isoamyl substituent. In phenylalanine and tyrosine it appears that the charge retention is preferentially on the aromatic ring system ($m/e = 91$ for phenylalanine and 303 for tyrosine) or on the C_5H_{10} ($m/e = 70$) ion. Evidence for this is that for phenylalanine the aromatic ring system is the base peak, and the C_5H_{10} has 42.5% relative intensity. Tyrosine has the opposite relationship, in which the aromatic system has 68.6% relative intensity, and C_5H_{10} is now the base peak.

Tryptophan (Table 9) occurs as both the mono- and diacyl derivatives, the diacyl derivative having a perfluorobutyryl group on the indole nitrogen. Both derivatives yield molecular ions and a characteristic M-115. The quinolinium ion is the base peak, but, in the case of the diacyl derivative, this ion includes the perfluoroacyl group. The monoacyl derivative elutes 15°C higher on a 4°C/min program on a 2 m long × 3mm-i.d. 1% SP2100 column than does the diacyl derivative.

The spectrum of methionine (Table 10) differs from that previously

TABLE 9
AROMATIC AMINO ACID 70-eV MASS FRAGMENTATION PATTERNS

Ion	Phe *m/e* (%)	Tyr *m/e* (%)	Trp_1 *m/e* (%)	Trp_2 *m/e* (%)
M^+	431 (0.2)	643 (0.1)	470 (4.7)	666 (5.8)
$M-CH_3$	416 (0.1)	628 (0.1)		651 (0.1)
$M-(CH_3)_2CH(CH_2)_3O$	344 (0.2)		383 (0.2)	579 (0.1)
$M-(CH_3)_2CH(CH_2)O_2C$	316 (13.7)	528 (10.2)	355 (2.0)	551 (2.5)
$M-CF_3$	362 (0.3)	574 (0.2)	401 (0.1)	597 (0.2)
$M-C_3F_7$	262 (0.1)			497 (0.1)
$(CH_3)_2CH(CH_2)_2$	71 (33.5)	71 (32.4)	71 (1.2)	71 (6.3)
$(CH_3)_2CH(CH_2)_2O_2C$	115 (0.4)	115 (0.2)	115 (0.6)	115 (0.7)
CF_3	69 (11.6)	69 (18.2)	69 (0.9)	69 (5.0)
C_2F_5	119 (14.2)	119 (3.5)		119 (0.1)
C_3F_7	169 (4.8)	169 (11.4)	169 (0.3)	169 (4.4)
C_3F_7CO	197 (0.2)			
$M-C_3F_7CO_2$		430 (13.1)		
$M-C_3F_7CONH_2$	218 (17.4)	[a]	257 (0.7)	453 (14.6)
$M-(CH_3)_2CH(CH_2)_2O_2C-C_3F_7CONH_2$	103 (18.4)	315 (4.2)	142 (0.6)	338 (0.5)
$M-(CH_3)_2CH(CH_2)_2-C_3F_7CONH$	148 (51.5)	360 (54.2)	187 (0.7)	383 (12.4)
$M-(CH_3)_2CH(CH_2)_2O-C_3F_7$	175 (0.8)	387 (0.1)		
$M-(CH_3)_2CH(CH_2)_2O-C_3F_7CO_2$		343 (9.0)		
$M-(CH_3)_2CH(CH_2)_2O-C_3F_7CONH_2$	131 (14.3)	[a]	170 (0.3)	366 (1.4)
$M-(CH_3)_2CH(CH_2)_2O_2C-C_3F_7CO$	119 (14.2)	331 (5.0)	158 (1.8)	354 (2.1)
$ArCH_2$	91 (100.0)	91 (4.1)	130 (100.0)	130 (4.4)
$ArCH_2-H$				129 (14.4)
$CH_2ArO_2CC_3F_7$		303 (68.6)		
$ArCH_2-HCN$			103 (1.7)	103 (0.6)
$ArCH_2COC_3F_7$				326 (100.0)
C_5H_{10}	70 (42.5)	70 (100.0)	70 (0.1)	70 (1.1)

[a] As this assignment and the preceding one have equal masses they are interchangeable.

observed (8) in that analogous ions have quite different intensities, as would be expected due to differences in ionization energies. The following intensities have been described (8): 61 (19.5) and 75 (39), while we find 61 (89) and 75 (30.7). The base peak has been observed to be $M—CH_2=CHSCH_3$, while we find the corresponding peak (*m/e* = 341) to have 15.8 relative intensity. The base peaks with these derivatives probably arose from the alkyl esters to yield *m/e* of 43 = 98.6% and *m/e* of 71 = 100%. Also there is a series of peaks with *m/e* of 298, 284, and 271 which could have arisen from either of the two losses shown in Table 10.

The mass spectra of derivatized cysteine and cystine have been determined, although these amino acids were not included in the quantitative determinations. A useful method for determining cysteine and cystine is to measure cysteic acid following perfomic acid oxidation. We experi-

TABLE 10
THE 70-EV MASS FRAGMENTATION PATTERN OF METHIONINE

Ion	*m/e* (%)
M^+	415 (7.4)
$M-CH_3$	400 (0.1)
$M-(CH_3)_2CH(CH_2)_2O$	328 (0.7)
$M-(CH_3)_2CH(CH_2)_2O_2C$	300 (2.1)
CH_3S	47 (4.1)
CH_3SCH_2	61 (89.1)
$CH_3SCH_2CH_2$	75 (30.7)
$M-CH_3S$	368 (0.3)
$M-CH_3SCH_2$	354 (1.2)
$M-CH_3SCH_2CH$	341 (15.8)
$M-C_6H_{12}O_2-CH_3S$	252 (10.1)
$M-C_6H_{12}O_2-CH_3SCH_2$	238 (3.1)
$M-C_6H_{12}O_2-CH_3SCH_2CH$	225 (0.6)
$M-CH_3SCH_2CH-(CH_3)_2CH$ or $M-CH_3S-C_5H_{10}$	298 (4.8)
$M-CH_3SCH_2CH-C_4H_9$ or $M-CH_3SCH_2-C_5H_{10}$	284 (3.9)
$M-CH_3SCH_2CH-C_5H_{10}$	271 (11.9)
$(CH_3)_2CH$	43 (98.6)
$(CH_3)_2CH(CH_2)_2$	71 (100.0)
$M-C_3F_7CO$	218 (0.3)
$M-C_3F_7CONH$	203 (0.1)

enced difficulty derivatizing cysteic acid to the trimethylsilyl or the heptafluorobutyryl derivatives in mixtures of amino acids on a microgram scale. The S-carboxyethyl derivative of cysteine (18) is however more stable to acid hydrolysis and is easily converted to a volatile derivative suitable for gas–liquid chromatography. The mass spectra of the *N*-perfluorobutyryl-*O*-isoamyl esters of cystine, cysteine, and S-carboxyethyl cysteine are reported in Table 11. The molecular ion is found for cystine and S-carboxyethyl cysteine but not for cysteine. A diagnostic feature of cystine is cleavage of the disulfide bond to yield $m/e = 386$. Further, an ion with m/e equivalent to loss of S from 386 to yield $m/e = 354$ is also present. The S-carboxyethylcysteine molecular ion fragments as shown in Table 11. Other unique ions for this compound are $m/e = 176$, and $m/e = 141$ as shown in Table 11. In addition to the abundant ions M-115 and M-69 for cysteine, there are diagnostic eliminations from the molecular ion, such as C_3F_7COS, to yield $m/e = 354$. Low intensity ions at $m/e = 568$ ($M-CH_3$) and $m/e = 496$ ($M-C_5H_{11}O$) are also present but are less than 0.1% of the base peak. Another unique peak for cysteine occurs at $m/e = 174$ $(CH_3)_2CH(CH_2)_2COOCH\ CH_2CS$).

The molecular ion and $M-CH_3$ ion were found in the lysine spectrum

TABLE 11
THE 70-eV MASS FRAGMENTATION PATTERNS FOR CYSTEINE, CYSTINE, AND S-CARBOXYETHYLCYSTEINE

Ion	Cysteine *m/e* (%)	Cystine *m/e* (%)	S-carboxyethyl-cysteine *m/e* (%)
M^+	538 (0.0)	772 (0.8)	529 (0.3)
$M-CH_3$	568 (trace)		514 (0.3)
$M-(CH_3)_2CH$		729 (0.1)	
$M-(CH_3)_2CH(CH_2)O$	496 (trace)		442 (2.7)
$M-C_6H_{10}O_2$			415 (0.3)
$M-C_6H_{11}O_2$	468 (8.7)	657 (0.1)	
$M-C_6H_{11}O_2-H_2O$	450 (1.5)		
$M-CF_3$	514 (0.5)	703 (0.1)	460 (0.6)
$M-C_3F_7CO$	386 (3.8)		332 (0.1)
$M-C_3F_7COS$	354 (0.8)		
$M-C_3F_7CONH_2$		559 (0.6)	316 (10.0)
$M-C_6H_{11}O_2-C_5H_{10}$		587 (0.3)	344 (1.1)
$M-C_3F_7CONH_2-C_5H_{10}$		489 (0.4)	246 (7.9)
$M-C_3F_7CONH_2-(CH_3)_2CH(CH_2)O$	283 (0.7)		229 (8.5)
$M-(CH_3)_2CH(CH_2)_2O_2C(CH_2)_2S$			354 (1.2)
$(CH_3)_2CH$	43 (100.0)	43 (100.0)	43 (100.0)
$(CH_3)_2CH(CH_2)_2$	71 (65.9)	71 (76.0)	71 (68.5)
C_3F_7	169 (7.4)	169 (5.4)	169 (0.8)
$C_3F_7CONHCH_2CH_2$	240 (6.5)		240 (1.1)
$(CH_3)_2CH(CH_2)_2CO_2CHCH$			141 (3.7)
$(CH_3)_2CH(CH_2)_2CO_2CHCH_2S$	174 (0.9)		
$(CH_3)_2CH(CH_2)CO_2(CH_2)_2SH$			176 (5.6)
$C_3F_7CONHCHCH_2COOH$	284 (1.0)	284 (5.3)	
M/2		386 (0.9)	
M/2-S		354 (16.3)	
$M-2(CH_3)_2CH(CH_2)_2O-C_3F_7CO$		401 (0.9)	158 (19.8)

but with a low intensity (Table 12). As previously reported (8), the ϵ-amino group is also acylated. Each of the four possible side-chain-length fragments with the ϵ-amino group acylated were found. The base peak in this spectrum corresponded to the analogous derivative (8) in that the alkyl ester and perfluoroacetamide are lost to yield the base peak.

The mass spectrum of a *N*-perfluorobutryl-*O*-alkyl derivative of arginine has not previously been reported. Table 12 shows that the guanido-group is diacylated and that the fragment ions include the diacylated guanido-group plus 1, 2 or 3 methylenic carbons. The ions resulting from the loss of a perfluoroacyl from the guanido-group with retention of 1, 2 or 3 methylenic carbons are also found. Presumably, it is the diacylated guanido-group that makes arginine sensitive to hydrolysis by traces of water in the acylation solvents and reagents.

TABLE 12
THE 70-EV MASS FRAGMENTATION PATTERNS OF BASIC AMINO ACIDS

Ion	Lys *m/e* (%)	Arg *m/e* (%)
M^+	608 (0.2)	832 (3.5)
$M-CH_3$	593 (0.1)	817 (0.7)
$M-(CH_3)_2CH(CH_2)_2O_2C$	493 (5.3)	717 (0.2)
$M-CF_3$	539 (0.4)	763 (1.4)
$M-C_3F_7$	439 (0.1)	663 (7.7)
$M-C_3F_7CONH$	396 (0.1)	
$M-C_3F_7CONHCNH$		593 (1.0)
$M-C_3F_7CONHCNHNCOC_3F_7$		382 (0.4)
$M-C_3F_7CONHCNHNCOC_3F_7CH_2CH$		355 (0.2)
$M-(CH_3)_2CH(CH_2)_2O_2C-C_3F_7CONH_2$	280 (100.0)	
$C_3F_7CONH(CH_2)_4$	268 (2.8)	
$C_3F_7CONH(CH_2)_3$	254 (0.6)	
$C_3F_7CONH(CH_2)_2$	240 (2.4)	
$C_3F_7CONHCH_2$	226 (10.1)	
$C_3F_7CONHCNH$		239 (1.6)
$C_3F_7CONHCNN$		252 (0.7)
$C_3F_7CONHCNNCH_2$		266 (16.8)
$C_3F_7CONHCNN(CH_2)_2$		280 (3.1)
$C_3F_7CONHCNN(CH_2)_2CH$		293 (0.7)
$C_3F_7CONHCNHNCOC_3F_7$		450 (0.4)
$C_3F_7CONHCNHNH_2COC_3F_7$		452 (1.1)
$C_3F_7CONHCNHNCOC_3F_7CH_2$		464 (0.4)
$C_3F_7CONHCNHNCOC_3F_7(CH_2)_2$		478 (3.2)
$C_3F_7CONHCNHNCOC_3F_7(CH_2)_3$		492 (10.4)
$(CH_3)_2CH$	43 (59.4)	43 (100.0)

DISCUSSION

The relative weight response (RWR) coefficients for the amino acids are not identical and range from 0.63 for valine to 1.24 for aspartic acid (Table 1). Since equal weights of the amino acids have been derivatized and injected, one might expect nearly equal coefficients due to the "equal per carbon atom response" of flame ionization detectors (FID) (14). However, some of the amino acids have multiple derivatizable groups. For instance, hydroxy amino acids have an additional perfluorobutyryl group, while dicarboxylic amino acids will have an additional isoamyl group. The additional mass of the isoamyl group on glutamic and aspartic acids yields a larger FID response than does the perfluorobutyryl group on alcoholic hydroxyls and amines, since fluoroalkanes yield a lower detector response than alkanes (15). Consequently aspartic and glutamic acid have a 20% higher RWR than norleucine. The additional perfluorobutyryl groups on serine, threonine, tyrosine and lysine

add little to the RWR. Hydroxyproline, however, gives an anomolously high response of 1.22. Arginine has a lower RWR than norleucine. Since NH_3 has a 700-fold lower response in the FID than CH_4 (16), we believe the lower RWR for arginine is not due to partial derivatization but to the lowered FID response of the guanido-group. CS_2 gives a low FID response (17), and this suggests that the sulfur of methionine would also give a low RWR. Valine and isoleucine also give low RWR's and, though the reason is not known, this is in agreement with prior observations (1,2).

SUMMARY

The derivatization and glc procedures described, if carefully adhered to, yield accurate and reproducible amino acid determinations, as shown by the data of this paper and by extensive studies on the composition of plant seed proteins (Felker, unpublished). Histidine, cysteine, cystine and tryptophan cannot be measured by these methods owing to the lability of the acyl histidine (9) and the acid lability of cysteine, cystine and tryptophan. Cystine, in addition, does not give a quantitative yield of the dimethyl ester. Thus special or modified conditions must ultimately be developed for these amino acids. Cysteine, cystine and tryptophan are similarly troublesome using amino acid analyzer procedures.

The advantage of the method is primarily that the cost of an amino acid analyzer system is avoided and that the method is potentially more rapid than conventional analyzer systems. Many samples can be derivatized simultaneously during a 5-hr period and glc profiles can then be obtained at the rate of one per hour. Further reductions in the time required for derivatization have been made (19), and we suggest that use of a heated sonication-bath might further reduce the time required for analysis.

REFERENCES

1. Gehrke, C. W., Roach, D., Zumwalt, R. W., Stalling, D. L., and Wall, L. L. (1968) Quantitative Gas-Liquid Chromatography of Amino Acids in Proteins and Biological Substances, Analytical Biochemistry Laboratories, Inc., Columbia, MO.
2. Moss, C. W., Lambert, M. A., and Diaz, F. J. (1971) *J. Chromatogr.* **60**, 134.
3. Zanetta, J. P., and Vincendon, G. (1973) *J. Chromatogr.* **76**, 91.
4. Perrin, D. D., Armarego, W. L. F., and Perrin, D. R. (1966) *in* Purification of Laboratory Chemicals, p. 158, Pergamon Press, London.
5. Perrin, D. D., Armargeo, W. L. F., and Perrin, D. R. (1966) *in* Purification of Laboratory Chemicals, p. 58, Pergamon Press, London.
6. Leibrand, R. J., and Dunham, L. L. (1973). *Res. and Develop.* Sept., 32.
7. Roach, D., Gehrke, C. W., and Zumwalt, R. W. (1969). *J. Chromatogr.* **43**, 311.
8. Gelphi, E., Koenig, W. A., Gibert, J., and Oro, J. (1969). *J. Chromatogr. Sci.* **7**, 604.
9. Bruce, T. C. (1963) *in* Methods in Enzymology (Colowick, S. P., and Kaplan, N. O., eds.), Vol 6, p. 606, Academic Press, New York.

10. Moore, S., and Stein, W. H. (1963) *in* Methods in Enzymology (Colowick, S. P., and Kaplan, N. O., eds.), Vol. 6, p. 819, Academic Press, New York.
11. Autret, M. (1972) *in* Amino Acid Content of Foods and Biological Data on Proteins, FAO, Nutritional Studies #24, Rome.
12. Canfield, R. E. (1963) *J. Biol. Chem.* **238**, 2691.
13. Budzikiewicz, H., Djerassi, C., and Williams, D. H. (1967) Mass Spectrometry of Organic Compounds, p. 50, Holden-Day, San Francisco.
14. Blades, A. T. (1973) *J. Chromatogr. Sci.* **11**, 251.
15. Blades, A. T. (1973) *J. Chromatogr. Sci.* **11**, 267.
16. Blades, A. T. (1972) *J. Chromatogr. Sci.* **10**, 693.
17. Rowland, M., and Riegelman, S. (1967) *Anal. Biochem.* **20**, 463.
18. Seibles, T. S., and Weil, L. (1967) *in* Methods in Enzymology (Colowick, S. P., and Kaplan, N. O., eds.), Vol. 11, p. 204, Academic Press, New York.
19. Cancalon, P., and Klingman, J. D. (1974) *J. Chromatogr. Sci.* **12**, 349.

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A Gas–Liquid Chromatographic–Isotope Dilution Analysis of Cysteine, Histidine, and Tryptophan in Acid-Hydrolyzed Protein

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A gas–liquid chromatographic–isotope dilution method for quantitative determination of cysteine (cystine), histidine, and tryptophan and a novel method of protein hydrolysis are described. Acid hydrolysis of protein, using lysozyme as a model, is accomplished in the absence of oxygen and in the presence of 1,2-ethanedithiol for protection of tryptophan. Following the disulfide reduction, cysteine is *S*-alkylated to protect it during hydrolysis. The resultant tryptophan, alkylated cysteine, and histidine are converted to the methyl esters and then *N*-acetylated to form volatile derivatives suitable for chromatography on a polar silicone column. Correction for losses is made by an isotope dilution assay. The 70-eV mass spectra of the derivatized amino acids are presented. Ultraviolet spectral data are shown for *S*-carboxyethylcysteine.

There are studies of the determination of amino acids in proteins by gas–liquid chromatography (cf. 1–3) but the determination of cysteine, histidine, and tryptophan from hydrolyzed proteins remains difficult. Cysteine and tryptophan are unstable during acid hydrolysis, while histidine is unstable during gas–liquid chromatography. There is an extensive body of knowledge concerning amino acid derivatization, the chemical modification of proteins, and ion-exchange chromatographic techniques for amino acid analysis. This knowledge and new methods and techniques have been utilized to permit the determination of cysteine, histidine, and tryptophan by gas–liquid chromatography (glc). Tryptophan can be protected during acid hydrolysis with thioglycolic (4), toluenesulfonic (5), or mercaptoethanesulfonic acid (6), but such reagents must be removed by column chromatography prior to glc. In this study, the use of the volatile protective reagent dithioethane (1,2-ethanedithiol) is described, thus permitting recovery of tryptophan following acid hydrolysis and its determination as *N*-acetyl-*O*-methyltryptophan. After *S*-alkylation of the protein, cysteine can be assayed in the hydrolysate as *N*-acetyl-*O*-methyl-*S*-carboxyethylcysteine and histidine as *N*-acetyl-*O*-methylhistidine. Using these hydrolysis and derivatization procedures and an isotope dilution method to correct for losses, the method has been shown to be applicable to lysozyme and chymotrypsin with 75 to 80%

recovery of tryptophan and 93 to 98% recoveries of cysteine and histidine, respectively. The procedure described here for tryptophan, cysteine, and histidine requires approximately 5 hr prior to hydrolysis and 4 hr after hydrolysis, with subsequent glc at the rate of three per hour. Thus, this procedure together with that previously described (1) permits a complete amino acid analysis on a fractional aliquot of the hydrolysate from 300 μg of protein.

MATERIALS AND METHODS

Methanol and acetyl chloride were purified as previously described (1), except the methanol was not stored over Linde 4A, since that causes a residue to remain upon drying. Purification of Mallinckrodt AR grade acetic anhydride was by distillation through a 60-cm Vigreaux column, and storage was in a ground glass-stoppered bottle at room temperature. Several hours prior to use approximately 30 mg of anhydrous $CaSO_4$ and 30 mg of anhydrous K_2CO_3 were added per milliliter of the acetic anhydride. This combination facilitates the acylation of the indole nitrogen in tryptophan and neither distillation nor treatment with P_2O_5 substituted for the anhydrous $CaSO_4$–K_2CO_3 treatment. Possibly, traces of acetic acid react with K_2CO_3 to yield potassium acetate and carbonic acid, which, upon dehydration by the $CaSO_4$, volatilizes as CO_2.

