STUDIES LEADING TO 'NON-DESTRUCTIVE' SINGLE KERNEL ESTIMATION OF AVAILABLE LYSINE, TRYPTOPHAN AND PROTEIN IN CEREALS

A Thesis

Submitted to the Faculty

of

Graduate Studies

The University of Manitoba

by

Charles Nkonge

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

Department of Plant Science

October, 1981

STUDIES LEADING TO 'NON-DESTRUCTIVE' SINGLE KERNEL ESTIMATION OF AVAILABLE LYSINE, TRYPTOPHAN AND PROTEIN IN CEREALS

BY

CHARLES NKONGE

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

And you, take wheat and barley, beans and lentils and spelt and put them into a single vessel and make bread of them. During the number of days that you lie upon your side, three hundred and ninety days, you shall eat it. (Ezek. 4:9)

ACKNOWLEDGMENTS

The author of this thesis wishes to extend his sincere thanks to the following:

Dr. G.M. Ballance, the major advisor, for his wise counselling at all stages of the work reported here, for his good understanding and his patience.

Dr. B.L. Dronzek for having the total amino acid analysis of the samples reported here done.

Drs. B.L. Dronzek, E.N. Larter and E.D. Murray for serving on his advisory committee and for their keen reviewing of the manuscript.

Dr. W. Bushuk, former head of the Plant Science Department, for accepting the author as a student in his department.

Professor W. Schulthess of the University of Nairobi for all the arrangements he made as concerns this study.

T. Nylen for being a good technician and putting up with all the rough times in the laboratory.

B. Luit, R. Batenchuk and the greenhouse staff for their invaluable services.

P. Ngai for his encouragement, B. Gasman and L. Griffith for being good office mates and L. Macri for being a good work mate.

B. Drebert for her excellent typing of this thesis.

Government of Switzerland for financial support and Ministry of Agriculture, Kenya for granting the study leave.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Nkonge, Charles, M.Sc., The University of Manitoba, October, 1981. Studies Leading to 'Non-Destructive' Single Kernel Estimation of Available Lysine, Tryptophan and Protein in Cereals. Major Professor: Dr. G.M. Ballance.

A 'non-destructive' single kernel estimation of available lysine, tryptophan and protein from cereals such as barley, rye, triticale and wheat is reported. To allow a more accurate subsampling of samples on a volume basis and to facilitate the available lysine and tryptophan analyses the cereal proteins from samples were first solubilized by enzymes. To achieve the above type of analysis the study that followed was carried out under five sections as follows: i) Protein Estimation, ii) Enzymic Solubilization of Cereal Proteins, iii) Available Lysine Estimation, iv) Tryptophan Estimation and v) 'Non-destructive' Single Kernel Analyses.

Protein Estimation. A phenol-hypochlorite based method was used for ammonia determination after the micro-Kjeldahl digestion of samples. Under the optimized assay conditions a minimum of 40% improvement in relative sensitivity over the previous methods was achieved. An inherent problem in this assay of having to treat only a few samples at a time was overcome. Protein values obtained by this method were in excellent agreement with the conventional micro-Kjeldahl titration

procedure values.

Enzymic Solubilization of Cereal Proteins. Five enzymes were evaluated for their ability to solubilize cereal proteins. Under selected conditions at least 95% of total protein from each of 22 different cereal varieties representing 7 cereal grain types was solubilized by at least one of the enzymes Pronase, papain or thermolysin. Both trypsin and α-chymotrypsin were found unsuitable for cereal protein solubilization.

Available Lysine Estimation. The assay that was used for the available lysine estimation was shown to be affected by both the buffer species that were present in the reaction mixture and the level of free amino acids other than lysine. The naturally occurring amino acids were found to fall into four groups according to their effect on the absorbance values of standard lysine samples. Under the new optimized assay conditions a minimum of 50% improvement in relative sensitivity was achieved. Different enzymes were found not equally suitable for cereal protein solubilization where available lysine was to be estimated even if they solubilized similar amounts of protein. Available lysine values obtained from papain 'supernatants' were closely correlated to total lysine values.

Tryptophan Estimation. The glyoxylic acid method was used for the tryptophan estimation. The working reagent (Reagent C) was composed of equal volumes of glacial acetic acid containing ferric chloride (Reagent A) and 30 N H_2SO_4 (Reagent B). The color forming ability of Reagent C with tryptophan was found to change with exposure to light of Reagent A containing no or suboptimum levels of acetic anhydride.

A minimum of 16% v/v of acetic anhydride in freshly prepared Reagent A was found to be optimum for maximum color formation of Reagent C with tryptophan. Under the new conditions of this assay a minimum of 20% improvement in relative sensitivity over previous methods was achieved. The tryptophan values that were obtained from the cereal protein 'supernatants' were similar to some of those that have been reported in literature.