Triethylamine was purified from a number of sources by resinifying yellow impurities with KOH pellets for 24 hr, followed by distillation from the KOH pellets and storage in an aluminum foil-wrapped glass-stoppered bottle over KOH pellets.

Acrylonitrile was redistilled in a nitrogen atmosphere and stored at 5°C in an amber bottle with 0.01% NH_4CO_3 as stabilizer.

Ethanedithiol was purchased from Aldrich Chemical Co. and used without further purification. Dithioerythritol was from Sigma Chemical Co. Both of these compounds were analyzed for thiol with Ellman's reagent (7) and found to be at least 80% reduced, based on two –SH per mole.

Hen egg white lysozyme, 3× crystallized, was obtained from Sigma Chemical Co. (St. Louis, Mo.). The desiccated lysozyme was dissolved in water to a concentration of 1 mg/ml. The concentration was then determined using an $E_{1cm}^{1\%}$ of 26.5 at 280 nm (8) which indicated the purity to be 85%. The amounts of cysteine, histidine, and tryptophan were then calculated using the amino acid composition and molecular weight data of Canfield (9). Absorbance measurements were with a Cary 15 spectrophotometer calibrated with a standard alkaline potassium chromate solution (10) and found to be within 2% of the standard at 280 nm.

The radioactive [^{14}C]cystine (30 mCi/mmol) was obtained from Schwarz/Mann (Orangeburg, N.Y.) while the [^{14}C]histidine (324 mCi/mmol) and tryptophan (57 mCi/mmol) were obtained from Amersham/

Searle (Arlington Heights, Ill.). The [^{14}C]cystine was shown to be radiochemically pure by tlc on silica gel G plates using propanol:acetic acid:water (4:1:1) as solvent, and its specific activity was confirmed by isotope dilution assay as described below. The specific activity of the tryptophan was determined by measuring the absorbance at 280 nm using a molar absorptivity of 6070 at 280 nm in methanol and by counting the radioactivity of a small aliquot of the same solution. A specific activity of 60.0 mCi/mmol was found in satisfactory agreement with that claimed. An attempt was made to estimate the specific activity of histidine in a similar manner using a molar absorptivity of 6310 in water at pH 0 at 211 nm. Ultraviolet-absorbing contaminants present in the [^{14}C]histidine precluded such measurements. The specific activity of the histidine was verified with the known histidine concentration in lysozyme.

Combined gc–ms was on an LKB-9000 mass spectrometer interfaced with a 1-m × 3.0-mm i.d. glass column packed with 1% OV-17 on 100/120 mesh Supelcoport. The helium flow rate was 25 ml/min, the ionizing energy was 70 eV, and the molecular separator and the ion source were at 240 and 290°C, respectively. Mass spectra were recorded with an on-line data acquisition and processing program. The oven temperature was initially 150°C and was programmed to 230°C at 5/min. The flash heater temperature was about 10°C higher than the oven temperature.

Exact mass measurements were obtained with a Varian MAT CH5/DF instrument using a direct probe, an ionization voltage of 70 eV, and perfluorokerosene as a standard.

Gas–liquid chromatography was with a Hewlett–Packard 402 gas chromatograph using an annular effluent splitter. The splitter contains Teflon parts with an upper temperature limit of 250°C, and this temperature restriction mandated a short column of 106 cm to separate *S*-carboxyethylcysteine, histidine, and tryptophan from the other amino acids. A peculiar feature of the annular splitter was an approximate threefold decrease in sensitivity after allowing for the split ratio. The decrease in sensitivity was attributable to the inside diameter of the flame ionization detector jet used with the splitter. The glc column used was 106 cm long, 2-mm i.d., with 5% SP2401 as the liquid phase on 100/120 Supelcon AW-DMCS. The column had 0.25-in. o.d., 3-mm i.d. pieces fused on the detector end to mate correctly with the splitter and a similar piece fused on the injection side to prevent the injection needle from striking the glass tube wall. The column was packed to within 1 cm of the site of needle penetration and had silanized glass wool on the injection port to prevent backflow of column packing to the injector when removing columns. The glass wool and the top 2 mm of packing were replaced daily if many injections were made.

The collection of peaks from glc was accomplished with a pasteur pipet inserted into Teflon tubing connected by means of a Swagelock

TABLE I

SPECIFIC ACTIVITIES OF AMINO ACIDS USED IN THE ISOTOPE DILUTION METHOD

Amino acid	Labeled amino acid added to protein			Labeled amino acid used for glc calibration			
	Specific activity (I) (μCi/μmol)	Activity added (cpm/10 μl)[a]	Mass added (X) (μg/10 μl)	Specific activity (II) (μCi/μmol)	Activity added (cpm/10 μl)[a]	Mass added (μg/10 μl)	Ratio a (I/II)
S-carboxyethyl-cysteine	30	31,595	0.129[b]	0.611	12,100	2.4[b]	49.1
Histidine	324	130,000	0.0336	0.254	13,450	3.7	1276
Tryptophan	57	54,400	0.101	0.475	25,090	5.45	120

[a] Counting efficiency was 88%.
[b] As cysteine equivalents.

ferrule to the effluent port. These Pasteur pipets were cut to an outside diameter that fitted snugly into the Teflon tubing. Differences in backflow pressure caused different flow rates and detector sensitivities, and this precluded using glass wool in the upper end of the pipets and required that the inside diameter of the pipets be uniform. After collection, the radioactive amino acids were washed into 6-ml scintillation vials with 1 ml of dioxane. Five milliliters of Bray's solution were added and the tubes were counted. Dioxane was found to decrease the counting efficiency by 2.5% and to leave only background counts in a second 1-ml dioxane wash after eluting 3700 cpm in the first 1-ml of wash.

Evacuation of hydrolysis tubes was with a Welch Duo-Seal No. 1405 two-stage vacuum pump connected with 0.5-in i.d. rubber tubing to a dry ice–ethanol cold trap. At approximately 20 in. from the hydrolysis tube, a "T" was inserted into the vacuum tubing to allow connection to a Bendix-type GTC-1000 thermocouple vacuum gauge. Between the vacuum gauge insertion and the hydrolysis tube, a heavy-duty Hoffman stopcock clamp was used to allow changing hydrolysis tubes without evacuation of the entire apparatus. As there was a pressure gradient from the hydrolysis tube to the pump, the vacuum transducer had to be near the hydrolysis tube. With this apparatus, freezing a sample, evacuating to 20 μm, thawing to 70–100 μm, refreezing, evacuating to less than 20 μm, and sealing in a flame requires about 15 min. For best results, the pump was allowed to run for a day before use.

Isotope Dilution Assay

Amounts of amino acids in the hydrolysate were determined by isotope dilution using the equation described by Rittenberg and Foster (11),

$$Y = \left(\frac{Co}{C} - 1\right) X,$$

where Y is the mass of the unlabeled compound, X is the mass of radioactive compound added, Co is the specific activity of the added radiochemical compound, and C is the specific activity of the mixture isolated. The initial and final specific activities need not be known, only the ratio Co/C. This ratio can be measured using a glc with an effluent splitter. The compound emerging from the glc was split so that a constant fraction is collected, and a constant fraction was burnt in the flame ionization detector. The counts collected divided by the peak area are then proportional to the specific activity. When the peak areas and collected counts were determined for both the initial and diluted compounds, the ratio Co/C could be determined. Since the mass of the radiochemical added was known, the amount present in the sample could be determined. The accuracy of this method depended upon the linearity of the splitter and upon a stable detector response. The "linearity" of annular splitters such as the H–P 402 standard splitter has been proven (12). The major source of error resides in a lowered detector response which could occur when crude samples are analyzed, leading to erroneously high values for the specific activity.

The isotope dilution method was most accurate if a dilution of at least 10-fold is obtained (11). Consequently two radioactive stocks were prepared for each amino acid, one of high specific activity, used undiluted to add radioactive tracer to the proteins, and another of sufficiently low specific activity to be measured by glc. The amounts and specific activities of the amino acids used are given in Table 1. Since the specific activity of the amino acid added to the protein and that of the standard used for glc calibration were different, a modification of the isotope dilution equation was used, with the terms having their above-described meanings and the multiplier a used to correct for specific activities:

$$Y = \left(\frac{aCo}{C} - 1\right) X.$$

Thus, a is a constant for each set of amino acid stocks and is the ratio of the specific activity of amino acid added to the protein divided by the specific activity of the amino acid used for glc calibration. Values for a are shown in Table 1.

Preparation of S-[^{14}C]Carboxyethylcysteine

A 2.5-ml aliquot of a 30 μCi/ml solution of cystine in 0.1 N HCl was dried under reduced pressure in a 13-ml conical centrifuge tube. A solution of 2 ml of 0.1 N NH_4HCO_3, pH 7.0, was added and the tube was sonically agitated for 10 min to dissolve the cystine. Cystine reduction was accomplished by addition of 50 μl of a 3.2% (v/v) aqueous solution of dithioerythritol. After 11 min of sonic agitation, 50 μl of a 7.0% (v/v) solu-

tion of acrylonitrile in water was added and agitation continued for 15 min. The mixture was shell frozen and lyophilized to dryness. The alkylated cysteine was then washed with 3 × 2 ml of diethyl ether to remove the dithioerythritol, and residual NH_4HCO_3 was removed by heating the tube to 80°C in a sand bath. After cooling, the contents were dissolved in 0.3 ml of 6 N HCl and transferred to a hydrolysis vial. The tube was washed with an additional 0.3 ml of 6 N HCl and this was added to the vial. The tube was evacuated to 15 μm, as described, and held at 110°C for 4 hr to hydrolyze the nitrile to the corresponding carboxylic acid.

Following hydrolysis, the *S*-carboxyethylcysteine in 6 N HCl was transferred to a conical centrifuge tube. The hydrolysis vial was washed twice with 0.7 ml of glass-distilled water and the washings were transferred to a conical centrifuge tube. The tube was dried *in vacuo*, 2 ml of glass-distilled water were added, and the tube again was dried to remove residual HCl. In a prior experiment, tlc on silica gel G (30:13:6, 1-butanol:acetic acid:water) showed contaminating radioactive and ninhydrin-positive spots. These contaminating materials were removed by the following procedure.

The *S*-carboxyethylcysteine was dissolved in 0.3 ml of water and applied, together with two 0.3-ml washes, to a 0.2-ml bed volume column of Dowex 1-acetate at a pH of approximately 5. The column was washed with 8.0 ml of glass-distilled water and the eluate was saved. A little of the *S*-carboxyethylcysteine as well as all of the contaminating ninhydrin-positive and radioactive compounds appear in this wash as judged by tlc. The *S*-carboxyethylcysteine was then eluted from the Dowex column with 10 ml of 1.0 N acetic acid and dried. A check of the column wash after concentration also showed the presence of *S*-carboxyethylcysteine. Accordingly, the concentrated wash was run over an identical Dowex 1 column. The 1 N acetic acid elutions were combined, dried, and taken up in 2.0 ml of 0.1 N HCl. Radiochemical purity was checked by tlc as before. Scraping the adsorbent from the plate and counting showed 87% of the radioactivity at the R_f of *S*-carboxyethylcysteine. The remaining counts tailed from that R_f. The overall yield was 40% as judged by counting an aliquot of the radiochemically pure *S*-carboxyethylcysteine. This low yield was probably due to the insolubility of cystine in NH_4HCO_3 at pH 7.0.

PROCEDURE

Twenty-five microliters of the protein stock solution were pipetted into 0.2 ml of distilled water in a 14-mm-diameter hydrolysis ampule. In the case of lysozyme, this aliquot, as determined by uv absorbance, contained 274 μg of lysozyme with 18.4 μg of cystine, 2.95 μg of histidine, and 23.4 μg of tryptophan. A 20% trichloroacetic acid solution (0.2 ml) was added and the tubes were centrifuged for 30 min at 1000 *g*. The super-

natant was removed, 2 ml of ether were added, and the contents of the tubes were mixed with a Vortex mixer. Following centrifugation, the diethyl ether was removed, and residual water and ether were evaporated at room temperature in a stream of nitrogen. Two milliliters of diethyl ether were added to the dry protein, and the contents of the tubes were mixed and centrifuged as before. The ether was removed, the protein was washed again with 2 ml of ether and dried in a stream of nitrogen to evaporate the ether. These washes removed the trichloroacetic acid from the protein. Next, 0.3 ml of 0.1 N NH_4HCO_3, pH 7.0, and 20 μl of a 3.2% (v/v) aqueous solution of dithioerythritol were added. Nitrogen was passed through the tubes. They were stoppered with No. 14 serum caps and shaken on a reciprocating shaker. After 35 min, 20 μl of a 7% (v/v) solution of acrylonitrile in water was added to the tubes, which were recapped and shaken an additional 25 min. The *S*-alkylation was stopped by adding 2.0 ml of hexane to the suspension, mixing briefly, and centrifuging. The hexane supernatant solution containing the acrylonitrile was removed and the hexane wash was repeated. The NH_4HCO_3 buffer and protein were taken to dryness at 60°C in a sand bath with a stream of nitrogen. Dithioerythritol causes interfering peaks on glc and was removed from the alkylated protein by 3 × 2-ml washes with diethyl ether. The tubes were placed in a 80°C sand bath to remove the remaining NH_4HCO_3. Residual NH_4HCO_3 was detected either by bubbling upon addition of the 6 N HCl or as needle-like crystals in the final acylation mix prior to glc.

Next, the labeled amino acids were added to the protein by first adding a small amount of water to the protein (0.1 ml) and then 10 μl of each of the standard labeled amino acids. This mixture was then dried at 50°C under a stream of N_2. The labeled amino acids, if dissolved in aqueous methanol, cannot be pipetted directly into the 6 N HCl because of volatile impurities (probably formaldehyde) which later condense with dithioethane and interfere with acylation.

A 0.5-ml aliquot of 6 N HCl and 25 μl of dithioethane (stench) were added to the protein. The outside of the tube was lubricated and inserted into the rubber tubing connected to the vacuum pump. The sample in the hydrolysis tube was next frozen in a dry ice–ethanol bath and evacuation was begun. When the vacuum gauge read 20 μm, the vial was allowed to thaw until a pressure of 70 to 100 μm was reached. The tube was refrozen and after two successive freeze–thaw cycles the frozen hydrolysis tube, at 20 μm pressure, was sealed with a flame. Hydrolysis was accomplished in a temperature-regulated heating block (Lab-Line Instruments, Melrose Park, Ill.) for 20 hr at 110°C.

At the end of the hydrolysis period, the tubes were allowed to cool, then centrifuged for 5 min to cause the immiscible dithioethane to form a globule in the bottom of the tube. The following procedure was con-

TABLE 2

REPRODUCIBILITY OF THE ISOTOPE DILUTION METHOD[a]

Material chromatographed	Peak area (1) (mg of paper)	Radioactivity (2) (cpm)	Ratio 2/1 (cpm/mg of paper)	Mean ± SD
Stock solution[b]	47.7	41,604	872	
	57.6	49,938	867	
	63.4	54,883	866	
	64.7	54,286	839	
	64.7	53,414	826	
	59.7	53,129	890	860 ± 23.2
Diluted stock solution I[c]	1,253	5,578	4.45	
	1,507	6,461	4.29	
	1,509	6,596	4.37	
	1,344	5,813	4.33	
	1,577	6,043	3.83*	
	1,532	6,681	4.36	4.36 ± .059[d]
Diluted stock Solution II[e]	98.4	1,856	18.9	
	160	2,885	18.0	
	136.6	2,377	17.4	17.5 ± 0.8
	154.2	2,609	16.9	
	129.8	2,193	16.9	
	142.6	2,431	17.0	

a As only 3.6 μg was derivatized from the stock solution[b] compared to 100.48 μg for the diluted stock solution I[c], recorder scales with 16-fold differences in sensitivity were used to record the peaks. The peaks on the less sensitive scale were then multiplied by 16.

[b] Solution as obtained from the supplier with a reported specific activity of 30 mCi/mmol.

[c] To 100 μg of unlabeled cystine was added 0.297 μCi of the above stock solution. This corresponds to 0.48 μg, assuming the manufacturer's specific activity to be correct.

[d] The mean and standard deviation were calculated without using the anomalously low value of 3.83.

[e] This was a working standard known from many previous assays to have a specific activity of 0.60 mCi/mmol.

* See footnote *d*.

ducted in a hood as the stench of even 20 μl of dithioethane is sufficient to cause complaints from adjacent laboratories. The top of the vial was broken and the 6 N HCl, avoiding the dithioethane, was pipetted into a 13-ml conical centrifuge tube with a ground glass joint. Glass-distilled water (0.7 ml) was added to the hydrolysis vial, and the contents were mixed and again centrifuged to sediment the dithioethane phase. The distilled water phase was transferred from the hydrolysis tube into the conical centrifuge tube. An additional 0.7 ml of glass-distilled water was added to the conical centrifuge tube, thus diluting it about threefold. It was then possible to remove the tube from the hood and take the conical centrifuge tubes to dryness on a Buchler Evapo-Mix at 60°C, using a heat gun or lamp to hasten evaporation from the tops of the connectors.

Following drying, 0.25 ml of glass-distilled water was added to the tubes, the contents were mixed and the water and a subsequent wash were transferred to 2-ml conical reaction vials (1). The 0.5 ml of distilled water and amino acids in the reaction vials were dried at 70°C with a stream of nitrogen in a sand bath. Next, 0.4 ml of a freshly (daily) prepared solution of acetyl chloride in methanol was added. The tubes were capped with a Teflon-faced culture tube cap, agitated sonically, and placed in a 70°C sand bath for 30 min. The tubes were cooled, then dried at 70°C with a stream of nitrogen, and 80 μl of acetic anhydride and 20 μl of triethylamine were added for acylation. The reaction mixture was capped, mixed, and placed in a sand bath at 100°C for 30 min. After cooling, the reaction mixture was reduced to dryness with a stream of nitrogen and, when almost dry, 30 μl of acetic anhydride were added. The reaction mixture was capped with a septum, mixed, and the *N*-acetyl-*O*-methyl esters of the amino acids were ready for glc.

Modifications of the procedure for denaturation, *S*-alkylation, hydrolysis, and glc must be made with caution. For example, ^{14}C-labeled cystine cannot be added prior to disulfide reduction and alkylation owing to the solubility of *S*-cyanoethylcysteine in ether. While the solubility was only 15 ng/ml, this was sufficient to yield an apparently greater dilution of the isotope. Attempts to remove dithioerythritol with an ether wash after hydrolysis contaminated the acid with peroxides, so that, upon taking the hydrolysate to dryness, the tryptophan was destroyed. Thus it was necessary to synthesize *S*-[^{14}C]carboxyethylcysteine and to add it just prior to hydrolysis.

We also attempted to modify the acylation conditions to avoid a brown color formed during acylation. This was done by heating the acylation mixture for longer times at lower temperatures, but it was found that when tryptophan was fully diacetylated, the reaction mixture was yellow in standards and darker brown for protein hydrolysates. In fact, when protein hydrolysates did not become brown during acylation for 30 min at 100°C, it indicated that either the K_2CO_3 or $CaSO_4$ was not anhydrous.

Acrylonitrile will also alkylate histidine (13) with prolonged heating at high pH, and thus the drying of the alkylation mixture and alkylated protein in NH_4HCO_3 buffer must be done cautiously. We compared lyophilized alkylation mixtures with a duplicate dried at 60°C with a stream of nitrogen. No differences were noted. The hexane wash following *S*-alkylation was not essential but it yielded cleaner chromatograms.

RESULTS

Accuracy and Reproducibility of the glc-Isotope Dilution Method

The precision of the method is shown by the data used to determine the specific activity of the ^{14}C-labeled cystine (Table 2). The manufacturer's reported specific activity of the stock solution was 30 μCi/

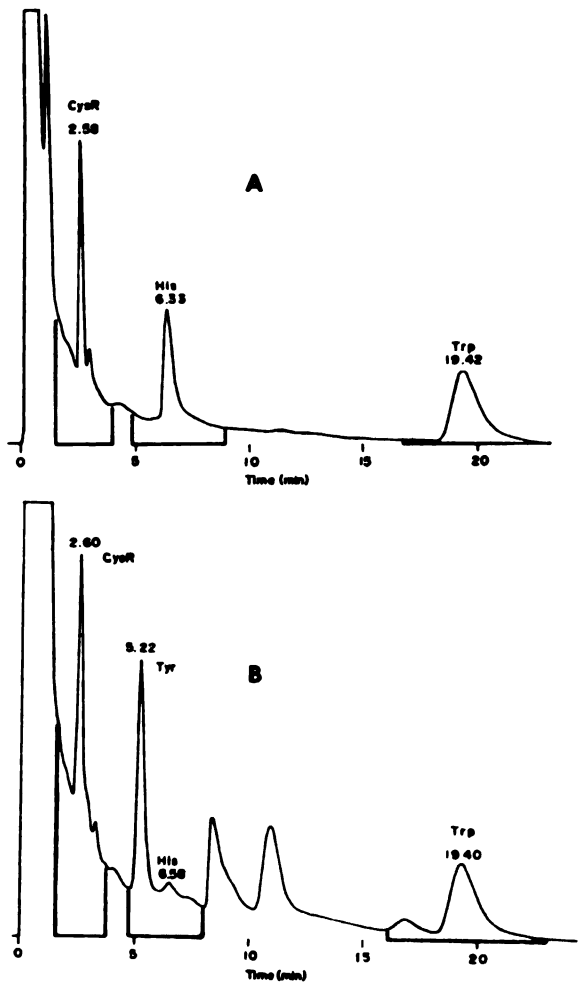

FIG. 1. Gas–liquid chromatography of approximately 0.7, 0.9, and 1.3 μg, respectively, of *S*-[^{14}C]carboxyethylcysteine, histidine, and tryptophan. (A) 1.07-m-long × 2-mm-i.d. glass column with 5% SP2401 on 100/120 AW-DMCS was used in combination with an annular splitter. The flow rates for nitrogen, hydrogen, and air were 60, 22, and 500 ml/min, respectively. Chromatography was done isothermally at 200°C, with the flash heater and detector both at 230°C. The collection of the peaks was done in "time windows" with the aid of a stopwatch. The bars under the peaks indicate the peak areas collected. The retention times in minutes are given above the peaks. (B) Gas–liquid chromatogram of an aliquot of the derivitized lysozyme hydrolysate. Approximately 36 μg total of amino acids were injected. All other conditions were as in (A).

μmol. After reduction, *S*-alkylation, and 6 N HCl hydrolysis, as would be done for an unknown, the resultant *S*-carboxyethylcysteine was chromatographed, using the effluent splitter, the peak was collected and counted, and the amount of *S*-carboxyethylcysteine (as peak area) was

determined. As seen in Table 2, the specific activity, in these arbitrary units, was 860 ± 23.3 counts/min per milligram of chart recorder paper. Next, 0.297 μCi of this stock, corresponding to 0.48 μg, assuming the manufacturer's reported specific activity to be correct, was diluted with 100 μg of unlabeled cystine. Derivatization and chromatography of the diluted stock yielded a specific activity, in arbitrary units, of 4.36 ± 0.059 cpm/mg of chart recorder paper (Table 2). The isotope dilution equation can be used to solve for X, the amount of labeled amino acid added,

$$X = \frac{Y}{(Co/C) - 1} = \frac{100}{(860/4.36) - 1} = 0.509\ \mu g,$$

and this corresponds to the value of 0.48 μg given by the manufacturer.

Next, another sample of [^{14}C]cystine, previously calibrated and known to have a specific activity of 0.60 μCi/μmol, was derivatized, chromatographed as above, and found to have an arbitrary specific activity of 17.5 cpm/mg of chart recorder paper (Table 2). The specific activity of the stock [^{14}C]cystine solution can be calculated as

$$(860/17.5) \times 0.60 = 29.5\ \mu Ci/\mu mol,$$

which again accords with the manufacturer's reported specific activity of 30 μCi/μmol. It should be noted that replicates for the values of Table 2 agree within 5% of the mean.

Chromatography of Amino Acid Standards

The glc recorder tracing for a standard mixture of *S*-carboxyethylcysteine, histidine, and tryptophan and for the corresponding acids from a hydrolysate of lysozyme are presented in Fig. 1. In a standard mixture, *S*-carboxyethylcysteine, histidine, and tryptophan are separated and yield well-formed peaks with a slight trailing edge. Since the chromatography was isothermal, *S*-carboxyethylcysteine yielded a sharp peak close to the solvent front, while the tryptophan peak was broader. If the monoacylated derivative of tryptophan was present, it would occur between histidine and tryptophan but closer to histidine. If the reaction mixture containing monoacylated tryptophan was held at room temperature, that peak would diminish in area, while the diacylated peak would increase.

The glc tracing of a lysozyme hydrolysate showed that most of the amino acids, other than the three being studied by this procedure, occurred in the solvent front. These of course, can be assayed by the procedure previously described (1). The exceptions are tyrosine, which occurs just prior to histidine, and a poorly shaped lysine peak. Between the lysine peak and tryptophan was a major peak which is not in the synthetic amino acid mixture of 17 amino acids. The very small peak immediately preceding tryptophan was perhaps a tryptophan acid hydrolysis degradation

TABLE 3

DETERMINATION AND CALCULATIONS OF CYSTEINE, HISTIDINE, AND TRYPTOPHAN IN LYSOZYME BY GAS-LIQUID CHROMATOGRAPHY ISOTOPE DILUTION

	Calibration standards				Lysozyme			
Amino acid[a]	(mg of paper)	(cpm)	(cpm/ mg of paper)	Average	(mg of paper)	(cpm)	(cpm/ mg of paper)	Average
S-carboxyethyl-cysteine	27.8	932	33.5		40.0	517	12.9	
	31.4	1187	37.8		50.8	691	13.6	
	29.6	1212	40.9		41.6	597	14.3	
	30.9	1252	40.5	38.2	47.7	718	15.1	14.0
Histidine	37.0	747	20.2		7.7	2434	316	
	44.7	1062	23.7		8.0	3034	380	
	42.5	1096	25.8		7.5	2763	368	
	35.8	1010	28.2	24.5	7.4	2542	343	352
Tryptophan	60.1	2116	35.2		54.1	1241	22.9	
	64.0	2253	35.2		66.9	1718	25.7	
	59.6	2209	37.1		56.7	1409	24.9	
	55.4	2126	38.4	36.5	58.9	1487	25.2	24.7

[a] By substituting the average values of C_0 and C in the isotope dilution equation and using the values for a, and X from Table 1 one can calculate:

		Theory	Yield (%)
Cysteine	$\left\lvert \frac{(49.1)(38.2)}{(14.0)} - 1 \right\rvert 0.129 = 17.2$	18.4	93.4
Histidine	$\left\lvert \frac{(1276)(24.5)}{(352)} - 1 \right\rvert 0.033 = 2.89$	2.95	97.9
Tryptophan	$\left\lvert \frac{(120)(36.5)}{(24.7)} - 1 \right\rvert 0.10 = 17.6$	23.4	75.4

product, although its specific activity was not as high as the main tryptophan peak. The bars underneath the peaks indicate where collection of the glc effluent took place and, as a general rule, collection was begun 1 min before the glc peak emerged and continued 1 min after the peak.

The data of Table 3 show the determinations of peak areas and radioactivity for the glc chromatogram of Fig. 1. The calculations in Table 3 show that cysteine, histidine, and tryptophan, respectively, were recovered with 93, 98, and 75% of theory. Theoretical values were obtained from the absorbance of unhydrolyzed protein at 280 nm and the calculation of the amount of amino acid that should be present in the hydrolysate. These percentages are not relative but are an absolute measurement of what should have been in the protein prior to hydrolysis. Histidine and cysteine are within the overall experimental error while tryptophan is substantially lower.

The replicates of Table 3 appear in the order in which they were chromatographed. Chromatography of the standard and of lysozyme were alternated beginning with the standard. This was necessary as the specific

TABLE 4

RELATIVE ABUNDANCE OF PROMINENT IONS IN THE 70-eV MASS FRAGMENTATION PATTERNS OF THE DIMETHYL ESTER OF ACETYLATED *S*-CARBOXYETHYLCYSTEINE

Ion	*m/e* (%)
M^+	263 (1.7)
$M-CH_3O$	232 (1.5)
$M-CO_2CH_3$	204 (66.0)
$M-CO_2CH_3-CH_2CO + H$	162 (7.9)
$M-2(CO_2CH_3)-CH_2CO$	102 (5.9)
$M-CH_2CO_2CH_3$	190 (12.2)
$M-(CH_2)_2CO_2CH_3$	176 (1.5)
$M-S(CH_2)_2CO_2CH_3$	144 (10.5)
$M-CH_2S(CH_2)_2CO_2CH_3$	130 (7.3)
$M-CHCH_2S(CH_2)_2CO_2CH_3$	117 (20.8)
$CH_2S(CH_2)_2CO_2CH_3$	133 (13.2)
$(CH_2)_2S(CH_2)_2$	88 (32.6)
$CH_2S(CH_2)_2$	74 (8.4)
CH_2CONH_2	59 (16.9)
CO_2CH_3	59 (16.9)
CH_2CO	43 (100.0)
$C_7H_8O_3S_1$	172 (48.1)
Unassigned	140 (10.8)
Unassigned	112 (23.0)

activity has a slow upward drift, particularly noticeable for protein hydrolysates. This upward drift was not due to better collection of radioactivity but to lowered detector response following repeated large protein hydrolysate injections.

Since the tryptophan value for lysozyme was low, it was decided to check free tryptophan recovery following acid hydrolysis. Thus 14.8 μg of tryptophan (as judged by the uv molar absorptivity) was added to a synthetic mixture of 10 μg of each of 17 amino acids. A value of 14.3 μg was found by the method reported here. When 14.8 μg of tryptophan was added directly to a glc reaction vial and chromatographed after derivatization, but without hydrolysis, a value of 14.4 μg was found. Thus the low tryptophan recovery from lysozyme hydrolysates was not due to losses during derivatization but, rather, to a preferential destruction of the peptidically linked tryptophan of lysozyme which was not accompanied by a compensatory destruction of the labeled free tryptophan added.

Mass Spectrometry

Gas–liquid chromatography of histidine, *S*-carboxyethylcystine, and tryptophan as the *N*-acetyl-*O*-methyl esters had not previously been

reported, and thus we wished to confirm the structures by mass spectrometry. Of particular interest were resolving the question of whether the imidazole nitrogen of histidine was acylated and establishing the nature of the two peaks resulting from incomplete derivatization of tryptophan. As will be shown, histidine existed as the diacyl derivative with an acyl group on the imidazole nitrogen as well as the α-amino group. Tryptophan may exist as both a monoacyl and diacyl derivative, having an acetyl group on the indole nitrogen as well as the α-amino group. However, using the derivatization process described in this paper, tryptophan existed solely as the diacyl derivative. The structure of our putative synthetic *S*-carboxyethylcysteine was also confirmed.

Mass fragmentation patterns of the *N*-acetyl-*O*-methyl esters of histidine, tryptophan, and *S*-carboxyethylcysteine were similar to the *N*-trifluoroacetyl-*O*-butyl esters (14) and the *N*-heptafluorobutyryl-*O*-isoamyl esters (1). Molecular ions were found for all derivatives studied here. Cleavage occurred between carbons 1 and 2 of the amino acid ester to lose the fragment CO_2CH_3 (*m/e* = 59) but the assignment was equivocal and could be CH_3CONH_2. Evidence from previous work with other derivatives (1,14) indicated that ester cleavage was favored over the CH_3CONH_2 loss. Cleavage also occurred at the amide linkage, resulting in loss of the acetyl group. Usually this loss occurs with hydrogen extraction, so that the net loss was 42 rather than 43. The loss of the acetyl group often occurs together with loss of the ester function to give a net loss of 101.

The acetylated dimethyl ester of *S*-carboxyethylcysteine yielded the ions shown in Table 4. Loss of the ester function was common, to yield an intense ion at *m/e* = 204. Ions resulting from loss of one ester and one acetyl function to yield *m/e* = 162 were seen, as were ions resulting from loss of both ester functions as well as the acetyl function (*m/e* = 102). Several small ions were present, which included the thioether linkage *m/e* = 74, 88, and 133. An intense ion occurred at *m/e* = 172 for which an exact assignment has not been made. Exact mass measurement of the *m/e* = 172 ion showed its mass to be 172.0193, corresponding to $C_7H_8O_3S_1$ with a calculated mass of 172.0193. Since nitrogen was not present in the *m/e* = 172 ion, cleavage must have occurred between the nitrogen and the α-carbon. If the nitrogen departed in the fragment $CH_3CONH_2(-59)$, this would have yielded an *m/e* = 204, which did occur and which had a relative intensity of 66%. The assignment of the *m/e* = 204 ion was equivocal, as this could have resulted from loss of the ester function. If, in conjunction with loss of CH_3CONH_2 ion, a methoxy ion was also lost (−31), this would yield an *m/e* = 173, and there is evidence for the methoxy loss as evidenced by the ion at *m/e* = 232. Loss of one hydrogen from the fragment resulting from a CH_3CONH_2 and the CH_3 loss would have yielded an *m/e* = 172 with an empirical formula of $C_7H_8O_3S$. That

TABLE 5

RELATIVE ABUNDANCE OF PROMINENT IONS IN THE 70-eV MASS FRAGMENTATION PATTERNS OF THE DIACETYLATED METHYL ESTER OF HISTIDINE

Ion	*m/e* (%)
M^+	253 (18.0)
$M—CH_3CO + H$	211 (16.0)
$M—CH_3O_2C$	194 (26.0)
$M—CH_3O_2C—CH_3CO + H$	152 (64.7)
$CH_3CONHCHCO_2CH_3$	130 (26.0)
$M—CH_3O_2C—2(CH_3CO) + 2H$	110 (50.0)
Im[a] + $CH_3CO—H$	110 (50.0)
Im + CH_2CH	95 (10.8)
Im + CH	81 (62.2)
Im + H	69 (15.0)
CH_3CO	43 (100.0)

[a] Im, imidazole, *m/e* = 68.

fragment could have existed as a linear or ring structure similar to that postulated by Gelpi *et al.* (14) for a cysteine-derived mass spectral ion.

The predominant ions of the 70-eV mass spectrum of the *N*-acetyl-*O*-methyl ester of histidine are shown in Table 5. The molecular ion as well as the molecular ion minus the acetyl and ester functions were prominent. The ion (*m/e* = 211) resulting from loss of an acetyl group from the molecular ion was present in histidine but was not present in either the tryptophan derivatives or in the *S*-carboxyethyl derivative. Acylimidazoles were very labile (15), and acylated histidine was the most difficult of amino acids to gas chromatograph, indicating that acetyl loss from the molecular ion was from the imidazole ring rather than the α-amino group. Of interest was the ion *m/e* = 110. This could have arisen from loss of both acetyl functions (−42) and loss of the ester function (−59) to yield *m/e* = 110, or it could have been assigned to the acylated imidazole ring. As the acylated imidazole ring was labile, we believe the *m/e* = 110 ion resulted from loss of both acetyl function and the ester function.

While Moodie (16) demonstrated an acylated histidine by direct probe, and Gelpi *et al.* (14) presented the gc–ms of the monoacylated histidine, there has been no prior gc–ms confirmation of a diacylated histidine structure. The gc–ms data presented in Table 5 show that the observed gc peak was diacylated.

The 70-eV mass spectra of the mono- and diacetylated methyl ester of tryptophan are given in Table 6. The spectrum of the diacetylated derivative was similar to that of the monoacetylated derivative. The molecular ions had similar intensities, as did the ions resulting from loss of the ester function, *m/e* = 201 and 243. As has been reported (1,14),

TABLE 6

RELATIVE ABUNDANCE OF PROMINENT IONS IN THE 70 eV MASS FRAGMENTATION PATTERNS OF MONO- AND DIACETYLATED METHYL ESTERS OF TRYPTOPHAN

Ion	Monoacyl tryptophan (*m/e* (%))	Diacyl tryptophan (*m/e* (%))
M^+	260 (5.2)	302 (6.0)
$M-CH_3O_2C$	201 (19.5)	243 (18.8)
$M-CH_3O_2C-CH_3CO + H$	159 (5.3)	201 (19.5)
$ArCOCH_3-H$	172 (0.0)	172 (10.7)
$Ar + CH_2NH$	159 (5.3)	159 (4.0)
Ar + CHNH	158 (2.8)	158 (2.0)
Ar + CH	143 (1.5)	143 (1.4)
Ar	130 (100.0)	130 (100.0)
Ar—HCN	103 (5.1)	103 (3.9)

tryptophan undergoes indole ring expansion using the methylene on carbon 3 to form the quinolinium ion. The quinolinium ion, *m/e* = 130, was base peak in both the mono- and diacetylated tryptophan mass spectra. In Table 6, the quinolinium ion is designated as Ar with *m/e* = 130.

The diacetylated tryptophan loses the acetyl group on the indole nitrogen but acetyl loss from the molecular ion was not observed. Loss of acetyl together with loss of ester were prominent in diacetylated tryptophan , 19.5% relative intensity versus 5.3% relative intensity for monoacetylated tryptophan. The loss of an acetyl group from the indole nitrogen was accompanied by H extraction from the acetyl to yield a loss of 42, rather than 43. Such H extractions have been demonstrated with trimethylsilyl derivatives of indoles using deuterated trimethylsilylating agents (Axel Ehmann, personal communication).

A characteristic ion for diacetylated tryptophan is *m/e* = 172, which is the acetylated quinolinium ion. Exact mass measurement of the *m/e* = 172 ion determined the mass to be 172.0764. The calculated mass for $C_{11}H_{10}NO$ is 172.0762, which is the empirical formula for the acetylated quinolinium ion. This ion was missing from the monoacetylated mass spectrum. The substituted quinolinium ion was not base peak as was the case with the perfluoroacyl amides (1,14). The acetyl amide should be more labile to ms conditions than the corresponding perfluoroacylated amide.

The uv spectra S-cyanoethylcysteine and *S*-carboxyethylcysteine have not previously been reported and are presented in Fig. 2 with cystine for comparison. The uv spectrum of *S*-carboxyethylcysteine is unusual in having a sharp peak at 256 nm and a shoulder at 251 nm. The *S*-cyanoethylcysteine uv spectrum had no corresponding peak. This 256-nm absorption in *S*-carboxyethylcysteine may have arisen from a cyclic structure in which the amino group cyclized with the carboxylic acid.

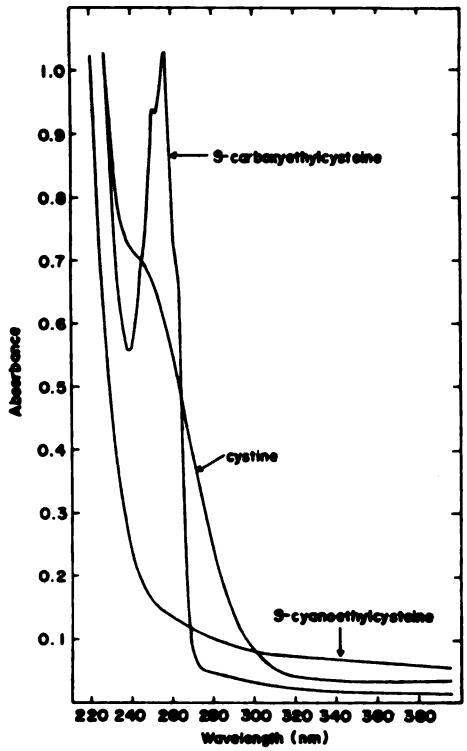

FIG. 2. Ultraviolet absorption spectra of *S*-carboxyethylcysteine, *S*-cyanoethylcysteine, and cystine. The *S*-carboxyethylcysteine and *S*-cyanoethylcysteine were dissolved in glass-distilled water at a concentration of 1 mg/ml. Cystine was used at a concentration of 0.5 mg/ml in water. The cystine concentration was achieved by adjusting the pH to 8.5 with NH_4OH. The uv spectra were measured and recorded with a Cary 15 spectrophotometer.

Evidence for this band occurred with glutathione with characteristic peaks at 268.5 and 261.0 nm in 12 N HCl (17), and these peaks have been ascribed to cyclic structures. In glutathione these absorption peaks occurred only in acidic solutions, while in *S*-carboxyethylcysteine the 256-nm peak occurs at pH 6 in distilled water.

DISCUSSION

A method has been developed for determining cysteine, histidine, and tryptophan in proteins using lysozyme as experimental material. This, together with previously published procedures for determination of the remaining 17 amino acids (1–3), made possible a glc assay for the 20 protein-associated amino acids. A number of procedures for determination of cysteine, histidine, and tryptophan was tested. For example, determination of cysteine as cysteic acid after performic oxidation of the protein

showed that as little as 20 μg of cysteic acid could have been derivatized using 50 μl of bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane at 150°C for 5 min. This derivative was not stable in a mixture of the other amino acids. As performic acid oxidizes tryptophan to *N*-formylkynurenine, tryptophan determination is precluded on performic acid-oxidized samples. Thus, a modification of Cavins and Friedman's (18) procedure for *S*-alkylation was adopted in which cystein was determined as *S*-carboxyethylcysteine. This procedure is compatible with histidine and tryptophan analyses.

Numerous methods for glc of histidine were attempted, including preparation of the trimethylsilyl derivative with chromatography on SP 2100, OV-17, or SP2401 and preparation and chromatography of the ethoxyformic anhydride derivative of the histidine methyl ester (16). The trimethylsilyl derivative was unsatisfactory since it yielded two fused peaks on glc whose stability decreased with increasing retention time. The ethoxyformic anhydride derivative of the methyl ester initially worked well, but this derivative was more sensitive to contamination in the injection port than the *N*-acetyl derivative.

Since aliphatic diamines can be chromatographed without derivatization on very basic columns (19), glc of histidinol on 1% Carbowax 20 M plus 0.25% KOH on 80/100 Gas Chrom A was attempted. Histidinol appeared, as a smaller peak than expected at 200°C on a 25-cm column with the above packing. Underivatized tryptamine gave a larger peak at about 160°C. Several workers have reported the efficacy of acetic anhydride as a chaser in the syringe for obtaining histidine as the perfluoroacyl anhydrides (2,3), so we attempted chromatography of the *N*-acetyl methyl ester of histidine. This was superior to other methods, particularly on a polar column such as OV-17 or SP 2401. An SP 2401 column was adopted since elution was at 20°C lower than OV-17.