'Non-destructive' Single Kernel Analyses. Using scaled down versions of the procedures presented in Sections I-IV of this thesis, it was shown that available lysine, tryptophan and protein could all accurately be estimated from distal parts of seeds. The proximal parts of the analyzed seeds were shown to germinate and grow to maturity without special treatments.

GENERAL INTRODUCTION

1. Importance of Cereal Grains

That the cereal grains account directly for 60% of the total world protein supply underlines their importance. In terms of overall production and consumption, the major cereal foods of the world are roughly in the order cited, wheat, rice, maize, sorghum, millets and barley (Shukla, 1975). It is thought that oat, rye and triticale may in future attain the production and consumption status of the other cereal crops. In the developing and underdeveloped nations, the cereals contributed directly to 82% of the total protein intake while in the industrialized nations they contributed 34% of the total protein consumption in the diet (Shukla, 1975).

2. Cereal Protein Quality and Quantity

Although the cereals are the most important source of dietary protein for the majority of the world population, they are found to be deficient in both protein quality and sometimes protein quantity. When the cereals are used as the only source of protein for man and other monogastric animals they are found not to support life very well. Sometimes a negative nitrogen balance results (Nelson, 1969). This is because the cereal proteins are characterized by amino acid imbalances and deficiencies. Even in biblical times it was possibly known that cereal grains would not supply all the nutritional requirements of man.

However when they were supplemented with legumes, the mixture could continue to be the only source of food for man for a long time (Ezek. 4:9).

Of about 20 naturally occurring amino acids 8 of them must be supplied in the diet of an adult human being if normal growth is to take place. The amino acids that must be supplied are lysine, tryptophan, phenylalanine, methionine, leucine, isoleucine, threonine and valine (Nelson, 1969). These amino acids must be supplied in the diet because the body has no ability to synthesize them from the other amino acids, and are hence termed essential. The cereal proteins are poor in protein quality because they contain less of one or more of the essential amino acids than is required by the body. When this is the case, the amino acid is said to be limiting. The essential amino acid which is least in amount relative to the body requirements is known as the 1st limiting amino acid.

For the cereal proteins, the 1st limiting amino acid has been shown to be lysine. On many instances the primary deficiency of lysine is further intensified by a secondary deficiency of other amino acids e.g. threonine and/or tryptophan (Kakade, 1974). Howe *et al.* (1965) in trials with weanling rats have shown that lysine is the 1st limiting amino acid in rice, wheat, millet, barley, sorghum, rye and oats. For corn, lysine and tryptophan are co-limiting (Nelson, 1969). In all these cereals except maize threonine appeared to be the 2nd limiting amino acid (Nelson, 1969).

The body requirement for essential amino acids is given by the ideal amino acid pattern formulated for the optimum utilization of protein (Shukla, 1975). This pattern should be the yardstick for the

cereal protein nutritional quality. The ideal level of lysine, the most limiting amino acid in the cereal proteins, is 5.0% on protein basis. When the cereal proteins have attained this ideal level of lysine, and assuming that no other amino acid becomes limiting, the cereal proteins could contibute all the dietary protein requirements for monogastric animals without supplementation.

It was estimated that 15% good-quality protein in the diet would be adequate for most young animals and 12% would satisfy the needs for mature animals (Mertz, 1968). At this level of good quality protein, when sufficient cereal is consumed to satisfy caloric requirements, the amount of protein ingested would be adequate for the essential amino acid requirements of the body (Howe *et al.*, 1965). Both barley (Balaravi *et al.*, 1976) and maize varieties (Nelson, Jr., 1970) with lysine values of 5.0% or more and protein contents of between 12 and 15% or higher, have been reported. High lysine sorghums with 3.34 and 3.13 g lysine per 100 g protein and protein contents of 15.7 and 17.2% respectively have been found from the world sorghum collection (Singh and Axtell, 1973). Thus the genetic potential of achieving improved levels of this essential amino acid have been identified for several cereals.

3. Cereal Protein Fractions and Lysine Content

The cereal proteins fall into four major categories based on their solubility: albumins -water soluble, globulins -soluble in dilute neutral salt solution, prolamines -soluble in 70% ethyl alcohol and glutelins -soluble in dilute alkali or acid solution (Johnson and Lay, 1974). The albumins and the globulins are the richest in lysine while

the prolamines are the poorest. The glutelin fraction of all the cereals tested has a considerably higher lysine content than the prolamine fraction, with wheat and corn glutelins both having a lysine content of 3.2 g per 100 g protein (Nelson, 1969). The prolamines of maize and sorghum have 0.1 and 0.2% respectively of lysine on a protein basis (Mosse, 1966; Nelson, 1969). The prolamines of wheat, rye and barley all contain larger percentages of lysine than do prolamines of maize (Mosse, 1966).

Since the amino acid lysine is the most nutritionally limiting in the cereals, improvement of nutritional quality of cereal proteins must begin with lysine (Johnson *et al.*, 1970; Nelson, 1969). For maize, lysine and tryptophan are co-limiting (Nelson, 1969) and hence should be considered simultaneously.

4. Genes Controlling the Lysine Content

The prolamine content of the major cereals has been reported to fall into three rather distinct groups. In group one is rice and oats containing 5-10% prolamine, while barley and wheat in group two contain 30 to 40 percent. Maize and sorghum in group three contain 50 to 60 percent prolamine (National Research Council, 1977). Since the lysine content and hence the protein quality in cereals is in general inversely related to their prolamine content, these cereal groups increase in protein quality as they decrease in prolamine content. The changes from the higher to lower prolamine content appear to occur in two steps. This has suggested that the regulation of the prolamine content in cereal grains is controlled by two major genes. In *Opaque*-2 maize (Mertz *et al.*, 1964) and in high lysine (*hl*) sorghum (Singh and

Axtell, 1973), mutations have reduced the content of prolamines to that found in barley. In barley, Risø 1508 (Rhodes, 1975) has a reduced prolamine content similar to that found in rice and oats.

These genes controlling the prolamine content in *Opaque*-2 maize (Mertz *et al.*, 1964), high lysine (*hl*) sorghum (Singh and Axtell, 1973) and the *Hiproly* barley (Hagberg *et al.*, 1970) are all recessive. Their effects on the triploid endosperm are realized when they are present in three doses. Expression of these genes except in *Hiproly* barley (Ma and Nelson, 1975) causes reduced synthesis of the lysine-poor prolamine fraction and an improved level of lysine-rich proteins within one or more of lysine-rich albumin, globulin or glutelin fractions (Kamra, 1971). The net result is an increase in lysine percent on a protein basis. Furthermore, the effect of the high lysine genes is only imparted in the kernel endosperm while the lysine content of the embryo of both normal and high lysine seeds remains the same (Nelson, 1969; Singh and Axtell, 1973). When some of the artificial mutations that increase the lysine content in the cereal proteins have been induced, they have been found to have similar effects on protein fractions as the natural mutations (Doll and Køie, 1977; Ahokas, 1978).

5. Selection Methods

At segregation, identification of high lysine seeds becomes easier because it is controlled by recessive genes. Characteristics controlled by recessive genes will either be fully expressed or not expressed at all. Both physical and chemical methods have been used in the identification of high lysine seeds. The physical methods that have been used depend on the marker genes that are associated with the high lysine

lines. For maize, opaqueness or floury kernels (Nelson, 1969) and in sorghum, floury kernels (Singh and Axtell, 1973) have been used for identification of high lysine kernels. In barley, shrivelled kernels (Doll and Køie, 1977) are associated with high lysine genes. Use of these physical methods for selection of the high lysine seeds, however, precludes the selection of kernels with more desirable characteristics (Johnson *et al.*, 1970). This is because these characteristics used for selection of high lysine seeds are at the same time undesirable. For this reason chemical methods are more favoured. However, chemical methods are generally destructive in nature.

To speed up breeding of varieties with high protein quality and other desirable characteristics, methods must be sought that accurately determine lysine, tryptophan and protein contents without destroying the kernels. These methods would make it possible for only those kernels that have been identified with the desirable characteristics to be directly used for further breeding work. Since most of the high lysine genes are recessive in nature, all those seeds identified for high lysine values will have already stopped segregating for this characteristic. The percent lysine on a protein basis of the high lysine cereals has also been reported to vary with different genetic backgrounds (Nelson, Jr., 1970). Accurate determination of the lysine values at the early stages of a breeding operation would help to eliminate these unsuitable backgrounds.

The term 'non-destructive' which has been used throughout this thesis will be defined and designated somewhat differently from the commonly accepted meaning of the expression. In this thesis 'non-

destructive' seed analysis refers to analyses which do not preclude direct propagation of a portion of the genetic unit that has been analyzed. Specifically, it refers to analyses of the distal portion of the seed while maintaining the embryo portion intact for potential propagation. Thus 'non-destructive' refers to maintenance of a particular combination of genetic characteristics found within a single seed.

At present protein can be determined accurately enough without destroying the samples (Dohan *et al.*, 1976). 'Non-destructive' kernel estimation of lysine has been done by use of basic dyes. However, since these dyes react with all the basic amino acids (Sharma and Kaul, 1971) the methods are not accurate enough. Recently Ahokas (1978) presented a method for analyses of both tryptophan and protein in kernel distal halves and small samples of barley meals by first extracting the protein from the samples with ethanolic hydroxide solution. However, the ethanolic hydroxide extracted only 64.3% of the total protein from the half kernels. The method was not suited for routine analysis because of critical timing of some operations.