The major difficulty with the method as here described was the 20 to 25% loss of peptidically linked tryptophan during acid hydrolysis. This loss does not seem attributable to incomplete hydrolysis or to uncompensated losses during derivatization but to the greater acid instability of peptidically linked tryptophan than free tryptophan, with the result that the isotope dilution method did not totally correct for losses of protein-derived tryptophan. The lability of tryptophan to acid is not totally understood. There are indications that ions such as Cu^{2+} and Fe^{3+} are responsible for the degradation in acid (20) but it has also been reported that aldehydes formed during hydrolysis react with the tryptophan (21). In a colorimetric assay for tryptophan described by Spies and Chambers (22) and based upon the Ehrlich reaction (23), *p*-dimethylaminobenzaldehyde was reacted with tryptophan under very strong acidic conditions in the presence of an oxidant. The colored product was complex and the mechanism appeared to be condensation of the aldehyde with carbon 2 of the indole

ring to form an acid-stable product (24). This would explain the effect of metal salts and aldehydes on tryptophan destruction but leaves unexplained the greater instability of peptidically linked tryptophan.

Subsequent to completion of most of these studies, we attempted to lower the redox potential of the hydrolysis solution to a point where aldehydes would not be formed to attack carbon 2 of the indole ring, and the oxidation of carbon 2 of the indole ring might have been prevented. The redox potential of alkyl thiols is −0.35 V at pH 7, but the potential is +0.03 V at pH 0. Thus we added 1 mg of tin metal (E^0 is −0.136 at pH 0) to the hydrolysis vial along with 25 μl of dithioethane. Immediately after placing the hydrolysis tube in the heating block, the tin metal was converted to stannous chloride and hydrogen gas. The stannous chloride was removed from the hydrolysis vial by taking advantage of the fact that dithioethane is a chelator of transition metals (25) at pH 2, while it does not chelate in 6 N HCl. Thus, after hydrolysis, the hydrolysate was removed from the dithioethane with a micropipet and taken to dryness several times to remove the bulk of the acid. A small volume of distilled water and 25 μl of dithioethane were added to the hydrolysate which caused the $SnCl_2$ to precipate. After centrifugation, the supernatant was taken to dryness, and the derivatives were prepared as usual. This procedure increased tryptophan recoveries to approximately 86% and eliminated the small peak on glc of the lysozyme hydrolysate immediately preceding tryptophan. This observation also lends credence to the fact carbonyl oxidation products may be responsible for tryptophan degradation.

Recently Nakai and Ohta (26) have reported that β-oxindolylalanine is the primary degradation product of tryptophan and that tryptophan degradation results from reaction of tryptophan with disulfides. Disulfides may well cause tryptophan degradation, but this cannot be the sole cause of tryptophan degradation, as carbohydrates also cause tryptophan degradation. Moreover, in our studies all thiols were alkylated prior to hydrolysis, yet degradation still occurred. The proposed structure for the tryptophan degradation product (26) suggested that the amino shift from the α-carbon on the tryptophan side chain to the β-carbon in the tryptophan degradation products occurred at the time of hydrolysis of the peptide bond, and this might explain the difference in lability between free and peptidically linked tryptophan.

We cannot rigorously exclude the possibility that the low tryptophan recovery was the result of incomplete hydrolysis, but (a) we found no significant differences in tryptophan recovery when the hydrolysis time was varied from 19 to 24 hr, (b) varying the dithioethane concentration from 10 to 50 μl per 0.5 ml of 6 N HCl had no effect on tryptophan recovery, and (c) increasing the number of freeze–thaw cycles for oxygen removal from one to five increased yield of tryptophan only about 5%.

An obvious solution to this difficulty would be the use of peptidically linked [^{14}C]-tryptophan rather than free [^{14}C]-tryptophan in the isotope dilution method.

A problem that merits further study is the varying difficulty of reducing disulfides to thiols. We found that dithioerythritol gives better reduction of TCA-denatured lysozyme than does mercaptoethanol but we have not studied other proteins.

The potential precision of the glc–isotope dilution method is great. The precision is probably limited by constancy of the flame ionization detector response. With improved detector stability, standard deviations of the order of 1% of the mean should be achieved. We have obtained improved stability by continuous operation of the detector.

In a protein hydrolysate, other amino acids interfered with the histidine derivative such that the histidine peak area was not always proportional to injection volume or other amino acid peak areas. Nonetheless, the isotope dilution method corrects for these losses. This indicates that the splitter must be on the detector side of the column and not on the injector side.

A modification of the method was found helpful when dealing with a low-cysteine protein, for example, soy protein which contains only 15% of the cysteine found in lysozyme. Hydrolysates of soy protein contained contaminating substances with retention times of 2.2 and 2.65 min at 200°C, which interfered with the methyl ester of *S*-carboxyethylcysteine having a rention time of 2.55 min. Preparation of the *n*-propyl esters, by the method identical to those described for the methyl esters, yielded peaks at 4.22, 5.40, 6.90, and 19.0 min at 210°C for derivatized *S*-carboxyethylcysteine, tyrosine, histidine, and tryptophan, respectively. The retention times of the unknown contaminants were 2.20 and 2.62 min and were thus easily eliminated. It was also found that substitution of chloroform:methanol (70:30, v/v) for the ether wash following *S*-alkylation gave improved recoveries of tryptophan from soy protein.

Despite the above-described difficulties, the potential accuracy, reliability, convenience, and rapidity warrant continued effort and development. As of now, a recovery correction must be made for tryptophan, but excellent recoveries of cystine and histidine are possible.

SUMMARY

A novel hydrolysis and gas–liquid chromatographic–isotope dilution amino acid analysis procedure is described which permits the determination of cysteine (cystine), histidine, and tryptophan in acid hydrolysates of protein. Acid hydrolysis is in 6 N HCl for 20 hr at 110°C in an inert atmosphere, using dithioethane as a protecting agent for tryptophan. Prior to hydrolysis, disulfides are reduced with dithioerythritol, and sulfhydryls

are alkylated with acrylonitrile. Labeled tryptophan, histidine, and *S*-carboxyethylcysteine are added, and the amino acids are methylated, *N*-acylated, and chromatographed on an SP-2401 column at 200°C. Recoveries of cysteine and histidine from lysozyme hydrolysates were, respectively, 93 and 98%, and tryptophan recovery was about 80%. Methods for improving tryptophan recovery are proposed.

The 70-eV mass spectral fragmentation patterns for the derivatized amino acids and the uv absorption spectrum for *S*-carboxyethylcysteine are presented.

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REFERENCES

1. Felker, P., and Bandurski, R. S. (1975) *Anal. Biochem.* **67**, 245.
2. MacKenzie, S. L., and Tenaschuk, D. (1974) *J. Chromatogr.* **97**, 19.
3. Moss, C. W., Lambert, M. A., and Diaz, F. J. (1971) *J. Chromatogr.* **60**, 134.
4. Matsubara, H., and Sasaki, R. M. (1969) *Biochem. Biophys. Res. Commun.* **35**, 1975.
5. Liu, T. Y., and Chang, Y. H. (1971) *J. Biol. Chem.* **246**, 2842.
6. Penke, B., Ferenczi, R., and Kovacs, K. (1974) *Anal. Biochem.* **60**, 45.
7. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70.
8. Sober, H. A. (1970) *in* Handbook of Biochemistry, Selected Data for Molecular Biology, p. C83. Chemical Rubber Co., Cleveland, Ohio.
9. Canfield, R. E. (1963) *J. Biol. Chem.* **238**, 2698.
10. Haupt, G. W. (1952) *J. Res. Nat. Bur. Stand.* **48**, 414.
11. Rittenberg, D., and Foster, G. L. (1940) *J. Biol. Chem.* **133**, 737.
12. Condon, R. D., and Ettre, L. S. (1968) *in* Instrumentation in Gas Chromatography. (Krugers, J. ed.) pp. 000–000, Centrex, Eindhoven, The Netherlands.
13. Bosshard, H. R., Jorgensen, K. H., and Humbel, R. E. (1969) *Eur. J. Biochem.* **9**, 353.
14. Gelpi, E., Koenig, W. A., Gibert, J., and Oro, J. (1969) *J. Chromatogr. Sci.* **7**, 604.
15. Bruce, T. C. (1963) *in* Methods in Enzymology (Colowick, S. P., and Kaplan, N. O., eds.), Vol. 6, p. 606, Academic Press, New York.
16. Moodie, I. M. (1974) *J. Chromatogr.* **99**, 495.
17. Isherwood, F. A. (1959) *in* Glutathione (Crook, E. M., ed.), p. 15, Cambridge University Press, Cambridge, England.
18. Cavins, J. F., and Friedman, M. (1968) *J. Biol. Chem.* **243**, 3357.
19. Amine Analysis, Bulletin 737 (1973) Supelco Inc., Supelco Park, Bellefonte, Pa.
20. Block, R. J., and Bolling, D. (1945) *in* The Amino Acid Composition of Proteins and Foods, p. 82, C. C. Thomas, Springfield, Ill.
21. Mitchell, H. H., and Hamilton, T. S. (1929) *in* The Biochemistry of the Amino Acids, ACS Monograph No. 48, p. 103, Chemical Catalog.
22. Spies, J. R., and Chambers, D. C. (1948) *Anal. Chem.* **20**, 30.

23. Ehrlich, P. (1901) *Med. Wochensch.* **151.**
24. Remers, W. A., and Brown, R. K. (1972) *in* The Chemistry of Heterocyclic Compounds, Indoles Part One (Houlihan, W. J., ed.), Vol. 25, p. 105, Wiley–Interscience, N. Y.
25. Harris, C. M., and Livingstone, S. E. (1964) *in* Chelating Agents and Metal Chelates, (Dwyer, F. P., and Mellor, D. P., eds.) Academic Press, New York.
26. Nakai, T., and Ohta, T. (1976) *Biochim. Biophys. Acta* **420**, 258.

EXPERIMENTAL III

A Colorimetric Micro-Kjeldahl Determination[1,2]

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Received:

Running title:

MICRO-KJELDAHL DETERMINATION

With the advent of new protein and amino acid micro-techniques such as fluorimetry, glc, and electrophoresis it has become desirable to develop a Kjeldahl assay of comparable sensitivity. A colorimetric technique useful in the microgram range for ammonia determination using the salicylate-dichloroisocyanurate reagent has been described (1), but with inadequate description of the reagent concentrations employed. Later papers reported on the automation of this ammonia assay using a Technicon "Auto Analyzer" (2, 3). We wish to report a modification of the salicylate-dichloroisocyanurate ammonia determination that can be performed on a Kjeldahl digest. The useful range of this assay is from 0.5 to 5 ug total N in the sample.

Methods

Up to 100 ul of nitrogen containing solution is pipetted into the digestion flasks (5136-G22 freeze drying ampoules, A. H. Thomas Co., Phila.) as well as 100 ul of concentrated H_2SO_4. The contents are mixed on a vortex mixer and the digestion flasks are then placed on a cold micro Kjeldahl digestion apparatus and heating begun. Bumping may occur on a preheated apparatus. Digestion is for only 10 minutes from the time fumes first appear in the flasks, since longer heating times with short neck flasks causes loss of NH_3. A longer neck flask might be preferable.

The flasks are allowed to cool and then 1.0 ml of water is added followed by 2.0 ml of 1.0 M phosphate buffer pH 12.5. The flasks are then stoppered with serum cap stoppers (8753-D22, A. H. Thomas Co., Phila.) as the next step will cause the pH to rise above the pk_a of NH_3 making it volatile. A 0.5 ml solution of 30% NaOH is then added through

the serum cap with a 1.0 ml plastic tuberculin syringe equipped with a 3 inch-22 gauge needle. After allowing the flask to cool 0.4 ml of the salicylate reagent followed by 0.3 ml of the dichloroisocyanuric acid reagent are added with minimal mixing in quick succession with the aid of two prefilled tuberculin syringes. The flasks are rotated by hand to mix the contents and are then placed in a reciprocating metabolic shaker in water at 37°C for 20 min. After cooling the absorbances are determined at 660 nm against a reagent blank.

REAGENTS

Salicylate Acid reagent. This contains 2.12 gm of sodium salicylate and 15 mg of nitroprusside in 25 ml of doubly distilled H_2O. The dichloroisocyanurate reagent contains 50 mg of sodium dichloroisocyanurate (Eastman Chemical Co.) in 10 ml of 1 molal sodium phosphate buffer pH 12.5. The pH 12.5 phosphate buffer is prepared by mixing 84 gm of Na_2HPO_4 with 152 gm of $Na_3PO_4.12H_2O$ per liter and adjusting to pH 12.5 with NaOH. Indicator paper is used to check the pH of the phosphate buffer solution since there would be appreciable error with a glass electrode at pH 12 with 1 M sodium.

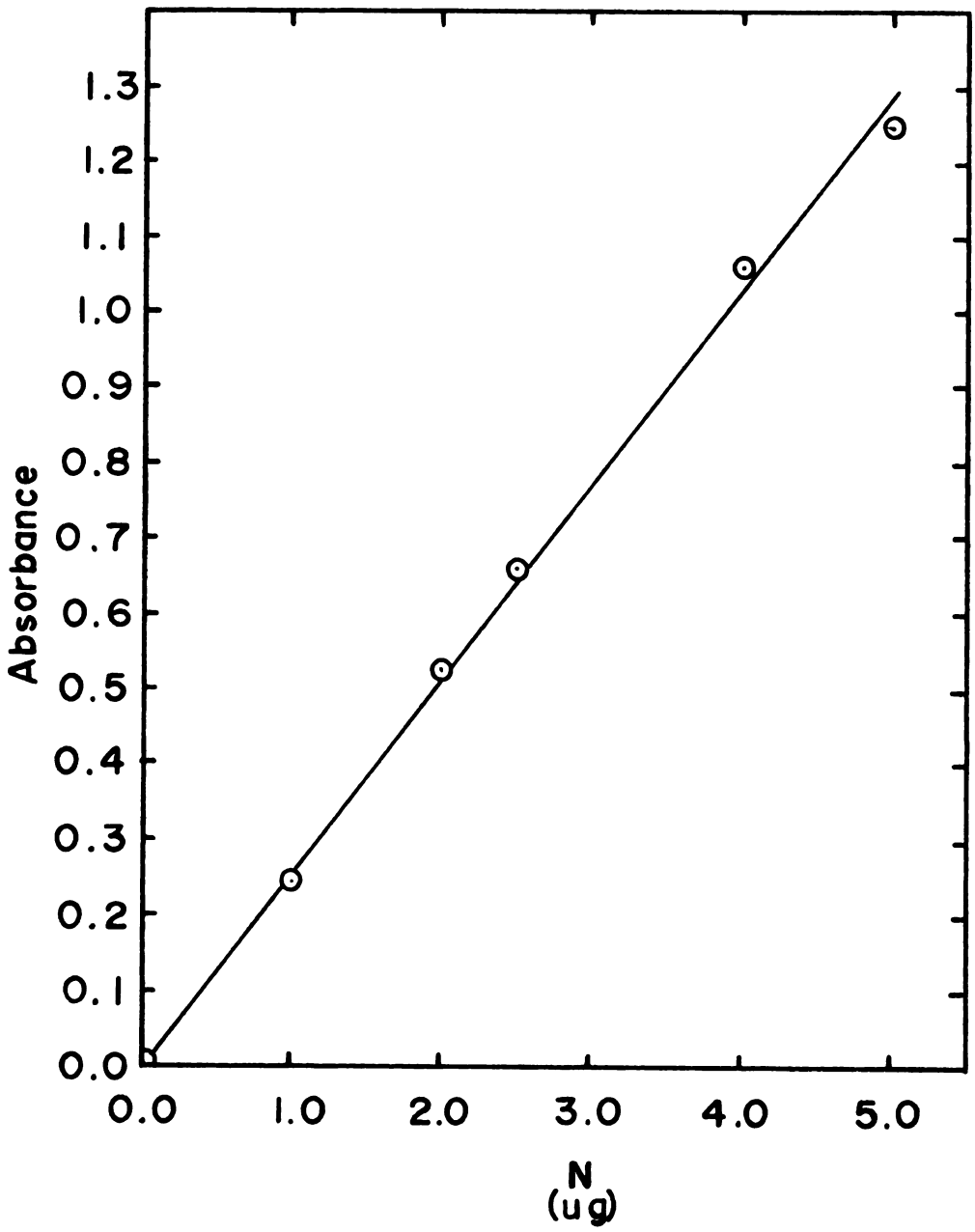
Results and Discussion

As Fig. 1 shows the assay to be linear and several fold more sensitive than the automated Kjeldahl assays (2,3). The increased sensitivity is due to less dilution of the digest by the reagents. Different concentrations and proportions of the salicylate and dichloroisocyanurate reagents were tried before the present procedure was adopted. As color development has been shown to be pH dependent, reaching a maximum at pH 12.5 (3), care should be taken in pipetting the

NaOH, H_2SO_4, and phosphate buffer, as the volumes and concentrations have been chosen to stoichiometrically neutralize and buffer the H_2SO_4 acid digest. It is also our experience that the salicylate and dichloroisocyanurate reagents should be made fresh daily. This is especially true for the dichloroisocyanurate reagent. When a 1 ml H_2O dilution of the digest was not used, the final colored solution often had a mass of precipating crystals in it. The initial 1.0 ml water dilution prevented this precipitation. Accordingly, it should be possible to dilute the phosphate buffer (which is near saturation) and use more of it, while adding the same buffer equivalents.

As the upper limit of the assay is approximately 30 μg of protein, an amount which is difficult to weigh, the assay is most useful in analyzing the nitrogen content of fluids.

Fig. 1. Standard curve for colorimetric Kjeldahl nitrogen determination. A glycine solution equivalent to 0, 1, 2, 2.5, 4, and 5 ug N was pipetted into the digestion flasks and the micro Kjeldahl N determination was conducted as described in the text.


Absorbance
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.0
1.0
2.0
3.0
4.0
5.0
N
(ug)

References

1. Reardon, J., Foreman, J. A., and Searcy, R. L. (1966) Clin. Chim. Acta. 14, 405.

2. Crooke, W. M., and Simpson, W. E. (1971) J. Sci. Fd. Agric. 22, 9.

3. Bietz, J. A. (1974) Anal. Chem. 46, 11, 1617.

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EXPERIMENTAL IV

Protein and Amino Acid Composition of Tree Legume Seeds

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ABSTRACT

Pods and seeds of tree legumes are widely used as food in developing countries since tree legumes can be grown with minimal inputs of capital and energy (1). The data presented here show that the protein content of the seed ranges from 16 to 69%, and methionine, cystine, and tryptophan content ranges from 44 to 121 mg/gN, 14 to 156 mg/gN, and 40 to 109 mg/gN respectively. The chemical score of the protein from Pithecellobium lobatum was 0.79 which compared to 0.58 and 0.47 for casein and soy protein respectively.

Introduction

Leguminous trees have been suggested as food sources since their production would require minimal inputs of capital, fuel, and energy (1). In this study we present data on the protein, and amino acid composition of the seeds of a number of leguminous trees that are currently being used as human food or for livestock. We have found variations in protein content of the seed ranging from 16 to 69%. Further, we have found wide variations in essential amino acid composition particularly methionine. This great variability in protein content and in amino acid composition indicates that there is sufficient genetic diversity in the legume family so that a breeding and selection program could greatly enhance the food value of tree legumes.

Experimental

The seeds for protein and amino acid analysis were obtained from several sources and we have used the botanical name provided by the collector. Parkia clappertoniana, P. roxburghii, Pithecellobium sonorae, P. saman, P. dulce, Prosopis juliflora, P. stephaniana, Leucaena leucocephala and Gleditsia triacanthos were obtained from the USDA, Northern Regional Research Laboratory, Peoria, Illinois. Parkia javanica, Pithecellobium lobatum, Acacia auriculaeformis, and Leucaena glauca were collected in Indonesia by Dr. Peter Murphy, Michigan State University. Acacia moniliformis and Prosopis chilensis were obtained through Mr. Howard Hyland, USDA, Northeastern Laboratory, Beltsville, MD, from Argentinean collectors.

In most cases it was necessary to remove the seed from its coat (endocarp). The seeds were crushed in a hydraulic press, wrapped in

miracloth and extracted in a soxhlet distillation apparatus for 24 hr. Following extraction the embryo could manually be removed from the endocarp. The seed embryo, which in the case of legumes is virtually the entire seed, was ground sufficiently fine to pass a 40 mesh sieve.

Since carbohydrates increase the destruction of hydroxy-amino acids during acid hydrolysis (2), we prepared a low carbohydrate crude protein fraction. The preparation of the protein fraction for analysis of amino acids, other than tryptophan, cystine and histidine, was performed as previously described (3). The protein extraction technique used for Cys, His, and Trp was as follows: A sample estimated to contain at least 20 mg of protein was homogenized in a Teflon-pestle tissue grinder in approximately 6 ml of 0.05 M sodium pyrophosphate pH 8.2, 0.4 M NaCl, and 0.005 M dithioerythritol. After grinding for 5 min, the suspension was centrifuged at 1,200 x g for 4 min. The supernatant fluid was transferred to a 30 ml Corex tube, and the pellet extracted twice more. This procedure is an improvement of that previously described (3).

The combined supernatent volume from the homogenate was measured, and an aliquot taken for Kjeldahl analysis. After addition and mixing of 3 g of solid trichloroacetic acid (TCA), the Corex tube was placed in a 95°C water bath for 3 min. The tube was then centrifuged at 15,000 X g for 3 min. The volume was measured and an aliquot was taken to measure TCA soluble nitrogen. The supernatant fluid was then discarded and the pellet was resuspended in 5 ml of 10% TCA. The resuspended pellet was sedimented at 15,000 X g for 3 min. The supernatant fluid was discarded and the 10% TCA wash, which is designed to remove NaCl, repeated once more. The TCA was then removed from the pellet by

resuspending in 5 ml of chloroform:methanol 28:72 (density of 1) and centrifuging at 15,000 X g for 3 min. The chloroform-methanol wash was repeated once more and the pellet dried in vacuo. (Peroxide contaminants in the previously described ether wash (3) may oxidize cystine and tryptophan.)