6. Aims of Research and Strategies Adopted

This study was carried out to evaluate the possibility of using existing methods with or without any modifications to perform fast and accurate 'non-destructive' single kernel estimation of available lysine, tryptophan and protein. Available lysine in this context means lysine molecules with free epsilon amino groups. The methods should be applicable to many cereal grain types. These methods could then be

used for the improved protein quality evaluation in the earliest stages of breeding programs while the population is still segregating. It was desirable that these methods also be applicable on large samples so that they could be used at the advanced breeding stages. The specific problems that were identified in this type of analysis centered around three parameters: 1) limited amount of sample, 2) protein insolubility and 3) compatibility of the assay systems to be used.

6.1 Limited Amount of Sample

In the cereal grains like barley, oats, rye, triticale and wheat on average the whole kernel would weigh 30-40 mg. If one-half of the kernel were used for analysis this would represent a sample size of 15-20 mg. Furthermore, assuming a protein content of 10-15%, this would mean that in effect the analysis for protein, available lysine and tryptophan estimations would have to be done on a total of 1.5-3.0 mg of protein. Directly associated with the analysis of such a small sample are:

a) Sensitivities of the Procedures to be Used. Highly sensitive procedures would be required to accurately estimate the protein and the two essential amino acids from each kernel.

b) Sample Preparation. Firstly, grinding of 15-20 mg of sample is not possible in the conventional laboratory mills without seriously affecting the recoveries. This necessitated special methods for grinding the samples. Secondly, even if sensitive enough methods were available, subdivision of the samples on weight basis would not be practical without seriously causing both sampling and weighing errors.

6.2 Protein Insolubility

Analyses are initially confounded by the insolubility of most of the cereal storage protein. However, if complete solubilization of the protein in the sample was possible, estimation of protein, available lysine and tryptophan in the solution could be more easily achieved. This could at the same time allow a more accurate subsampling on volume basis.

There are not many single solvents that will give a complete solubilization of cereal proteins without interfering with some of the assays to be performed. Use of acid or base (which are the most common methods) to solubilize or hydrolyze the protein would lead to destruction of lysine or tryptophan and some other amino acids. For these reasons it was decided to examine the potential of using proteolytic enzymes to achieve protein solubilization.

6.3 Compatibility of the Assay Systems

To speed up the analyses any methods that could be used to directly estimate protein, and the two essential amino acids from the hydrolyzate without any further pretreatment of sample would be most desirable.

6.4 Work Scheme

To accomplish the 'non-destructive' single kernel analyses the study that follows was divided into five major sections as follows:

Section I - Protein Estimation

Section II - Enzymic Solubilization of Cereal Proteins

Section III - Available Lysine Estimation

Section IV - Tryptophan Estimation

Section V - 'Non-destructive' Single Kernel Analyses

All the studies that were done in Sections I to IV used samples larger than what would be available from the distal half seeds. This was found necessary for both accuracy and convenience. It was at the same time recognized that these assays must be scaled down without loss of accuracy but with the required sensitivities. In Section V the scaling down and the direct application of these assays to distal half seeds were examined.

GENERAL MATERIALS

1. Chemicals

Tribasic sodium phosphate, anhydrous sodium bicarbonate, anhydrous sodium carbonate, ethyl acetate, acetic anhydride, stannous chloride dihydrate, n-propanol, sodium nitroprusside dihydrate, anhydrous dibasic sodium phosphate, sodium hydroxide, sodium potassium tartarate, calcium chloride dihydrate and petroleum ether were certified A.C.S. grade; cupric chloride dihydrate, ethylene glycol monomethyl ether (methyl cellosolve) and sodium salicylate were certified grade; hydrochloric acid, sulphuric acid and glacial acetic acid (assay 99.8%) were reagent A.C.S. grade; methanol was technical grade and anhydrous ammonium sulfate was certified primary standard grade. All the above chemicals and reagents were supplied from Fisher Scientific Company.

Sodium borate decahydrate and ferric chloride hexahydrate were Baker analyzed reagents from May and Baker Company. L-lysine monohydrochloride and DL-tryptophan were supplied from Sigma Chemical Company; 2-chloro-3,5-dinitropyridine was obtained from Eastman Kodak Company, U.S. Ninhydrin was a Pierce reagent grade from Pierce Chemical Company, while commercial liquid bleach (Javex) was obtained from Bristol-Myers, Canada Limited. The Kjeldahl catalyst mixture was a product of Pope Kjeldahl Mixtures, Dallas, Texas and contained on weight basis 0.3% TiO_2, 0.3% $CUSO_4$, 10% K_2SO_4 with the remainder being pumice. The

reagents that were used in this study were made from the above chemicals. Any other chemicals and reagents used have been mentioned in the text.

2. Apparatus

All the samples were weighed on either top loading Mettler P1210 or analytical Mettler H35 balances. The samples were ground in a Udy Cyclone mill as described in the text. A Beckman digital pH meter model 76 equipped with a general purpose electrode was used for the pH readings.

Large volume pipettings were accomplished by use of 5.0 ml or 10.0 ml automatic pipettes obtained from Lancer Brunswick, U.S., while small volume pipettings were done with either 0.20 ml or 1.0 ml automatic pipettes (Pipettman Gilson). All the centrifugations were done in a Beckman model J2-21 centrifuge. Absorbance readings were recorded in a Zeiss PMQ spectrophotometer and QMIII monochrometer. Any other apparatus or equipment used in this study have been mentioned in the text.

I. PROTEIN ESTIMATION

1. Review of Literature

There are several methods for the quantitative determination of protein. Of these there is no completely satisfactory one to determine concentration of protein in a given sample. The choice of the method to be used will depend on the nature of the protein under test, the other components in the sample that would interfere as well as the desired speed, accuracy and sensitivity of the assay. Some of the methods that have been commonly used are discussed below.

In this study, protein estimations in enzymic hydrolyzates and solid cereal samples were to be done. The method that was going to be used had to be sensitive, fast and accurate because it was to be applied to enzymic hydrolyzates of single distal half kernels in a breeding operation.

1.1 Estimation by Absorbance Measurements at 280 nm

Estimation of the amount of protein at 280 nm is based on the fact that both tyrosine and tryptophan strongly absorb light at this wavelength. Since the concentrations of these amino acids are constant in any one protein, the concentration of the protein should be proportional to the absorption at 280 nm (Kalckar, 1947). A number of proteins in solution at a concentration of 1 mg/ml have absorbance readings of about 1.0 in 1 cm path length cuvettes at this wavelength (Clark, Jr., 1963).

Although this method is rapid it cannot be successfully applied in a situation where many different proteins are present. This is because different proteins may contain different amounts of tyrosine and tryptophan.

1.2 Estimation by Absorbance Measurements at 215 and 225 nm

Proteins strongly absorb ultra-violet (uv) light in the region of 200-220 nm (Ahokas, 1978) mainly due to the peptide bonds. Thus a method was developed for the estimation of protein in dilute solutions from uv absorption at 215 and 225 nm (Waddell, 1956). Later this method was successfully used for the estimation of protein in kernel halves as well as small samples of barley meal (Ahokas, 1978). However since the method measures a property of peptide bonds, accurate values may not be obtained in protein with different degrees of hydrolysis.

1.3 Estimation by Ninhydrin Reagent

This method is based on the reactivity of the ninhydrin reagent with the free alpha amino groups in proteins (Cocking and Yemm, 1954; Yemm and Cocking, 1955) to produce a chromophore measurable at 570 nm. Since the reaction involves the amino groups, the values obtained will depend on the degree of hydrolysis of a particular protein. The protein values that were obtained by Lein *et al.* (1973) using this method on cereal protein hydrolyzates were found to correlate well with the micro-Kjeldahl values.

1.4 Estimation by Biuret Test

This reaction is specific for compounds containing two or more peptide bonds. The color formed is due to the coordination complex

of the copper atom and four nitrogen atoms, two from each of the peptide chain (Clark, Jr., 1963). The biuret test although fairly reproducible requires relatively large amounts of protein (1-20 mg) for color formation (Clark, Jr., 1963). Using this method, values very close to the micro-Kjeldahl ones (Lein *et al.*, 1973) have been obtained. The values obtained by this method depend on the degree of hydrolysis of the particular protein sample.

1.5 Estimation by Lowry Method

The Lowry *et al.* (1951) method combines the properties of both peptide bonds and the amino acids tryptophan and tyrosine to estimate the amount of protein.

The color formed by the Folin-Ciocalteau reagent used in this procedure is due to the reaction of protein with alkaline copper and the reduction of the phosphomolybdate-phosphotungstate salts in the reagent by the tyrosine and tryptophan of proteins. Samples containing as little as 5 μg of protein can be readily analyzed (Clark, Jr., 1963). The accuracy of this procedure again depends on using proteins of similar composition.

1.6 Estimation of Protein after Conversion to NH_4^+

All the above five methods are applicable to samples in solution form. When estimation of total protein in either soluble or solid sample is required, a standard method that is commonly used is the Kjeldahl digestion, or its scaled down form, the micro-Kjeldahl digestion. The sample is first heated, usually with concentrated sulphuric acid in the presence of a catalyst at a elevated temperature to convert

the protein or proteinaceous material to NH_4^+. The ammonia in the acid digest can be estimated in various ways. A protein value is obtained by multiplying the nitrogen value by a nitrogen to protein conversion factor obtainable from the protein's amino acid composition.