Protein hydrolysis for amino acids other than Cys, His, and Trp was as previously described (3) using approximately 5 mg of protein and 1.0 ml of 6 N HCl. Prior to hydrolysis 200 μg of norleucine was added as an internal standard. After hydrolysis, duplicate aliquots of 0.4 ml were taken for Kjeldahl analysis and duplicate aliquots of 20 μl were derivatized for GLC. The Kjeldahl analyses were performed as previously described (4).

For GLC analysis, duplicate, and alternating injections were made from each of the two derivitizations so there were a total of 4 replicates per hydrolysate. A standard amino acid mixture was also derivatized and analyzed immediately before and after the unknowns so that relative weight responses (RWR) (3), could be established. This method of cutting out and weighing peaks on the recorder chart paper must be used if an integrating recorder capable of measuring asymmetric peaks is not available. The amino acid composition was then calculated from the average of the four replicates, the RWR coefficients, and the Kjeldahl analyses.

Determination of cysteine, histidine and tryptophan was as previously described (5) following S-alkylation of protein prepared as described above. Nitrogen determinations could not be made on the hydrolysate, owing to the residual ammonium bicarbonate buffer. Thus, Kjeldahl analysis were performed on the dried protein. The protein (400-600 μg)

was weighed on a Cahn electrobalance and transferred to hydrolysis vials. N-propanol (50 µl) was then added to promote wetting by the NH_4HCO_3 buffer. Analyses of Trp, Cys, and His were performed in triplicate using isotope dilution of the n-propyl esters as previously described (5). Column contamination was eliminated before each analysis by a 10 µl injection of acetic anhydride and a sufficient delay to permit emergence of histidine-like contaminants.

Nitrogen determinations were performed by a colorimetric micro-Kjeldahl assay employing the dichloroisocyanurate-salicylate reagent (submitted for publication).

Results

Since the nitrogen content of the seed powder and of the buffer extract were known, the percentage of nitrogen extracted by the buffer was calculated. These data are presented in Table 1. It can be seen that, with one exception, at least 70% of the nitrogen was extracted by the procedure described. The nitrogen in the supernatant fluid after TCA precipitation and centrifugation was also measured and an appreciable amount of the extracted nitrogen was not TCA precipitable (Table 1). This was unexpected since the conditions described will precipitate greater than 90% of the extractable nitrogen from soybeans. It was of interest to know whether the nitrogen not precipitable by TCA consisted of amino acids and alkaloids or of protein. Glycosylated proteins are TCA soluble (6). Consequently, the TCA supernatant fluid from the pods of Prosopis juliflora and from the seeds of Leucaena leucocephala were chosen for a nitrogen distribution study as these both had a large percentage of TCA soluble nitrogen. From Table 2 it can be seen that

approximately 20% of the TCA soluble nitrogen was non dialyzable. In a preliminary experiment sugar and amino acid analysis of a hydrolysate of non-dialyzable material showed the presence of arabinose, galactose and galacturonic acids, as well as hydroxyproline and a number of other amino acids, suggesting that the non-dialyzable material contained glycoproteins. The presence of much more ammonia in the hydrolysate then could be accounted for as amides of glutamic and aspartic acid is reminescent of other legume glycoproteins (7). The TCA precipitable substance was 62% protein by dry weight as an average for all the samples. The remainder presumably was polysaccharide, but this was not studied.

GLC chromatographic profiles of Cys, His, and Trp in a hydrolysate of legume protein are shown in Fig. 1. This profile of Prosopis chilensis protein is similar to that of lysozyme (5) except the legume amino acids were chromatographed as the n-propyl esters instead of the methyl esters. The propyl esters are preferable since the two peaks preceding S-carboxyethyl cysteine (CySR) are separated from CySR, while they are not with the methyl esters. It should be noted that, with the exception of the other amino acids on the chromatogram, Tyr, Lys, and Arg, the chromatogram is free of contaminating peaks.

As can be seen from Fig. 1 a coincidence of retention times between the standard amino acids, and the corresponding amino acids from a protein hydrolysate was not observed. The protein hydrolysate amino acids tended to emerge later than standards. The retention time offset was not a constant but was roughly proportional to retention time. This

difference in retention time could be due to the 10 fold more material injected for the protein hydrolysate assay than for the standard amino acid mixture.

The results of the protein and amino acid composition determinations are presented in Table 3. The protein values were from Kjeldahl analyses of the Soxhlet-extracted seed without the stony endocarp. The fact that the protein content varies from 16 to 69% indicates sufficient genetic variability to encourage a breeding and selection program. In general the larger seeds seemed to have a lower protein content than the smaller ones.

The values for the seed protein amino acid analysis are compared with the FAO provisional values in Table 3. It is apparent that the majority of the legumes are low in the sulphur amino acids-methionine and cysteine, as well as in tryptophan. The single exception is the protein from Pithecellobium lobatum the Djenkol bean (8). While the Djenkol bean is about 15% low in methionine, it has a much higher level of cysteine than do the remainder of the seed proteins. Since cysteine can substitute for a portion of the methionine requirement, the large amount of cysteine in the Djenkol bean could perhaps overcome the methionine deficiency. Again, this variability in amino acid composition indicates that there is sufficient genetic variability in the family to permit improvement by selection and breeding.

The amino acid composition data presented here for several Parkia species (African locust bean) are in fair agreement with that of Fetuga et al. (9) and Busson (10). The amino acid composition previously

reported for Prosopis, known as mesquite or algaroba (11) are in good agreement with our data except for the hydrophobic amino acids. In the earlier study the acid hydrolysis of the Prosopis protein was for only 4 hr (11), and this would explain their lower values for the hydrophobic amino acids. Hydrophobic residues are sometimes not hydrolyzed until 72 hr (12), and increasing the 20 hr hydrolysis time used here might increase the yield of the essential amino acids valine, and isoleucine in Prosopis seed. The amino acid composition of the protein from Prosopis pods is better than that of the seed. However, in terms of nutrition, it must be remembered that: the total N in the pod is, on a weight basis, an order of magnitude lower than that in the seed; the extractability of nitrogen from the pod is lower than that of the seed; the percent of dialyzable buffer-extractable nitrogen is several fold higher in the pod than the seed; and an accurate analysis of pod protein is more difficult owing to the problem of measuring small peak areas. The low yields of TCA precipitable nitrogen is not unique to Prosopis pods as the pods of Acacia moniliformis and the pulp of Pithecellobium dulce yielded no discernible TCA precipitate.

Difficulties were encountered with the GLC analyses for methionine. Sometimes the methionine peak area on GLC from the protein hydrolysate declined with successive injections over a 6 hour period. During this same period the peak area for methionine in standards did not decline relative to the other amino acids. In such case the highest and first peak area is used to calculate the methionine values and these values are listed in parenthesis in Table 3. Perhaps the methionine antioxidant of March (13) could have prevented this destruction.

In terms of repeatability, the standard deviation, as percent of the mean, for all the amino acids minus Cys, His and Trp averaged 6% for four runs done on the same day.

Discussion

Data are presented on the protein and amino acid composition of the major seed proteins of tree legume seeds. The seeds chosen for analyses have been, or are presently used, as food for humans or livestock in some area of the world (1). Since the use of these trees is mainly in poor, developing countries there is a paucity of biochemical and nutritional data on these seeds. The results reported here are in good agreement with the protein (14) and amino acid content of Prosopis (11) and the amino acid (10) and nutritional data (9) of Parkia species. Amino acid composition data of the seeds of Acacia, Gleditsia, Pithecellobium and Leucaena have not, to our knowledge, been reported.

That these seeds came from plants of unknown nutritional status may be important. Since sulphate is a limiting nutrient on some soils where legumes are grown (15), and since sulfate deficient legumes have been reported to have lowered levels of methionine, cysteine, and tryptophan rich proteins (16), it is possible that the low levels of cysteine, methionine and tryptophan we have observed are the results of the nutritional rather than genetic status of the plant.

The data presented here show that there is a great deal of diversity among the tree legumes. For instance, the protein content ranges from 16 to 69% of dry weight, and the methionine, cysteine and tryptophan content respectively range from 44 to 121 mg/gN, 14 to 156 mg/gN, and 40 to 109 mg/gN. The diversity we have found is in a very small sample

of potential breeding stock. For example, only 2 of a possible 20 species of Prosopis and one out of 90 varieties of Leucaena leucocephala have been analyzed.

The use of tree legumes as food or fodder is not without problems. For instance Leucaena contains an alkaloid, mimosine, which is a depilatory if eaten in excess (17) although ferrous sulphate has been shown to counteract the depilatory action in pigs (18). The Djenkol beans (Pithecellobium lobatum), although a prized Indonesian food (8) will cause cystitis if eaten in excess. The causative agent is thought to be an oil (8). If cattle are given a diet consisting solely of Prosopis pods they may die with a ball of compacted pods and unbroken seeds in their rumen (19,20). Perhaps if the Prosopis seeds which contain 69% of the protein in the pod (21) and are indigestible because of the seed coat, were broken prior to feeding, sufficient nitrogenous substances would be released into the rumen to allow digestion of the fibrous pods. Pig feeding trials, with ground mesquite pods as 50% of the ration, show no toxicity (21).

Although the number of species analyzed in this work is small some new data emerge. First, a seed with 69% protein in the seed embryo is promising since this protein content is as high as any reported in 759 species in a systematic sampling of the plant kingdom (22). Second, the protein from Pithecellobium lobatum has an amino acid composition that should make it much more nutritious than the average legume or cereal. For instance the chemical scores, derived by comparison with hen's egg

protein (23), are 0.79, 0.58, and 0.47 for P. lobatum protein, casein, and soy protein respectively. It seems obvious that intensive studies, including for example, feeding studies of P. lobatum protein, should be made with this extraordinary group of plants.

Literature Cited

1. Felker, P., and Bandurski, R. S. 1976 Manuscript submitted for publication

2. Roach, D. & Gehrke, C. W. 1970 J. Chromatog. 52, 393.

3. Felker, P. & Bandurski, R. S. 1975 Anal. Biochem. 67, 245.

4. Ma, J. A., & Zuazaga, G. 1942 Indus. Eng. Chem. 14, 280.

5. Felker, P. 1976 Anal. Biochem. in press.

6. Jansen, E. F., Jang, R., & Balls, A. K. 1952 J. Biol. Chem. 196, 247.

7. Brown, R. G., Kimmins, W. C., & Lindberg, B 1975 Acta. Chem. Scand. B29, 843.

8. Ochse, J. J. in "Vegetables of the Dutch East Indies" 1931 p. 426. Archipel Drukkerij Buitenzorg Publishers Java

9. Fetuga, B. L., Babatunde, G. M. & Oyenuga V. A. 1974 Br. J. Nutr. 32, 27.

10. Busson, F., Jaeger, P., Lunven, P., & Pinta, M. in "Plantes Alimentaires de l'Ouest Africain" 1965 p. 282 Inter-Agency Publication (Ministere de la Cooperation, Ministere d'Etat charge de la Recherche Scientifique et Technique, Ministere des Armees) Marseille France

11. Figueiredo, A. A. "Lebensmittelchemische relevante Inhaltstoffe der Schoten der Algarobeira" Ph.D. Thesis, Wurzburg, Germany.

12. Moore, S. "The Precision and sensitivity of Amino Acid Analysis" 1972, 629 in Chemistry and Biology of Peptides (Meienhofer, J., ed.) Ann Arbor Sci., Ann Arbor, Mich.

13. March, J. F. Anal. Biochem. 69, 420.

14. Walton, G. P. 1923 USDA Dept. Bulletin No. 1194, 1.

15. Martin, W. E. 1958 Calif. Agric. 10

16. Blagrove, R. J., Gillespie, J. M., & Randall, P. J. 1976 Aust. J. Plant Physiol. 3, 173

17. Brewbaker, J. L. & Hylin, J. W. 1965 Crop Sci. 5, 348

18. Malynicz, G. 1974 Agr. J. 25, 12

19. Alder, H. E. 1949 J. Amer. Vet. Med. Ass. 115, 263.

20. Hendershot, J. M. 1946 Amer. Ved. Med. Assn. J. 108, 74

21. Garcia, F. 1916 N. Mex. Agr. Exp. Sta. 28th Ann. Rpt. 77

22. Jones, Q., & Earle, F. R. 1966 Econ. Bot. 20, 127

23. Autret, M. (1972) in "Amino-Acid Content of Foods and Biological Data on Proteins". FAO Nutr. Stud. No. 24, p. 57 and 133.

Table 1

Extractability and TCA precipitability of legume seed proteins

	N in seed powder extractable by buffer (%)	Extracted N not precipitable by TCA (%)
Parkia clappertoniana	28	38
roxburghii	91	33
javanica	84	23
Pithecellobium sonorae	106	36
saman	81	50
dulce	97	43
lobatum	65	50
Acacia moniliformis	91	27
auriculaeformis	75	36
Prosopis chilensis	85	16
juliflora (seeds)	79	22
juliflora (pods)	70	73
Leucaena leucocephala	106	42
glauca	69	16
Gleditsia triacanthos	86	16
Glycine max	90	9

Table 2

Distribution of Trichloroacetic acid soluble nitrogen after dialysis.

	Prosopis juliflora		Leucaena leucocephala	
	N (mg)	%	N (mg)	%
Hexane extracted meal	3.2	100	3.8	100
Combined buffer extract	2.2	69	3.9	103
Hot TCA soluble	2.1	64	1.5	40
Not dialyzable	.42	13	.29	7.7
Dialyzable	1.5	47	1.2	31.

Table Legends

Table 1 The fact that two of the values for % N are greater than 100 % is experimental error. The final TCA concentration was approximately 15 %.

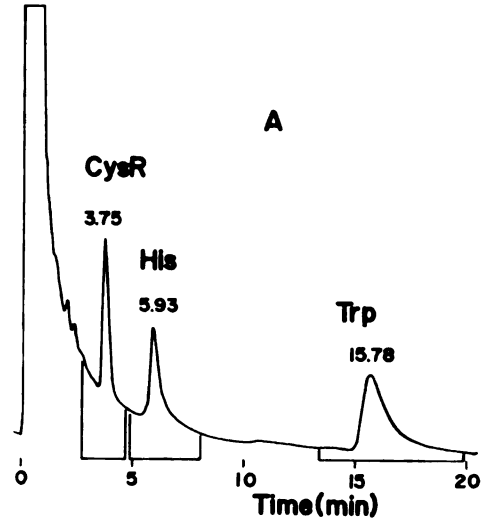
Table 2 Hexane-extracted 40 mesh powder from Prosopis juliflora pods without the seeds and Leucaena leucocephala seeds were used for this study. Dialysis was done three times, 24 hrs each time, against 20 volumes of glass distilled water.

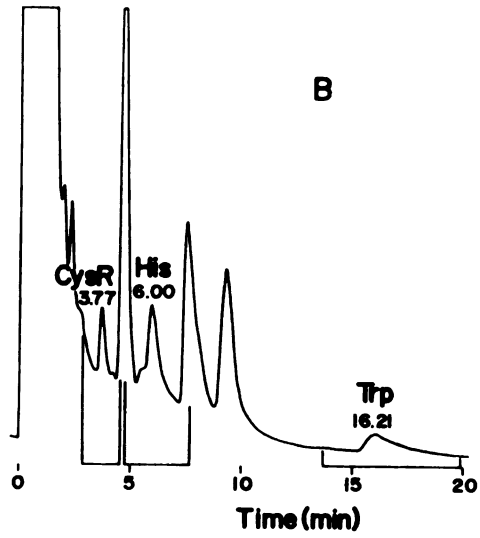
Table 3 Insufficient material was available for the tryptophan, cystine, and histidine analysis of Prosopis stephaniana. Protein was calculated for the defatted seed minus seed coat as N x 6.25. Amino acid values are the average of four replicates from the same hydrolysate. If the methionine value declined in time with each replicate the first value is listed in parenthesis.

Table 3
Protein and Amino Acid composition of Tree legume seeds.
(mg/gN)

	Prot. (%)	ala	gly	val	thr	ser	leu	ile	pro	met	phe	asp	lys	tyr	glu	arg	trp	cys	his
Acacia auriculaeformis	38	307	296	300	224	391	648	308	378	74 (90)	335	764	551	357	1144	496	46	35	164
Acacia moniliformis	55	320	236	258	205	333	584	270	410	50	242	687	311	354	894	556	56	49	115
Gleditsia triacanthos	58	297	309	321	207	325	450	271	285	64 (100)	242	608	371	243	1453	631	51	88	123
Leucaena glauca	39	317	297	305	209	332	540	289	310	77	321	707	385	273	1167	563	86	28	184
Leucaena leucocephala	46	295	286	263	226	367	484	266	319	59	314	652	377	298	1095	631	62	34	161
Parkia clappertoniana	16	291	283	294	236	417	585	340	339	95	351	943	500	404	1121	284	53	92	93
Parkia javanica	44	318	296	324	201	327	522	263	315	44 (55)	325	613	460	298	1161	366	40	19	150
Parkia roxburghii	41	303	276	288	170	297	494	239	299	50 (71)	307	568	385	278	1088	356	43	22	127
Pithecellobium saman	38	333	286	270	253	392	607	266	363	81 (97)	300	673	493	357	808	400	73	53	136
Pithecellobium sonorae	44	305	292	197	231	370	704	245	391	82	312	780	618	374	1150	500	66	49	154
Pithecellobium dulce	28	351	291	296	289	352	607	308	369	121 (146)	321	709	519	342	888	441	81	27	157
Pithecellobium lobatum	16	321	286	384	279	394	538	321	398	121	285	669	335	300	618	167	109	156	176
Prosopis chilensis	69	278	233	242	152	255	432	221	350	47	247	475	240	208	1041	645	77	62	163
Prosopis juliflora (seeds)	60	274	249	203	130	254	441	200	363	57	246	503	250	204	1261	730	61	14	83
Prosopis juliflora (pods)	6.5	339	318	317	232	329	533	299	347	76	272	611	330	263	848	330	112	38	136
Prosopis stephiania	56	352	282	321	168	375	594	306	283	78	271	696	351	283	1179	569			
FAO Provisional Score				270	180		306	270		144	180		270	180			90		

Fig. 1. A gas-liquid chromatogram of approximately 1 μg of S-[^{14}C] carboxyethylcysteine, ^{14}C-histidine, and ^{14}C tryptophan chromatographed as the N-acetyl-O-n propyl esters. Chromatography condition are identical to those reported in (4) except that the oven temperature was 215°C. The bars under the peaks indicate the times of radioisotope collection. The retention times are given in minutes above the peaks. B. Gas-liquid chromatogram of an aliquot of derivatized Prosopis chilensis seed protein. Approximately 50 μg of amino acids were injected.


A
CysR
3.75
His
5.93
Trp
15.78
0
5
10
15
20
Time(min)


B
CysR
3.77
His
6.00
Trp
16.21
0
5
10
15
20
Time (min)

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EXPERIMENTAL V

MINIMAL ENERGY INPUT AGRICULTURE

What Crops do we grow when the oil is gone?

The tree legumes may be ideal.

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Neither the developed, nor certainly, the developing nations can hope to sustain agricultures based upon massive inputs of fossil fuel energy. Thus, it is important to develop agricultures with higher yields, particularly of protein, but with lessened input of fertilizer, irrigation and tillage (1,2,3,4,5). To this end we have conceptualized a model crop plant. The features sought for in this model were 1) minimal soil and nutrient loss, 2) little or no need for irrigation, 3) maximum yield, 4) ability to fix nitrogen and 5) production of large amounts of high quality protein. We next made an assessment of food producing plants to identify those having morphological, physiological and ecological attributes that fitted them to the model and found that tree legumes have the necessary characteristics. Further, we have shown, by protein and amino acid analysis, that there is sufficient genetic diversity among the tree legumes to permit selection for a protein of amino acid composition superior to, even, milk casein!

Minimization of soil and nutrient loss: This is probably best attained by utilizing an untilled perennial tree or shrub crop with some grass cover. The importance of perennial cover in conserving mineral nutrients is demonstrated by the Hubbard Brook project (6) showing that mineral and salt efflux from an area denuded of forest vegetation was 40 fold higher than the control for nitrate, 15 for potassium, 4 for calcium and magnesium, and 2 fold for sodium. The estimated time for loss of 8 inches of surface soil is 37 years with non-contour tillage and 2234 years with no tillage (6). An additional feature of a perennial soil cover is that a no tillage system does not require fuel and machinery, and the backup support required by mechanization.

Need for irrigation: Sufficient tolerance to drought to obviate the need for irrigation is an important factor in reducing needs for capital and energy input (4,5). To this end, CAM metabolism, a CO_2 storage mechanism allowing a plant to conserve water by closing its stomates during the day and opening them at night (7), is helpful. Other attributes of plants adapted to arid environments would be valuable, and in particular, a tap root that could penetrate to ground water. Some trees have tap roots that penetrate to 60 meters (8).