1.6.1 Estimation of Ammonia by Titration. The classical method for the estimation of ammonia in Kjeldahl digests involves converting NH_4^+ to NH_3 (volatile) under alkaline conditions, retrapping the ammonia so released in a boric acid solution followed by titration with a standard acid. This method, apart from being time consuming, is relatively insensitive and can be applied to large samples only. As a result alternative procedures have been sought.

1.6.2 Estimation of Ammonia by Nessler's Reagent. In an alkaline solution ammonia will react with Nessler's reagent (mercuric potassium iodide) to yield a reddish-brown colloid which absorbs strongly over a broad wavelength range (Burns, 1969). The method can be used for samples containing 1-1000 µg N (Bailey, 1967_b). Although the sensitivity of this method is high, sometimes higher sensitivities may be required. For this a more attractive method has been the Berthelot reaction.

1.6.3 Use of Berthelot Reaction to Estimate Ammonia. The Berthelot (phenol-hypochlorite) reaction (Patton and Crouch, 1977) in which ammonia, hypochlorous acid and alkaline phenol react to yield an intensily blue chromophore is both highly specific for ammonia and extremely sensitive (Patton and Crouch, 1977). In this assay N concentrations in the range of 0.2-10 µg/ml have been reported to be closely linearly related (Bietz, 1974).

Various combinations of different phenol reagents and sources of available chlorine have been used for the estimation of ammonia (Patton and Crouch, 1977; Yamaguchi *et al.*, 1970; Reardon *et al.*, 1966) in the Berthelot reaction based methods.

1.7 General Deductions

Of the methods that have been discussed, the estimation of ammonia after a micro-Kjeldahl digestion was in the view of the author the most suitable method for the protein estimation in the cereal grains. Because the assay was to be applied to a 'non-destructive' single kernel analysis, Berthelot reaction was found more applicable because of its sensitivity. This method was therefore examined for the estimation of nitrogen after micro-Kjeldahl digestions of the samples.

2. Introduction

The Berthelot reaction based colorimetric method of Reardon *et al.* (1966) with the reagent proportions of Wall and Gehrke (1975) was used for the ammonia nitrogen estimation in the micro-Kjeldahl digests. However, although this method was chosen for use in this study because of its high sensitivity, only one or two samples could be treated at a time if the Koops *et al.* (1975) procedure is followed. Wall and Gehrke (1975) as well as Reardon *et al.* (1966) did not comment on this problem.

For many protein determinations a procedure that could treat many samples at the same time would be of great use. It was therefore decided to determine whether the colorimetric conditions in this assay could be modified to allow analyses of many samples at a time while retaining or improving the assay's sensitivity.

3. Materials and Methods

3.1 Micro-Kjeldahl Digestion Conditions

The samples were accurately weighed (50-100 mg) into specially made micro-Kjeldahl flasks with a 100 ml calibration marks, to which the catalyst mixture (1.0 g) and concentrated sulfuric acid (3.0 ml) were added. The digests were heated for 20 minutes beyond the point when the solutions had cleared. They were then cooled and diluted to 100 ml with distilled water.

Standard nitrogen solutions which were used for establishing optimum assay conditions were prepared using oven-dried (3 h at 103° C) ammonium sulfate. The ammonium sulfate (47.16 mg) was substituted for the sample in the above digest conditions. After digestion and dilution to 100 ml, this yielded a solution of 100 µg N/ml. Blank digests were prepared in an identical manner, but containing just catalyst and acid. These were used for dilution of the 100 µg N/ml solution to suitable values.

3.2 pH Measurements

A Beckman digital pH meter was used for the pH readings. Corrections for sodium error at the high pH values were made according to the electrode manufacturer's instructions.

3.3 Reagents for Ammonia Estimation

1) The working buffer was prepared from two stock solutions. Solution A was composed of Na_2HPO_4 (0.20 M), NaOH (0.20 M), sodium potassium tartarate·$4H_2O$ (0.36 M) prepared in distilled deionized water. Solution B was 2.50 M NaOH. The working buffer was prepared by mixing

A and B on a 1:1 volume basis.

2) Salicylate-nitroprusside reagent was prepared by dissolving sodium salicylate (20.0 g) and sodium nitroprusside (30.0 mg) in distilled deionized water and diluting to 100 ml.

3) Hypochlorite solution of the appropriate concentration was prepared prior to use by dilution with distilled deionized water of a commercial solution (5.2% w/v, available chlorine as determined by method 6.111 of the A.O.A.C. Official Methods of Analysis, 12th ed., 1975).

3.4 Standard Procedure for Ammonia Estimation

To 0.50 ml of the diluted digest containing 2-10 µg N, 1.50 ml of working buffer was added. After addition of 0.40 ml of salicylate-nitroprusside reagent, the solution was mixed and placed in a water bath and allowed to reach the incubation temperature before 0.20 ml of the hypochlorite reagent was added. The complete reaction mixture was mixed and incubated for a further 30 minutes in a shaking water bath. The sample was then diluted with 10 ml of distilled water and the absorbance at 660 nm was measured.

3.5 Evaluation of the Accuracy of the Standard Procedure

To evaluate the accuracy of this colorimetric procedure, values obtained were compared to the conventional micro-Kjeldahl titration procedure values. Samples used in the comparison were derived from cereal and legume grains ranging in crude protein from 7-29% (N X 5.7). Samples were individually ground to fine grist in a Udy Cyclone mill prior to micro-Kjeldahl digestion.

3.6 Distillation and Titration Method for Ammonia Estimation

Distillation of the ammonia from micro-Kjeldahl digests was carried out according to the A.O.A.C. Method 47.023. The HCl used for titration was standardized according to A.O.A.C. Methods 50.014 and 50.015 (Official Methods of Analysis, 12th ed., 1975).

4. Results and Discussion

4.1 Optimization of the Colorimetric Assay Conditions

4.1.1 Salicylate-Nitroprusside Reagent. The Wall and Gehrke (1975) sodium salicylate and sodium nitroprusside concentrations of 15 g/100 ml and 30 mg/100 ml, respectively were initially used to evaluate the optimum pH as well as hypochlorite concentration for the maximum color formation. Later when the concentrations of both sodium salicylate and sodium nitroprusside were re-examined, it was found that 30 mg/100 ml was the optimum for sodium nitroprusside (Fig. 1) but 15 g/100 ml of sodium nitroprusside was not optimum. The optimum sodium salicylate concentration was found to be 20 g/100 ml (Fig. 2), and this concentration was adopted for all the subsequent investigations.

4.1.2 The Effect of pH on Absorbance Values. In spite of the fact that the alkaline reagent proportions of Wall and Gehrke (1975) were found to buffer at a pH of around 13.1 for the micro-Kjeldahl digest-colorimetric assay proportions used here (Materials and Methods), low color yields were obtained. Many factors were interacting at the same time. Although eventually the low color yields were traced to the wrong hypochlorite concentrations and other factors reported here, it also

Figure 1. Effect of sodium nitroprusside concentration on color yield for 4 µg N ⊙—⊙ and 10 µg N ●—●.