Maximum yield: Forest ecosystems are among those with the highest net annual productivity (9). This seems due to multiple layers of leaves, plus the rapidity with which a complete, light-absorbing leaf canopy can be established. An additional feature of tree ecosystems is that while their net annual productivity is high, fertilizer requirements are often barely demonstrable as the nitrogen is recycled through organic matter (10) (11). Since the productivity is so great there is the possibility that this growth can be siphoned off into edible portions or used for animal feed.

Ability to fix nitrogen: A feature of the model is that the plant should be a nitrogen fixer. This is important since seeds of nitrogen fixing plants are often higher in protein than those of non-fixers, and since nodulation would reduce needs for fertilizer nitrogen.

Production of Protein: The last feature of the model is that the plant system should have a high yield of protein, and that the amino acid composition should be as close as possible to that recommended for human nutrition even though other foods will, of course be eaten to complement the legume amino acid profile.

"The Ideal Crop"

Using the above parameters it became apparent that tree legumes may be the "ideal crop" for reducing capital and energy expenditures. Perennial tree legumes compose an entire subfamily of the Leguminosae, the Mimosoideae, and part of the subfamily Caesalpinioideae. They do not require tillage and most are nodulated (12) with reported nitrogen fixation rates of 200 $\pm$ 45 kg N per ha. per year (13). It is not known whether nitrogen fixation stops during drought periods, as it does with annuals, or if it is continuous. Some of the tree legumes are quite drought resistant (14), and have large yields of pods (15). Data presented in this paper establish that tree legumes produce large amounts of high quality protein.

The requisite attributes, and the closeness with which the mesquite tree (Prosopis) approximates them is shown in Fig. 1. Prosopis has palatable low tannin pods which have been used both for human and livestock food (16,17, 18). Our experiments show there is 69% protein in the seed after removal of the seed coat. P. chilensis also has a high seed to pod ratio, an important feature, as the seed has more protein per unit weight than the pod. Busson (19) has reported a value of 350 mg methionine/gm of N in Prosopis africana which is several fold higher than required by humans. Several authors (12) (20) have reported nodulation in the genus. The root system commonly extends 20 meters to the water table (21) and occasionally to the much greater depths of 60 and 80 meters (8,21). The yield data of Fig. 1 are extrapolated from the expected yield of a 10 yr old tree at the recommended planting density (14). Later work from S. Africa estimates that pod yields of

9,000-14,000 kg/ha might be conservatively expected (22). Such yields are high considering that a 100 bu/acre corn crop is 5,000 kg/ha. The report that Prosopis has crassulacean acid metabolism (CAM) (23) allowing it to close its stomates in the day and open them at night, thus better tolerating drought, requires confirmation. The fact that Prosopis is tolerant to frost at 40° S Lat. in Argentina (15) suggests the range to which it might be adapted.

We have chosen Prosopis as the example for our model since there is more information on Prosopis than other tree legumes. However, under water limiting conditions it is not typical of either a temperate or tropical forest and many other leguminous trees are used as food and could serve as examples. We have obtained seeds from a number of leguminous trees and analyzed them for their protein and amino acid composition (24,25). The results of that survey are presented in Table I.

Seed Proteins

Table I lists only the essential amino acids of the seed proteins. The amino acid composition of the proteins are compared with the Food and Agricultural Organization (FAO) provisional scores for adequacy in human nutrition (26). Each X indicates the amino acid is 15% below the recommended value. FAO values for soybean protein are given for comparison. Our data on the amino acid composition of Prosopis are comparable to those of Figueirdo (27), with the exception of the hydrophobic amino acids. This can probably be explained by their milder hydrolysis procedures, i.e. 4 hours with 3N HCl. The amino acid composition of the Parkia spp. are also in fair agreement with the work of Fetuga et al.

(28) and Busson (19). Interestingly, the amino acid composition of the storage protein of P. lobatum shows that it is of very high quality having a chemical score (29) of 0.79 compared to 0.59 and 0.47 for casein and soy protein respectively.

It is to be noted that almost all the legumes are low in sulfur amino acids and in tryptophan. However, there is considerable genetic diversity in protein and amino acid composition indicating that selection for a seed high in protein and having a favorable amino acid profile is possible. It might also be possible to increase the sulfur amino acid levels in the seeds with applications of sulfate fertilizer, as plants grown under sulfate deficient conditions have been shown to have decreased sulfur amino acid levels in the leaves (30), and seeds (31) and lower proportions of sulfur rich proteins (32). That the protein composition varies from 16 to 69% is encouraging, since it again indicates genetic diversity. Our data on protein and amino acid composition represent only a fraction of the species known. There are 17 species (16) of Prosopis from S. America and several from North America (33) of which we have analyzed only two. Considering that Brewbaker (34) has 90 varieties of the species Leucaena leucocephala alone, the enormous genetic potential becomes apparent.

It must be mentioned though, that some legume seeds contain toxic substances. For instance, Leucaena has mimosine in the leaves, a depilatory when fed in excess to animals (35), and Pithecellobium lobatum (Djenkol bean) causes hematuria when eaten in excess (36). Nonetheless, P. lobatum is regarded as a delicacy in Indonesia (36). Not all leguminous trees have toxic principals. For example Prosopis (37) and Parkia filicoidea (38) are reported to be toxin free.

Uses of Tree Legumes

If leguminous trees are ideal crop plants it is legitimate to ask why they are not widely used as food sources in Europe and N. America. That is unknown but the Indians of the American Southwest and Mexico (39) and the Indians in S. America (16) (40) widely used Prosopis as a human and livestock food. Similarly Parkia is used in the African Sahel (19,38,41), and Pithecellobium lobatum and Parkia speciosa are greatly used in Malaysia (36) (42) as is Prosopis on the Indian subcontinent (43,44).

The geographical locations, uses, habitat, and yield of leguminous trees are given in Table II and show their widespread uses. They are used as food for humans or livestock in every major savannah in the world.

For example, in Hawaii in 1910, Prosopis was regarded as the most valuable introduced tree on the island (45), because of its use as a livestock food. In the North American Sonoran desert ethnobotanists and anthropologists found that Prosopis was the most important food of the Seri Indians (39). In Argentina, the center of diversity for the genus, Prosopis is widely used as a food for humans and livestock as well as for firewood (16). In Brazil where Prosopis has been recently introduced, there is considerable study on the chemical composition of the pods (27) and references to its use by the Indians in pre-colonial times as a food source in Chile, Peru, and Argentina.

In South Africa where Prosopis and Gleditsia have been introduced, a Forestry Department Bulletin lists methods of cultivation, planting densities and yield projections (22). This bulletin was produced at the

request of farmers who desired information on cultivation methods for these fodder trees. There is also a summary of a 10 year UNESCO project on the cultivation of Prosopis in south and eastern Africa, and in India (46). In this report, Prosopis yields of 20 tons of edible beans per hectare were reported.

In India where Prosopis is both native and introduced (20,43), the leaves and pods are useful in the arid areas as a livestock food. Pithecellobium species are also used in India as forage (43).

In Malaysia and Indonesia several Parkia species and Pithecellobium lobatum are used as human food and rarely as livestock food (36,42). No yield data or attempt at developing these trees has taken place in Malaysia and Indonesia. This is unfortunate as our data show that the isolated P. lobatum protein has a higher total sulfur amino acid content than casein in which the sulfur amino acids are the limiting ones.

The use of leguminous trees in Africa as human and livestock food has been amply documented in books by Dalziel (38), Irvine (41), and Busson (19). The genus Parkia (African locust bean) is the major food-producing tree legume in the Sahel. Nutritional studies on a cultivar of a single species has been recently completed (28) indicating the seed is low in tryptophan and methionine. A fermented product from Parkia is eaten and is known as Soumbara or dawa-dawa (47). Other leguminous trees grown and eaten in the Sahel are Prosopis africana and Pentaclethra macrophylla (19,38,41).

It is unfortunate that with the exception of the yield data on Leucaena leaves used as a fodder in Hawaii (34) the other yield data are merely estimates. It is encouraging to note that, without correction

for below ground biomass, the C_3 leguminous tree Leucaena (34) has a net annual primary productivity comparable to the highest reported (9) for any plant ecosystem.

Summary

We have presented a plant model, for reducing expenditures of capital, fossil fuels, and machinery, adaptable to intensifying agriculture in countries with limited resources. We have studied the protein and amino acid composition of the seeds of a number of leguminous trees from around the world. This survey indicates a genetic diversity that makes possible the selection and breeding for specific seed proteins of desirable amino acid composition. A brief review of the uses, yields, and occurrence of nodulation in the tree legumes has also been presented, showing that its use is widespread and that the yield is higher than that of conventional annual crops.

We feel that a research program should be initiated to determine if the yield potential of mesquite is in fact 20,000 kg/ha; to determine, through feeding studies, how nutritious these seed proteins are; to determine the effect of sulfate fertilization on the sulfur amino acid content in the seed; and to determine the suitability of tree legume systems as crop plants in different parts of the world. Improved varieties of Prosopis should be evaluated in the U.S.A. from east to west coast in areas of light frost. Further north in the U.S.A. Gleditsia triacanthos varieties, Millwood and Calhoun, (15) should be evaluated. The most pressing areas of the world, where tree legumes would be of advantage, because of shortages of industrial inputs, would be the Indian Desert and Ganges Plain,, the dry areas of Argentina, Chile, and Peru, and the sub-Saharan Desert region.

Leguminous trees alone are not the answer to food problems in the semi-arid tropics. The solution resides in an integrated approach to population control, animal grazing, and water allocation to list but a few factors. However, leguminous trees do play a role in food production and data presented here indicates that Leguminous trees may present a relatively unexploited potential.

References and Notes

1. D. J. Greenland, Science, 190, 841 (1975)

2. D. Pimentel, L. E. Hurd, A. C. Bellotti, M. J. Forster, I. N. Oka, O. D. Sholes, and R. J. Whitman, Science 182, 443 (1973)

3. G. Borgstrom, in "The Hungry Planet", The Macmillan Co. Publisher, New York, 1965

4. G. H. Heichel, Amer. Sci. 64, 64 (1976)

5. A. Makhijani, in "Energy and Agriculture in the Third World", Ballinger Publishing Co., Cambridge, MA (1975)

6. G. E. Likens, F. H. Bormann, N. M. Johnson, D. W. Fisher, R. S. Pierce, Ecol. Monographs 40, 23, 1970; J. V. Martin, Farm Bur. Res. Comm. Mtg. Jefferson City, Missouri 1975; U.S. Dept. Agric. Tech. Release No. 51 (Rev.) 1975

7. I. P. Ting in "CO_2 Metabolism and Plant Productivity" R. H. Burris and C. C. Black, Ed. Univ. Park Press, Baltimore MD 251 (1976)

8. W. S. Phillips, Ecology 44, 424 (1963)

9. P. G. Murphy, in Primary Productivity of the Biosphere, H. Lieth, R. H. Whittaker, Ed. (Springer-Verlag, NY 1975), p. 227

10. J. L. Mason, Can. J. Plant Sci. 49, 149 (1969)

11. W. W. Moschler, G. D. Jones, and R. E. Adams, Soil Sci. Soc. Amer. Proc. 34, 683 (1970)

12. E. K. Allen, and O. N. Allen, Proc. 9th Int. Bot. Congress 1, 585 (1959)

13. E. R. Orchard, and G. D. Darb, 6th Int. Soil Sci. Cong. Paris IV, 305, (1956)

14. E. E. M. Loocke, Farming S. Africa 2, 250, 7 (1947)

15. J. R. Smith in "Tree Crops - A Permanent Agriculture", Devin Adair Co. Publish. New York 36,39,76,80,87 (1953) and S. B. Chase, J. For. 45, 715 (1947)

16. A. Burkart in "Las Leguminosas Argentinas" Acme Agency Publishers, Buenos Aires, Argentina 133 (1943)

17. R. S. Felger, and M. B. Moser, Kiva 37, 1, 53 (1971)

18. J. L. Schuster in "Literature on the Mesquite (Prosopis L.) of North America" Special Rpt 26, Int. Ctr. Arid, Semi Arid Land Studies, Texas Tech. Univ., Lubbock, TX

19. F. Busson, P. Jaeger, P. Lunven, and M. Pinta in "Plantes Alimentaires de l'Ouest Africain" Inter-Agency Publication (Ministere de la Cooperation, Ministere d'Etat charge de la Recherche Scientifique et Technique, Ministere des Armees) Marseille France 272 (1965)

20. R. K. Gupta, and G. S. Balera Indian Forester 280 (1972)

21. O. T. Solbrig, and P. D. Cantino, J. Arnold Arb. 56, 2, 185 (1975)

22. A. Juriaanse in "Are they fodder trees" Pamphlet 116 of Forestry Dept. Private Bag X93 Pretoria Transvaal S. Africa 2 (1973)

23. Y. D. Gaur, Experienta 24, 239 (1968)

24. P. Felker, and R. S. Bandurski, Anal. Biochem. 67, 245 (1975)

25. P. Felker, Anal. Biochem in press

26. W. R. Aykroyd, and J. Doughty in "Legumes in Human Nutrition" FAO Nutritional Studies 19, 106 and 117 (1964)

27. A. A. Figueiredo in "Lebensmittelchemische relevante Inhaltstoffe der Schoten der Algarobeira" Ph.D. thesis, Wurzburg, Germany (1975)

28. B. L. Fetuga, G. M. Babatunde, and U. A. Oyenuga, Br. J. Nutr. 32, 27 (1974)

29. M. Autret in "Amino Acid Content of Foods" FAO Nutritional Studies No. 24, Rome Italy 5 and 26 (1970)

30. E. T. Mertz, and H. Matsumoto, Archiv. Biochem. Biophys 63, 50, (1956)

31. A. Stabursvik, and O. M. Heide, Plant and Soil 41, 549 (1974)

32. A. Millerd, Ann. Rev. Pl. Phys. 26, 68 (1975)

33. M. C. Johnston, Brittonia 14, 72 (1962)

34. J. L. Brewbaker, D. L. Pluckhett, and V. Gonzalez Hawaii Agric. Exp. Sta. Res. Bulletin, 166 Univ. of Hawaii (1972)

35. J. L. Brewbaker, and J. W. Hylin, Crop Sci. 5, 348 (1965)

36. J. J. Ochse, in "Vegetables of the Dutch East Indies", Archipel Drukkerij Buitenzorg - Java 426 (1931)

37. R. Felger. Personal communication and F. Garcia, N. Mexico Agric. Exp. Sta. 28th Annual Rpt. 77 (1916)

38. J. M. Dalziel, in "The Useful Plants of West Tropical Africa" Crown Agents for the Colonies, Publish London 217 (1937)

39. R. S. Felger, and M. B. Moser, Ecol. Fd. Nutr. 5, 13, (1976)

40. G. Azevedo, Mundo Agricola 15, 53 (1966)

41. F. R. Irvine in "Woody Plants of Ghana" Oxford Univ. Press, London 348 (1961)

42. H. N. Ridley in "Flora of the Malay Peninsula", Vol. 1, L. Reeve and Co. Publ. London 652 (1922)

43. Wealth of India, Publications and Information Directorate, CSIR, New Delhi, 8, 140 and 245 (1969)

44. S. I. Ali, in Flora of W. Pakistan, E. Nasir, Ed. Univ. of Karachi, Pakistan, 36, 29, (1973)

45. E. V. Wilcox, Hawaii Agric. Exp. Sta. Honolulu Hawaii Press Bulletin 26 (1910)

46. J. S. Douglas, World Crops 19, 4, 20, (1967)

47. B. S. Platt, Fd. Tech. 18, 662 (1964)

48. G. P. Walton, USDA Bulletin No. 1194, 1 (1923)

49. This article Table 1

50. H. L. Bentley, USDA Division of Agrostology, 10 (1898)

51. R. O. White, G. Nilsson-Leissner, and H. C. Trumble in "Legumes in Agriculture" FAO Studies #21, 315 and 251 (1953)

52. W. R. Stanton, in "Grain Legumes in Africa" FAO Public., Rome Italy 133 (1966)

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Table Legend

Table 1. Amino acid composition of the trichloroacetic acid precipitable albumin and globulin fraction from seed embryos. The Food and Agricultural Organization (FAO) provisional scores for adequacy in human nutrition in mg/gm N are; valine 270, threonine 180, isoleucine 270, lysine 270, methionine 144, cystine 126, and tryptophan 90. Phenylalanine, tyrosine, and leucine are not listed in this table as they are above the FAO provisional score. The seeds are graded with one X for each 15% they fall below the score value. Thus XX represents 30% below the recommended value and XXX represents 45% etc. The seeds were obtained from: A) the USDA Northern Regional Research Laboratory, Peoria, IL, B) from Dr. Peter Murphy, Dept. Botany and Plant Pathology, Michigan State University and collected in Indonesia, and C) Mr. Howard Hyland, USDA Northeastern Laboratory, Beltsville, MD from collectors in Argentina.

Table 2. Characteristics of tree legumes used as food and forage.

TABLE 1

ESSENTIAL AMINO ACID DEFICIENCY PROFILES IN TREE LEGUME SEEDS

PLANT	% PROTEIN	AMINO ACID						
		VAL	THR	ILE	LYS	MET	CYS	TRP
PARKIA CLAPPERTONIANA[A]	16					XXX	XX	XXX
ROXBURGHII[A]	41		X	X		XXXXX	XXXXXX	XXXX
JAVANICA[B]	44			X		XXXXX	XXXXXX	XXXX
PITHECELLOBIUM SONORAE[A]	44	XX		X		XXX	XXXX	XX
SAMAN[A]	38	X		X		XXX	XXXX	XX
DULCE[A]	28					X	XXXXXX	X
LOBATUM[B]	16					X		
ACACIA MONILIFORMIS[C]	55	X		X		XXXXX	XXXXX	XXX
AURICULAEFORMIS[B]	38					XXXX	XXXXX	XXXX
PROSOPIS STEPHANIANA[A]	56		X			XXXX	no data available	
CHILENSIS[C]	69	X	XX	XX	X	XXXXX	XXXX	X
JULIFLORA(SEEDS)[A]	60	XX	XX	XX	X	XXXX	XXXXXX	XXX
JULIFLORA(PODS)[A]	6.5					XXXX	XXXXX	
LEUCAENA LEUCOCEPHALA[A]	46	X		X		XXXX	XXXXX	XX
GLAUCA[B]	39					XXXX	XXXXX	
GLEDITSIA TRIACANTHOS[A]	58			X		XXXX	XX	XXX
GLYCINE MAX	38					XXXX	XXX	X

TABLE 2 - USES OF TREE LEGUMES

GEOGRAPHICAL LOCATION	LEGUMINOUS TREES PRESENT	HABITAT	USES	PROTEIN IN (EMBRYO)%	METHIONINE VALUE % OF SOYBEAN*	NODULATION IN GENUS	YIELD OF PODS
Southwestern USA and Mexico	Prosopis (several species) (18,15)	Arid-Savannah	Livestock-previously used by Indians as food staple (17,18,15)	60% (48,49)	72% (49)	Yes (12,20)	ca. 2,000 kg/ha pods unmanaged Arizona desert. (50)
	Pithecellobium (51)		Pods used as livestock food (51)		153% (49)	Yes (12)	
Desert regions of Argentina	Prosopis (20-25 species) (16)	Arid-Savannah	Pods eaten by humans (16) Wood, charcoal, alcoholic beverages	69% (49)	60% (49)	Yes (12)	
	Acacia moniliformis (51)	Arid-Savannah	Livestock food (51)	55% (49)	63% (49)	Yes (12)	
Brazil	Prosopis (introduced) (27,40)	Arid-Savannah	Human and livestock food (27,40)	13% (Pod) (27) 32% (embryo and seedcoat)		Yes (12,20)	
Sahel	Parkia roxburghii (19,38, 41)	Arid-Savannah	Human and livestock food (19,38,41)	41% (embryo) (49)	63% (49)	Yes (12)	350-500 kg/ha unmanaged (52)
	Parkia clappertoniana (19,38,41)		Human and livestock food (19,28,38,41)	16% (embryo) (49)	120% (49)	Yes (12)	
	Pentaclethra macrophylla (19,38,41)	Arid-Savannah	Human and livestock food (19,38,41)	28% (19)	111% (19)	No (12)	
	Prosopis africana (19,38,41)	Arid-Savannah	Limited human use (19,38, 41)	19.1% (19)	443% (19)	Yes (12,20)	
South Africa	Prosopis (introduced) (14,22)	Savannah	Livestock food (14,22)			Yes (12,20)	20,000 kg/ha (46) 9,000-14,000 kg/ha conservatively (22)
	Gleditsia triacanthos(14,22)	Savannah	Livestock food (14,22)	58%(49)	81% (49)	No (12)	11,500 kg/ha (22)
	Ceratonia siliqua (14,22)	Savannah	Livestock food (14,22)			No (12)	
India	Prosopis (20,43,44)	Arid-Savannah	Wood, leaves and pods as livestock food (20,43,44)			Yes (12,20)	
	Acacia (20)	Arid-Savannah	Livestock browse (44)			Yes (12)	
	Pithecellobium dulce (43)	Savannah	Livestock and human food (43)			Yes (12)	
Hawaii	Leucaena spp (34)	moist-tropical	Leaves used as forage (34)	46% (49)		Yes (12)	30,000 kg/ha**(34)
	Prosopis spp (45)	Arid-Savannah	Pods used for livestock for food (45)			Yes (12)	4,000-20,000 kg pods/ha (15)
Malaysia	Pithecellobium lobatum (26,36)	moist-tropical	Food delicacy-no livestock consumption (26,36)	16% (49)	153% (49)	Yes (12)	
	Parkia speciosa (26,36, 42)	moist-tropical	Feed delicacy-no livestock stock consumption (26,36,42)			Yes (12)	
	Leucaena glauca (36,26)	moist-tropical	Leaves used forage (36)	46% (49)	97%(49)	Yes (12)	
	Parkia javanica (36)	moist-tropical	Human consumption (36)	44% (49)	56% (49)	Yes (12)	

*This calculation uses an average methionine value of 79 mg/gm N (29).

** 12% moisture

Legend for Figure

Figure 1. A tree legume showing characteristics desirable for a minimal energy-input agriculture. References are given in the text.

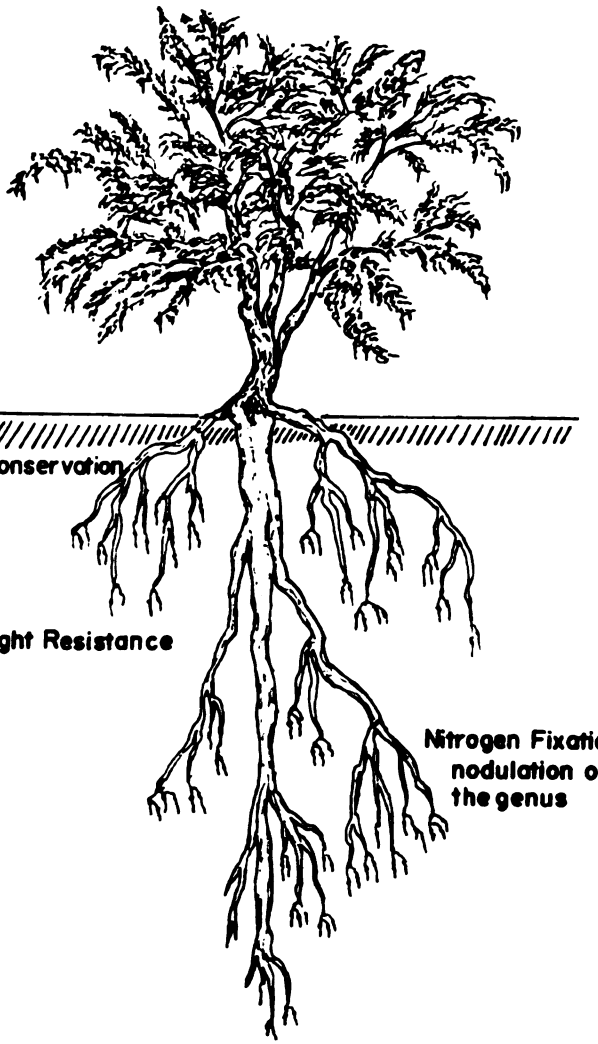
ATTRIBUTES OF AN IDEAL FOOD CROP TREE

Prosopis as a Model Tree for Minimal Inputs of Energy, Machinery, and Capital

High Yield of Pods
18,000 kg/ha

Minimal Water Needs
CAM metabolism

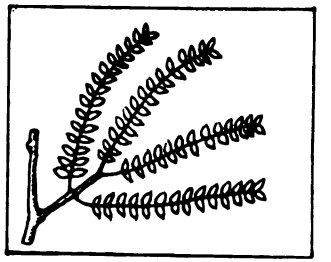
Frost Resistant to 40°S lat.

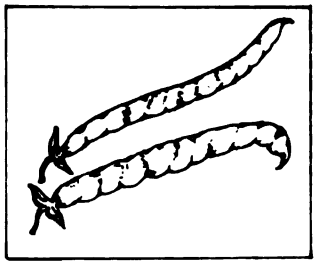


Minimal Tillage and Nutrient Conservation
with perennial soil cover

Extensive Root System for Drought Resistance
and nutrient sufficiency

Nitrogen Fixation
nodulation occurs in
the genus





A Nutritious Food
palatable low tannin pods
70% protein in seed embryo
high seed/pod ratio
high methionine in seed

APPENDIX TO V

An expansion of the literature on minimization of nutrient and soil loss will be given in this appendix since only a few examples were cited in the preceding paper. A partial explanation for the dramatic soil nutrient losses reported by Likens et al. (1970) comes from mineral nutrition studies of orchard and forest crops. Oberly and Boynton (1966) in a review on mineral nutrition of apples, cited work showing that nitrate or ammonia determinations did not indicate the availability of nitrogen in the soil, but that the total soil nitrogen, and carbon to nitrogen ratio of the orchard sod cover regulated nitrogen availability to the plant. Thus the sod cover acted as a nitrogen buffer absorbing nitrogen when carbohydrate is high and releasing it to the plant when the carbohydrate level is low. Olsen et al. (1970) found that most leaching of nitrate in annual crops occurred in the spring before the crop was dead and harvested. In contrast Aldrich (1931) found that apple tree roots continued to absorb nitrogen even during the winter months in Maryland. Apple trees also conserve their nitrogen during annual senescence by absorbing 20-40 % of the leaf nitrogen into the wood (Murneck and Logan, 1932). This conservation of nitrogen is perhaps why a 224 kg N/ha addition to 4 yr old pines causes only a 3.3 % increase in pine biomass (Baker et al., 1974) while a 218 kg N/ha application is required to double the yield of a 76 kg N/ha application in corn (Anon, 1970). The presence of mycorrhizae in forest soils which has been shown to increase heights of pine trees by 30 % over controls (Marx et al., 1976) is another reason why forest soils conserve nutrients.

Recently Pimentel et al. (1976) have presented dramatic data showing soil loss from annual crops.

Not only can tree legumes fix nitrogen as cited in the previous thesis section, but the fact that some tropical soils are as high in nitrogen and humus as many of the most productive temperate soils, despite their extremely high litter decomposition rate, is attributed to a 50 % cover by tree legumes (citation in Williams, 1967).

CONCLUSIONS

It would be impossible to develop a new agricultural system in one thesis research program. Nonetheless, sufficient data have been adduced to indicate that tree legumes are worthy of consideration as an alternative to conventional annual crops. The literature now available indicates that the use of tree legume crops would minimize soil and nutrient loss, obviate the need for fertilization and irrigation, and provide substantial yields of high protein content food or feed. In that sense, the objective of this thesis has been attained.

Much new knowledge will have to be obtained before tree legumes can be further incorporated into agricultural systems. For example, better yield data must become available, a better understanding of the phenomenon of alternatively heavy and light fruit yields by some trees(mast) and a better knowledge of what, if any, tillage and fertilization might be needed. Most of these areas lie outside the scope of a doctoral dissertation but, at least, the relevant literature is discussed in the thesis and below. I have concentrated on those aspects of the problem relating to the protein content and amino acid composition of tree legume fruits currently being used as food or feed. The following conclusions are divided into two parts, that dealing with amino acid assay techniques and that dealing with tree legumes in a minimal energy input agricultural system.

The method reported here for quantitative glc of the isoamyl esters of the perfluoroacyl derivatives of amino acids requires a long derivatization time but is, none the less valuable because of the methodologies of reagent preparation, new in vacuo techniques, and because of its delineation of the capabilities and problems of this technique. Mackenzie and Tenaschuk (1974, 1975) have improved upon this technique by preparing the esters in isobutanol-HCl at 120 C for 20 min and by introduction of the use of the isobutyl esters which give much better separation of glutamic acid and tyrosine. March's (1976) introduction of 2,6 di-t-butyl-p-cresol(BHT) is reported to prevent degradation of methionine in derivatized samples and has particular utility for crude samples with low methionine levels. The use of the sensitive nitrogen detector instead of a flame ionization detector might prove useful for amino acid analysis of crude materials. A combination of in vacuo and reagent preparation techniques of Felker and Bandurski (1975), of the use of the direct isobutyl esterification of Mackenzie and Tenaschuk (1976), and of the use of March's (1976) antioxidant would probably be the best glc amino acid technique available to date for the amino acids other than cystine, histidine and tryptophan.

The analysis of cystine, histidine, and tryptophan by glc-isotope dilution as reported here has as its major drawback the downward drift of the flame ionization detector response during analysis of crude samples. This drift requires alternating injections of standards and unknowns in order to maintain calibration and will probably prevent the widespread use of glc-isotope dilution as described in this thesis. Other engineering problems that need to be worked on are the linearity and availability of

effluent splitters, and the design of inexpensive collection tubes with uniform internal diameters to minimize differences in back pressure, and thus detector sensitivity, during collection of radioisotopes. It is unfortunate that engineering difficulties limit glc-isotope dilution analysis, since a rapid, simple, and accurate determination of specific activities could greatly stimulate many areas of biochemistry.

The improvement in yields of tryptophan by addition of tin during acid hydrolysis with dithioethane, demonstrates the importance of the redox potential in tryptophan recovery. Tin increased tryptophan recovery by 12% to 86% and prevented the yellow coloration of the hydrolysates in lysozyme. Longer hydrolysis times should be studied since lysozyme has hydrophobic-acid resistant peptide linkages.

It would also be interesting to study tryptophan recovery in the presence of carbohydrate such as glucose which is known to reduce tryptophan recoveries in the presence of the hydrolysing agents toluenesulfonic acid, and β-mercaptoethanesulfonic acid. Since aldehydes can condense wtih tryptophan to form acid resistant polymers (Remers and Brown, 1972) and since glucose lowers tryptophan recoveries from acid hydrolysates it would be interesting to compare the action of inositol, and glucose on tryptophan degradation to determine the importance of the glucose aldehyde in tryptophan degradation. If glucose but not inositol causes tryptophan degradation it would be interesting to study effects of reductants, both during and before acid hydrolysis, on carbohydrate induced tryptophan degradation. Characterization of the products resulting from acid hydrolysis of a ^{14}C tryptophan containing peptide with glucose by tlc, and glc, and of non-labeled tryptophan peptides and glucose by GC-MS might help elucidate the mechanism of glucose introduced tryptophan degradation in acid hydrolysis. GC-MS of the compound emerging slightly

before tryptophan in dithioethane acid hydrolyzed lysozyme on glc might also prove interesting as this peak disappears with the addition of tin when tryptophan recoveries go up 10-12%. A study of the importance of the completeness of dithioethane reduction on tryptophan recovery and ways of maintaining dithioethane reduction would certainly would not be enjoyable.

A study of quantitative disulfide reduction and S-alkylation using proteins of known amino acid composition with difficultly reducible disulfide bonds should be undertaken to ascertain the general applicability of this method to proteins. The possibility of using alkylation reagents that would lend more stability to the thioether linkage such as 4-vinyl pyridine (Friedman et al.,1970) should be investigated as well as the glc properties of the resultant alkylated cysteine.

The possibility of using pivalyl anhydride, which is less susceptible to Sn2 attack than acetic anhydride (Staab, 1956) should be evaluated as a histidine imidazole acylating reagent. Since anhydrous conditions are especially important for histidine, perhaps the derivatization manipulations, and reagent storage should be done in a dry-box.

The possibility of mating the glc-isotope dilution assay for cystine, histidine, and tryptophan with the method for the other 17 amino acids would yield take considerable effort, and would require,1) a column packing capable of separating all the amino acids and yet polar enough to yield a sharp histidine peak, 2) combining the use of the isobutyl esters with acetic anhydride to acylate the histidine imidazole as outlined by MacKenzie and Tenaschuk (1974) and March (1976),3) use of isotope dilution methodology to correct for losses of cystine, histidine, and tryptophan either during hydrolysis, preparation of the esters in 120 C butanol-HCl, or on the column, 4) effluent splitters and collectors of sufficient stability to be usable under the changing temperature,

pressure, and flow conditions of the temperature program needed to separate 20 amino acids, and 5) establishment of conditions such that the ε- amino of lysine and other free amino groups will not be alkylated during the thiol alkylation.

The results of amino acid analysis are often expressed as amount of amino acid per unit of nitrogen necessitating a nitrogen as well as an an amino acid analysis. At the time glc of amino acids was attempted the semi-micro Kjeldahl analysis of Ma and Zuazaga (1942) was used which required a minimum of 3 mg of protein or amino acids. Since only 50 ug of amino acids were required for glc this meant that 60 times the amount of protein required for glc was necessary because of the Kjeldahl requirement. Thus a modification of an existing automated procedure was made and is reported in this thesis permitting Kjeldahl analysis on 3 to 30 ug of protein.

Potential Use of Tree Legumes in a Minimal Energy Input Agriculture

The last two experimental sections of the thesis concerning Minimal Energy Input Agriculture, and Protein and Amino Acid Composition of Tree Legume Seeds have been discussed in detail but a few points should be emphasized.

Under arid and semi-arid conditions, sunlight, CO_2, and nitrogen will probably not limit plant production by tree legumes. One would expect the availability of ground water and precipitation to govern the yield of tree legumes under such conditions. Measuring the yields of leguminous trees in a moisture gradient, for example, from the windward to leeward side of a tropical island, would yield a calibration curve showing the relationship of water availability to yield.

Moisture availability could be quantitated by measuring a) the precipitation b) the soil moisture profile with neutron activation probes, and c) the potential evapotranspiration with a class A pan (The potential evapotranspiration could also be calculated from temperature, humidity, radation, and windspeed measurements).

A plot of yield versus moisture availability might make the prediction of yields possible, and enable prediction of the suitability of tree legumes for new sites. The literature indicates the lower yield limit would be about 18 kg of Prosopis pods per tree for 150 mm of yearly precipitation at a water table depth of 20 m (Garcia, 1916), and the upper limit might be 90-140 kg of pods per tree recieving 250-500 mm of precipitation per year (Jurriaanse, 1973) at unspecified water table depths.

The estimated yields (18,000 kg/ha) of leguminous trees may appear to be unreasonably high but it is important to realize that in semi-arid and arid climates the effective growing season is almost the entire year. By comparison most of the growth of corn in Michigan occurs in June, July and August i.e. ¼ of the year, and since the yield of corn grain in Michigan is approximately 6,000 kg/ha, it seems possible that annual mesquite pod yields of 18,000 kg/ha might be attainable under favorable mositure regimes.

Data on tree legume pod yield is inadequate but actually yield in terms of mass per unit of land area may not be the most important yield in the future. The important data might be the food calories and protein produced per unit of energy, capital, or machinery invested. When tree legume yields are measured in these terms their usefulness may be more apparent.

There are many unstudied aspects of tree legume production such as their susceptibility to insect damage to the pods, the tendency of trees, in general,to have irregular yields, and the hard seed coat of tree legumes. These difficulties appear to be relatively minor and should be amenable to standard agronomic and horticultural techniques. Furthermore new silvicultural techniques capable of reducing the time to flowering in trees by several-fold (Prof. J. Hanover, personal communication) will make tree based crop systems more amenable to crop production and crop improvement.

Much of the information on tree legumes contained in this thesis concerns tree legumes in arid climates, but this does not mean that tree legumes are restricted to arid climates. The nitrogen fixing ability of tree legumes give them a selective advantage on low organic matter arid soils. Since trees are generally unresponsive to nitrogen on temperate soils (Baker et al.,1974) tree legumes would not have a selective advantage on high organic matter temperate soils. Moist tropical forest soils have high organic matter degradation rates that would provide tree legumes with a selective advantage, and indeed some tropical moist forests have 50 % leguminous tree cover with soil organic matter contents of 12 %, almost twice as high as normal temperate soils (Williams, 1967). Tree legumes in other than arid climates are exemplified by Gleditsia triacanthos (honey locust)- a temperate tree legume, and Parkia speciosa, and Pithecellobium lobatum which are adapted to heavy rainfall tropical regions in Thailand, Malaysia, and Indonesia.

The ultimate objective of this thesis was the identification of agricultural systems that would require minimal inputs of fossil fuels,

capital, mechanization and still yield large amounts of high quality protein. To that end 15 species in six genera of leguminous trees were identified that were used either for human and/or livestock food, and the protein and amino acid composition of the seeds was determined. It has been found that Prosopis chilensis has a protein content in the true seed of 69 %, and that Pithecellobium lobatum has a chemical score 20 percentage points higher than casein. Unfortunately the Prosopis chilensis protein had many amino acid deficiencies and the Pithecellobium lobatum seed had only 16 % protein with only half of the extractable nitrogen being TCA precipitable. Most of the tree legumes have protein and amino acid contents intermediate between Prosopis chilensis and Pithecellobium lobatum with their protein contents similar to Glycine max and their amino acid compositions similar to Phaseolus vulgaris. The broad range of protein and amino acid values could prove very useful in a breeding program. Since some of the tree legumes came from poor arid soils with little capacity to bind sulfate and since sulfate fertilization has been shown to increase the levels of sulfur amino acid rich proteins(Millerd,1975) a study of the effect of sulfate fertilization on the sulfur amino acid composition of tree legumes from arid areas holds much potential.

Reliable replicated yield data for the pods of tree legumes is nonexistent,with only Prosopis having sufficient qualitative estimates to be able to ascribe average yield values. The reported yields of Prosopis pods range from ca 2,000 kg/ha in the New Mexico-Arizona desert with 150 mm of rainfall (Garcia, 1916; and Bentley 1898) to about 18,000 kg/ha in S. Africa (Loock, 1947),with 11,000 kg/ha under 250-500 mm of precipitation (Jurriaanse, 1973) being an average figure. The protein content of the entire pod of 12 % (Figueirdo, 1975) is much lower

than the 60-69 % protein content in the seed but is still higher than the protein content of cereals.

The palatability and usefulness of the mesquite pods is established, since Prosopis pods were the most important food of the Seri Indians (Felger, 1976). Azevedo (1966) cites the importance of Prosopis pods as a food in pre-colonial S. America, and Burkart (1943) describes half a dozen Prosopis species of exceptional palatability and utility.

Not only was Prosopis a primary food plant in arid climates but it served as this without the input of fossil fuels, capital, and 20th century transportation, and agricultural mechanization. While the living standard of Indians utilizing Prosopis as a food may not have been high, Prosopis provided an "unfailing resource" which prevented mass starvation (Felger and Moser, 1976).

Much of the data necessary to judge the potential of tree legumes for minimal energy input agriculture is tenuous and fragmentary. Nonetheless I believe there is enough information to warrant an intensified investigation to quantify yields and delimit tree legume uses. Specifically I believe there are four major areas of research needed to evaluate tree legumes:

1. Establishment of a collection of tree legume varieties and development of horticultural techniques applicable to tree legumes including: grafting and vegetative propagation techniques; hybridization techniques and use of artificial pollination to increase seed set; development of resistance to insects and plant diseases; studies of frost tolerance; spacing requirements for orchard plantings; and response to accelerated growth conditions to shorten time to pod bearing.

2. Understanding of factors influencing nitrogen fixation including: confirmation of nitrogen fixation using acetylene or ^{15}N enrichment assay; effect of water stress on nitrogen fixation; the culture of nitrogen fixing organisms from tree legumes; and the effect of molybdenum, sulfate, phosphate, lime, and pH on the rate of nitrogen fixation in tree legumes.

3. Evaluation of the nutritional quality of tree legume seeds and pods including: feeding of ground honey locust and mesquite pods and seed meal to ruminants and non-ruminants; study of the toxic principles from Pithecellobium lobatum, and the toxicity of the alkaloids mimosine, and paucine; study of the protein from P. lobatum with the chemical score of 0.79; and the effect of sulfate soil levels on the methionine and cysteine nutritional deficiencies in the seed.

4. Calibration of yield with soil moisture profile, potential evapotranspiration, and rainfall data in order to predict yields in possible new sites.

BIBLIOGRAPHY

BIBLIOGRAPHY

Adams, R. F. (1974) Determination of amino acid profiles in biological samples by gas chromatography. J. Chromatog 95,189-212.

Adler, H. E. (1949) Indigestion from an unbalanced Kiawe (Mesquite) bean diet. Amer. Vet. Med. Assn. Journal 115,263.

Aldrich, W. W. (1931) Nitrogen intake and translocation in apple trees following fall, winter, and spring sodium nitrate applications. Proc. Amer. Soc. Hort. Sci. 28,532-538.

Ali, S. I. (1973) Mimosaceae in "Flora of West Pakistan" No. 36 Nasir. E., and S. I. Ali Ed. Univ. of Karachi, Karachi, Pakistan. p. 29.

Anon (1928) The honey locust contest. J. Heredity 19:217-224.

Anon (1929) Awards of honey locust prizes. J. Heredity 20:468.

Anon (1965) Gleditsia (honey locusts) in "Silvics of forest trees of the United States. Agric. Hdbk 271,197-201.

Anon (1970) Fertilizer recommendations for vegetables and field crops in Michigan. Ext. Bulletin E-550 Cooperative Extension Service, Michigan State University.

Atkins, A. O. (1942) Yield and sugar content of selected thornless honey-locusts. Alabama Exp. Sta. 53 Ann. Rpt. 25-26.

Autret, M. (1972) in Amino acid content of foods and biological data on proteins. FAO Nutritional Studies #24 Rome.

Azevedo, G. D. (1966) Vagens da algarobeira na alimentacao humana. Mundo Agricola 15,53-54.

Baker, J. B., G. L. Switzer, and L. E. Nelson (1974) Biomass production and nitrogen recovery after fertilization of young loblolly pines. Soil. Sci. Soc. Amer. Proc. 38,958-961.

Bentley, H. L. (1898) Grasses and forage plants of Central Texas. USDA-Div. Agrostology Bulletin 10,36.

Blagrove, R. J., and J. M. Gillespie (1975) Isolation, purification, and characterization of the seed globulins of Lupinus angustifolius. Aust. J. Pl. Physiol. 2,13-27.

Block, R. J., and D. Bolling (1945) in "The amino acid composition of proteins and feeds." C. C. Thomas Publish. Springfield, IL. USA. 82-83.

Bosshard, H. R., K. H. Jorgensen, and R. E. Humbel (1969) Preparation and properties of cyanoethylated insulin. Eur. J. Biochem. 9, 353-362.

Boynton, D., and G. H. Oberly (1966) Apple nutrition in "Temperate to Tropical Fruit Nutrition". Norman Childers Ed. Rutgers Univ. Press. 1-50.

Brewbaker, J. L., and J. W. Hylin (1965) Variations in mimosine content among Leucaena species and related mimosaceae. Crop. Sci. 5, 348-349.

Brewbaker, J. L., D. L. Plucknett, and V. Gonzalez (1972) Varietal variation and yield trials of Leucaena leucocephala (Koa haole) in Hawaii. Hawaii Agr. Exp. Sta., Univ. of Hawaii Res. Bull. 166,1-28.

Briggs, W. R., and H. V. Rice (1972) Phytochrome: chemical and physical properties and mechanism of action. Ann. Rev. Pl. Physiol. 23, 304.

Brown, R. G., W. C. Kimmins, and B. Lindberg (1975) Structural studies of glycoproteins from Phaseolus vulgaris. Acta. Chem. Scand. B 29,843-852.

Bruice, T. C. (1963) Acyl imidazoles in methods in enzymol. VI. (Colowick, S. P., and N. O. Kaplan Ed.) Acad. Press. Publ. New York, N.Y. 606-610.

Burkart, A. (1943) Las leguminosas argentinas. Acme Agency Publishers, Buenos Aires, Argentina.

Burkill, I. H. (1966) "A dictionary of the economic products of the Malay Peninsula". Ministry of Agric. and Co-operatives, Gov'ts of Malaysia and Singapore, vol II, 1697.

Burr, B. and F. A. Burr (1976) Zein synthesis in maize endosperm by polyribosomes attached to protein bodies. Proc. Nat. Acad. Sci. USA. 73,515-519.

Bush, A. (1972) Symbol of the southwest. Herbarist 38,39-48.

Busson, F., P. Jaeger, P. Lunven, and M. Pinta (1965) "Plantes Alimentaires de l'Ouest Africain". Inter-Agency Publication (Ministere de la Cooperation Ministere d'Etat charge de la Recherche Scientifique et Technique, Ministere des Armees) Marseille, France.

Busson, F., J. Perisse, and P. Jaeger (1958) Die bestimmung der aminosauren in den samen von Parkia biglobosa durch chromatographic an harzsaulen. Hoppe-Seyler's. Zeit. fur Physiol. Chemie. 310,1-3.

Cancalon, P., and J. D. Klingman (1974) An improved procedure for preparing the n-butyl-trifluoroacetyl amino acid derivatives and its application in the study of radioactive amino acids from biological sources. J. Chromatog. Sci. 12,349-355.

Cavins, J. F., and M. Friedman (1968) Specific modification of protein sulfhydryl groups with α-β unsaturated compounds. J. Biol. Chem. 243,3357-3360.

Charreau, C., and P. Vidal (1965) Influence de L'Acacia albida del sur le sol, nutrition minerale et rendements des mils pennisetum au senegal. L'Agronomie Trop. 67,600-626.

Chase, S. G. (1947) Propagation of thornless honeylocust. J. Forestry 45,715-722.

Cheshire, R. M., and B. J. Miflin (1975) The control of lysine biosynthesis in maize. Phytochem. 14,695-698.

Cleland, W. W. (1964) Dithiothreitol, a new protective reagent for SH groups. Biochem 3,480-482.

Coulter, J. R., and C. S. Hann (1968) A practical quantitative gas chromatographic analysis of amino acids using the n-propyl N-acetyl esters. J. Chromatogr. 36, 42-49.

Coulter, J. R., and C. S. Hann (1971) Gas Chromatography of amino acids in "New Techniques in Amino Acids, Peptide and Protein Analysis" A. Niederwieser and G. Pataki Ed. Ann Arbor Sci. Publ. Ann Arbor, Mich.

Dalziel, J. M. (1937) in "The useful plants of west tropical Africa". Crown Agents for the Colonies Publish. London.

Derbyshire, E., D. J. Wright, and D. Boulter (1976) Legumin and vicilin, storage proteins of legume seeds - a review. Phytochem 15, 3-24.

Dollahite, J. W. (1964) Management of the disease produced in cattle on an unbalanced diet of mesquite beans. Southwestern Vet. 17, 293-296.

Douglas, J. S. (1967) 3-D Forestry World Crops, 19,20-24.

Felger, R. S., and M. B. Moser (1971) Seri use of mesquite Prosopis glandulosa var torreyana.

Felger, R. S., and M. B. Moser (1976) Seri Indian food plants: desert subsistence without agriculture. Ecol. Fd. Nutr. 5,13-27.

Felker, P., and R. S. Bandurski (1975) Quantitative gas-liquid chromatography and Mass spectrometry of the N(O)-perfluorobutyryl-O-isoamyl derivatives of amino acids. Anal. Biochem. 67,245-262.

Fetuga, B. L., G. M. Babatunde, and V. A. Oyenuga (1974) Protein quality of some unusual protein foodstuffs: studies the African locust-bean seed (Parkia filicoidea Welw.) Br. J. Nutr. 32,27-36.

Figueirdo, A. A. (1975) "Lebensmittelchemische relevante in haltstoffe der schoten der algarobeira". Ph.D. thesis, Wurzburg, Germany.

Flynt, T. O., and H. L. Morton (1969) A device for threshing mesquite seed. Weed Sci.17,302-303.

Forbes, R. H. (1895) The mesquite tree: its products and uses. Ariz. Agric. Exp. Sta. Bulletin 13,1-26.

Friedman, M., L. H. Krull, and J. F. Cavins (1970) The chromatographic determination of cystine and cysteine residues in proteins as S-β-(4-pyridylethyl) cysteine. J. Biol. Chem. 245,3868-3871.

Garcia, F. (1916) Mesquite beans for pig feeding. N. Mexico. Agric. Exp. Sta. 28th Ann. Report. 77-82.

Gaur, Y. D. (1968) Preliminary studies on titrable acidity in xerophytic plants: Salvadora persica Linn. and Prosopis juliflora D.C. Experientia 24,239-240.

Gehrke, C. W., and K. Leimer (1971) Trimethylsilylation of amino acids derivatization and chromatography. J. Chromatogr. 57,219-238.

Gehrke, C. W., and H. Takeda (1973) Gas-liquid chromatographic analysis of tryptophan in proteins. J. Chromatogr. 76,77-89.

Gehrke, C. W., and H. Takeda (1973) Gas-liquid chromatographic studies on the twenty protein amino acids: a single column separation. J. Chromatog. 76,63-75.

Gelpi, E., W. A. Koenig, J. Gibert, and J. Oro (1969) Combined gas chromatography-mass spectrometry of amino acid derivatives. J. Chromatog. Sci. 7,604-613.

Goodwin, T. W., and R. A. Morton (1946) The spectrophotometric determination of tyrosine and tryptophan in proteins. Biochem. J. 40,628-632.

Gortner, R. A., and E. R. Norris (1923) The origin of the humin formed by the acid hydrolysis of proteins. VII Hydrolysis in the presence of ketones. J.A.C.S. 45,550-553.

Gupta, R. K., and G. S. Balara (1972) Comparative studies on the germination, growth, and seedling biomass of two promising exotics in Rajasthan desert. Ind. Forester. 280-285.

Hendershot, J. M. (1946) Ketosis in the Hawaiian Islands. Amer. Vet. Med. Assn. Journal 108,74-75.

Himes, C. A., and S. J. Pscodna (1976) Michigan Agric. Statistics, Mich. Crop Reporting Service, Mich. Dept. Agric. p.22.

Hirs, C. H. W. (1967) Determination of cystine as cysteic acid in "Methods in Enzymology". S. P. Colowick and N. O. Kaplan Ed. Acad. Press N.Y. Vol 11, 59-62.

Hill, J. E., and R. W. Breidenbach (1974) Proteins of soybean seeds, Isolation and characterization of the major components. Plant. Physiol. 53,742-746.

Homer, A. (1915) A method for the estimation of the tryptophane content of proteins, involving the use of baryta as hydrolyzing agent. J. Biol. Chem. 22,369-389.

Hopkins, F. G., and S. W. Cole (1901) A contribution to the chemistry of proteids. Part I. A preliminary study of a hitherto undescribed product of tryptic digestion. J. Physiol. 27,418-428.

Hugli, T. E., and S. Moore (1972) Determination of the tryptophan content of proteins by ion-exchange chromatography of alkaline hydrolysis. J. Biol. Chem. 247,2828-2834.

Hull, H. M. (1958) The effect of day and night temperature on growth, foliar wax content, and cuticle development of velvet mesquite. Weeds 6,133-142.

Irvine, F. R. (1961) in "Woody Plants of Ghana". Oxford Univ. Press. London, England.

Jansen, E. F., R. Jang, and A. K. Balls (1952) The inhibition of purified, human plasma cholinesterase with diisopropyl fluorophosphate. J. Biol. Chem. 196,247-253.

Johnson, V. A., and C. L. Lay (1974) Genetic improvement of plant protein, J. Agr. Fd. Chem. 22,558-566.

Jones, Q., and F. R. Earle (1966) Chemical analysis of seeds II: oil and protein content of 759 species. Econ. Bot. 20,127-155.

Jones, R. A., B. A. Larkins, and C. Y. Tsai (1976) Reduced synthesis of zein in vitro by a high lysine mutant of maize. Biochem. Biophys. Res. Commun. 69,404-410.

Jurriaanse, A. (1973) Are they fodder trees. Dept. Forestry, Private Bag X93, Pretoria, S. Africa, Pamphlet 116,1-32.

Kakade, M. L. (1974) Biochemical basis for the differences in plant protein utilization. J. Agr. Fd. Chem. 22,550-555.

Karkhanis, Y. D., D. J. Carlo, and J. Zeltner (1975) A simplified procedure to determine tryptophan residues in proteins. Anal. Biochem. 69,55-60.

Kossel, A. and S. Edlbacher (1914) Einige bemerkungen uber das histidin. Hoppe Seylers Zeit. fur Physiol. Chemie. 93,396-400.

Lanza, M., P. Regli, and F. Busson (1962) Contribution a l'etude chimique de la pulpe de fruit de Parkia biglobose Benth. Medecine Tropicale 22,377-384.

Larkins, B. A., and A. Dalby (1975) In vitro synthesis of zein-like protein by maize polyribosomes. Biochem. Biophys. Res. Comm. 66,1048-1054.

Liu, T. Y, and Chang (1971) Hydrolysis of proteins with p-toluenesulfonic acid. J. Biol. Chem. 246,2842-2848.

Loocke, E. E. M. (1947) Three useful leguminous fodder trees. Fmg. S. Africa 2,7-12.

Ma, J. A., and G. Zuazaga (1942) Micro-Kjeldahl determination of nitrogen. Indus. Eng. Chem. 14,280-282.

Mackenzie, S. L., and D. Tenaschuk (1974) Gas-liquid chromatography of N-heptafluorobutyryl isobutyl esters of amino acids. J. Chromatogr. 97,19-24.

Mackenzie, S. L., and D. Tenaschuk (1975) Rapid formation of amino acid isobutyl esters for gas chromatography. J. Chromatog. 111, 413-415.

Malynicz, G. (1974) The effect of adding Leucaena leucocephala meal to commercial rations for growing pigs. Papua New Guinea Agr. J. 25,12-14.

March, J. F. (1975) A modified technique for the quantitative analysis of amino acids by gas chromatography using heptafluorobutyric n-propyl derivatives. Anal. Biochem. 69,420-442.

Martin, W. E. (1958) Sulfur deficiency widespread. California Agric. 10-12.

Marx, D. H., W. C. Bryan, and C. E. Cordell (1976) Growth and ectomycorrhizal development of pine seedlings in nursery soils infested with the fungal symbiont Pisolithus tinctorius. Forestry Sci. 22,91-100.

Matsubara, H., and R. M. Sasaki (1969) High recovery of tryptophan from acid hydrolyzates of proteins. Biochem. Biophys. Res. Comm. 35,175-181.

Means, G. E., and R. E. Feeney (1971) Chemical modification of proteins. Holden-Day Inc. 161,166.

Melchior, W. B., and D. Fahrney (1970) Ethoxyformylation of proteins. Reaction of ethoxyformic anhydride with α chymotrypsin, pepsin, and pancreatic ribonuclease at pH 4. Biochem. 9, 251-258.

Millerd, A. (1975) Biochemistry of legume seed proteins. Ann. Rev. Plant. Physiol. 26,53-72.

Mitchell, H. H., and T. S. Hamilton (1929) in "The Biochemistry of the Amino Acids". Chemical Catalog Co. Publish. ACS monograph 48, 102-104.

Moodie, I. M. (1974) Gas-liquid chromatography of amino acids, a solution to the histidine problem. J. Chromatogr. 99,495-505.

Moore, S., and W. H. Stein (1963) Chromatographic determination of amino acids by the use of automatic recording equipment in "Methods in Enzymology". S. P. Colowick and N. O. Kaplan Ed. Acad. Press N.Y., vol. 6,820-822.

Moss, C. W., M. A. Lambert, and F. J. Diaz (1971) Gas-liquid chromatography of twenty protein amino acids on a single column. J. Chromatogr. 60,134-136.

Murneek, A. E., and J. C. Logan (1932) Autumnal migration of nitrogen and carbohydrate in the apple tree. Missouri Agr. Exp. Sta. Res. Bulletin 171.

Nakai, T., and T. Ohta (1976) β-3-oxindolylalanine: the main intermediate in tryptophan degradation occurring in acid hydrolysis of protein. Biochim Biophys. Acta. 420,258-264.

Noltmann, E. A., T. A. Mahowald, and S. A. Kuby (1962) Studies on adenosine triphosphate transphosphorylases. J. Biol. Chem. 237,1146-1154.

Ochse, J. J. (1931) in "Vegetables of the Dutch East Indies". Archipel Drukkerij Buitenzorg-Java.

Oelshlegel, F. J., J. R. Schroeder, and M. A. Stahmann (1970) A simple procedure for basic hydrolysis of proteins and rapid determination of tryptophan using a starch column. Anal. Biochem. 34, 331-337.

Olsen, R. J., R. F. Hensler, O. J. Attoe, S. A. Witzel, and L. A. Peterson (1970) Fertilizer nitrogen and crop rotation to movement of nitrate nitrogen through soil profiles. Soil Sci. Amer. Proc. 34,448-452.

Orchard, E. R., and G. D. Darb (1956) Fertility changes under continued wattle culture with special reference to nitrogen fixation and base status of the soil. Proc. 6th Int. Soil Sci. Congress, Paris IV,45,305-310.

Penke, B., R. Ferenczi, and K. Kovacs (1974) A new hydrolysis method for determining tryptophan in peptides and proteins. Anal. Biochem. 60,45-50.

Pimentel, D., E. C. Terhune, R. D. Hudson, S. Rochereau, R. Samis, E. A. Smith, D. Denman, D. Reifschneider, and M. Shepard (1976) Land degradation: effects on food and energy resources. Science 194:149-155.

Remers, W. A., and R. K. Brown (1972) Indoles Part One in The Chemistry of Heterocyclic compounds. Vol 25, p. 105. Wiley-Interscience, N.Y.

Ridley, H. N. (1922) Flora of the Malay Peninsula. vol I. L. Reeve & Co. Ltd. Publ., London.

Roach, D., and C. W. Gehrke (1969) Direct esterification of the protein amino acids. Gas liquid chromatography of the N-TFA n-butyl-esters. J. Chromatog. 44,269-278.

Roach, D., C. W. Gehrke, and R. W. Zumwalt (1969) Quantitative gas-liquid chromatography of histidine. J. Chromatogr. 43,311-321.

Romero, J., S. M. Sun, R. C. McCleester, F. A. Bliss, and T. C. Hall (1975) Heritable variation in a polypeptide subunit of the major storage protein of the bean, Phaseolus vulgaris L. Plant. Physiol. 56, 776-779.

Ruggli, P., R. Ratti., and E. Henzi (1929) Uber benzoylderivate des diamino-athylens und ihre unwandlung in imidazolone. Helv. Chim. Acta 12,332-347.

Sasaki, T., B. Abrams, and B. L. Horecker (1975) A fluorometric method for the determination of the tryptophan content of proteins. Anal. Biochem 65,396-404.

Schonau, A. P. G. (1969) A site evaluation study in black wattle (Acacia mearnsii). Annale Universiteit van Stellenbosch 44,2A,79-214, Republic S. Africa

Schuster, J. L. (1969) "Literature on the mesquite (Prosopis L.) of North America". Special Rpt. 26 Int. Ctr. Arid & Semi-Arid Land Studies, Texas Tech. Univ. Lubbock, TX.

Shahrokhi, F., and C. W. Gehrke (1968) Quantitative gas-liquid chromatography of sulfur containing amino acids. J. Chromatogr. 36, 31-41.

Smith, A. K., and S. J. Circle (1972) Chemical composition of the (soybean) seed. in "Soybeans: Chemistry and Technology". A. K. Smith and S. J. Circle Ed. Avi Publ. Co. Westport, Conn.

Smith, J. G. (1900) Fodder and forage plants. USDA-Division of Agrostology. Bulletin 2,56-57.

Smith, J. R. (1953) "Tree Crops-A Permanent Agriculture". Devin-Adair Publ. Co., New York, N.Y.

Smyth, D. G., O. O. Blumenfield, and W. Konigsberg (1964) Reactions of N-ethylmaleimide with peptides and amino acids. Biochem. J. 91,589-595.

Sober, H. A., R. A. Harte, and E. K. Sober (1970) in "Handbook of Biochemistry, selected data for molecular biology". 2nd Ed. Chem. Rubber Co. Publ. Cleveland, Ohio. p. B66.

Solbrig, O. T., and K. S. Bawa (1975) Isozyme variation in species of Prosopis (Leguminosae). J. Arnold Arb. 56,398-412.

Solbrig, O. T., and P. D. Cantino (1975) Reproductive adaptations in Prosopis (Leguminosae, Mimosoideae). J. Arnold. Arb. 56,185-210.

Spies, J. R., and D. C. Chambers (1949) Chemical determination of tryptophan in proteins. Anal. Chem. 21,1249-1266.

Spies, J. R., and D. C. Chambers (1948) Chemical determination of tryptophan. Anal. Chem. 20,30-39.

Staab, H. A. (1956) Transacylierungen II. Uber die sterische beeinflussung der hydrolytischen und aminolytischen spaltung reaktionsfahiger N-acyl-verbindungen. Chem. Ber. 89,2088-2093.

Stohrer, G. (1976) Tryptophan oxidation to dioxindole alanine spirolactone. J. Heter. Chem. 13,157-158.

Strickland, E. H., M. Wilchek, and C. Billups (1973) Circular dichroism of modified tryptophan residues, β-3-oxindolyl-L-alanine. Biochem. Biophys. Acta. 303,28-35.

Sun, S. M., B. U. Buchbinder, and T. C. Hall (1975) Cell-free synthesis of the major storage protein of the bean, Phaseolus vulgaris. Plant. Physiol, 56,780-785.

Supelco (1971) G. C. analysis of amino acid TMS derivatives. 5,1.

Supelco, Inc. (1973) Amine Analysis. Bulletin 737, Supelco Park, Bellefonte, PA.

VanEtten, C. H., W. F. Kwolek, J. E. Peters, and A. S. Barclay (1967) Plant seeds as protein sources for food or feed. Evaluation based on amino acid composition of 379 species. J. Agr. Fd. Chem. 15,1077-1089.

Walton, G. P. (1923) A chemical and structural study of mesquite, carob, and honey locust beans. USDA Dept. Bulletin, 1194,1-19.

Wealth of India (1966) Parkia. Pulbications and Information Directorate CSIR New Delhi, India, vol 7,264-265.

Wealth of India (1969) Pithecellobium, Prosopis. Publications and Information Directorate CSIR New Delhi, India. 8,140-143, & 245-249.

Wieghardt, T., and H. J. Goren (1975) The reactivity of imidazole nitrogens in histidine to alkylation. Bioorg. Chem. 4,30-40.

Wilcox, E. V. (1910) The algaroba in Hawaii. Hawaii Agric. Exp. Station. Press Bulletin 26,1-8.

Williams, W. A. (1967) The role of the Leguminosae in pasture and soil improvement in the Neotropics. Trop. Agric. Trinidad. vol 44, 103-115.

Wolf, W. J. (1972) Purification and properties of the (soybean) proteins in "Soybeans: chemistry and technology". A. K. Smith and S. J. Circle Ed. Avi Publ. Co. Westport, Conn.

Wolf, W. J., and D. R. Briggs (1956) Ultracentrifugal investigation of the effect of neutral salts on the extraction of soybean proteins. Arch. Biochem Biophys. 63,40-49.

Zanetta, J. P., and G. Vincendon (1973) Gas-liquid chromatography of the N(O) heptafluorobutyrates of the isoamyl esters of amino acids. J. Chromatogr. 76,91-99.

Zarger, T. G. (1956) Status of tree crop investigations in the Tennessee Valley Region. Report 47th Ann. Mtg. North. Nut Growers Assn. 57-68.

Zumwalt, R. W., D. Roach, and C. W. Gehrke (1970) Gas-liquid chromatography of amino acids in biological substances. J. Chromatogr. 53,171-193.