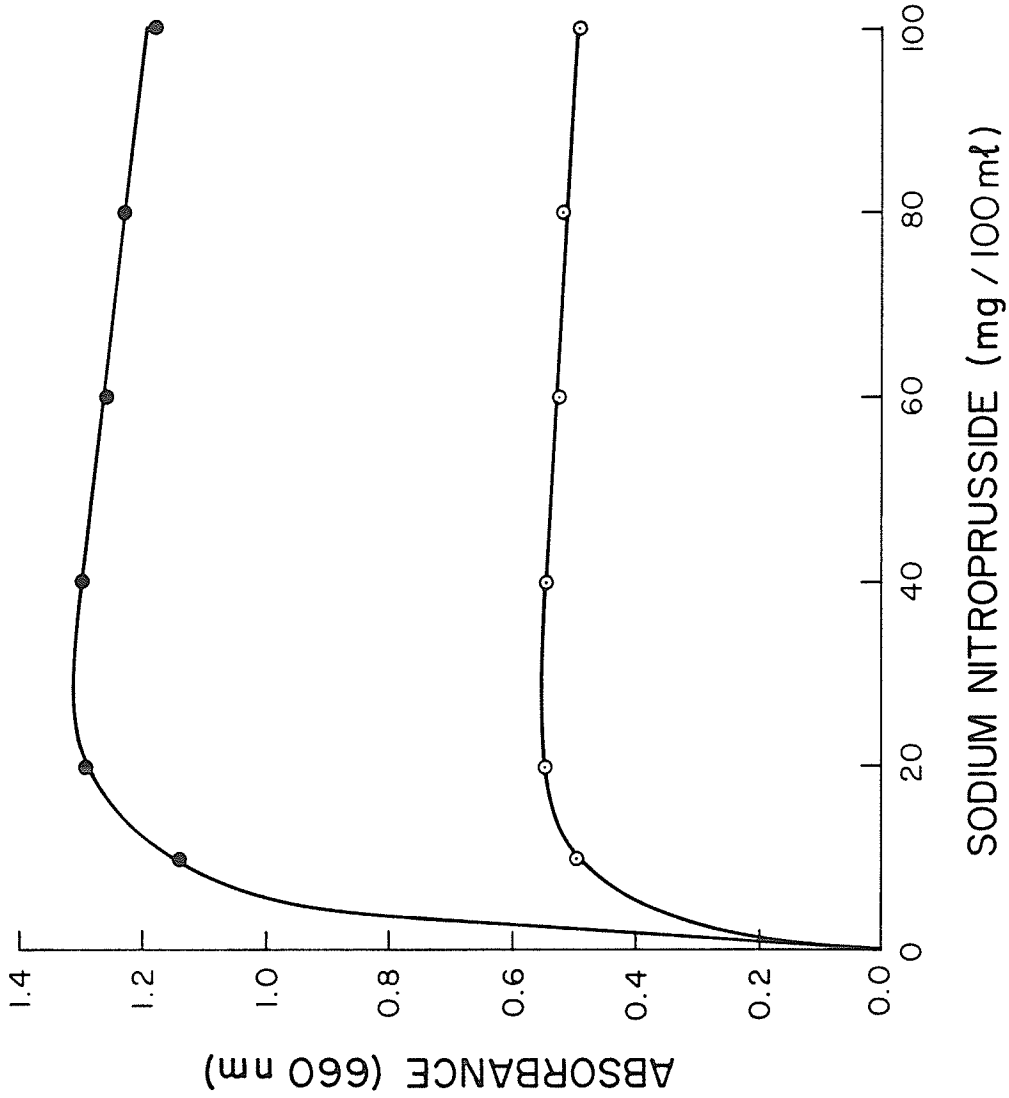
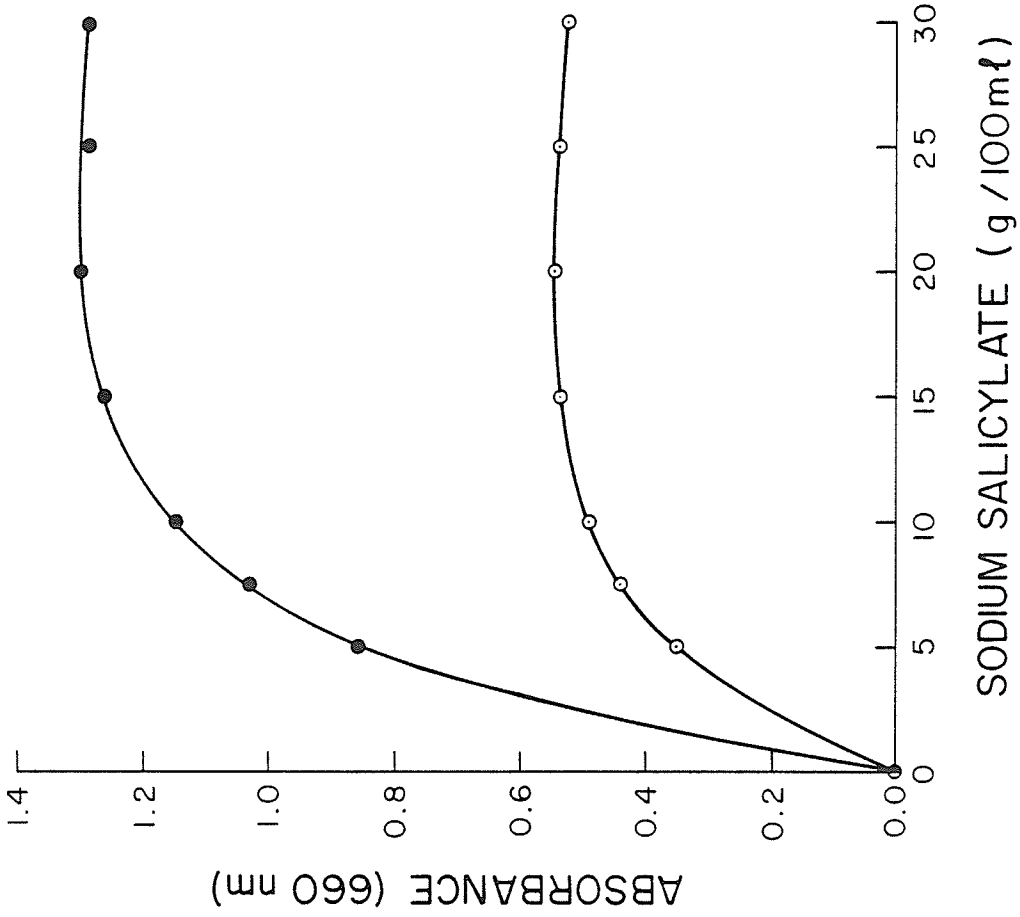

ABSORBANCE (660 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
SODIUM NITROPRUSSIDE (mg / 100 mℓ)

Figure 2. Effect of sodium salicylate concentration on color yield for 4 µg N ⊙—⊙ and 10 µg N ●—● .


ABSORBANCE (660 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
SODIUM SALICYLATE (g/100ml)

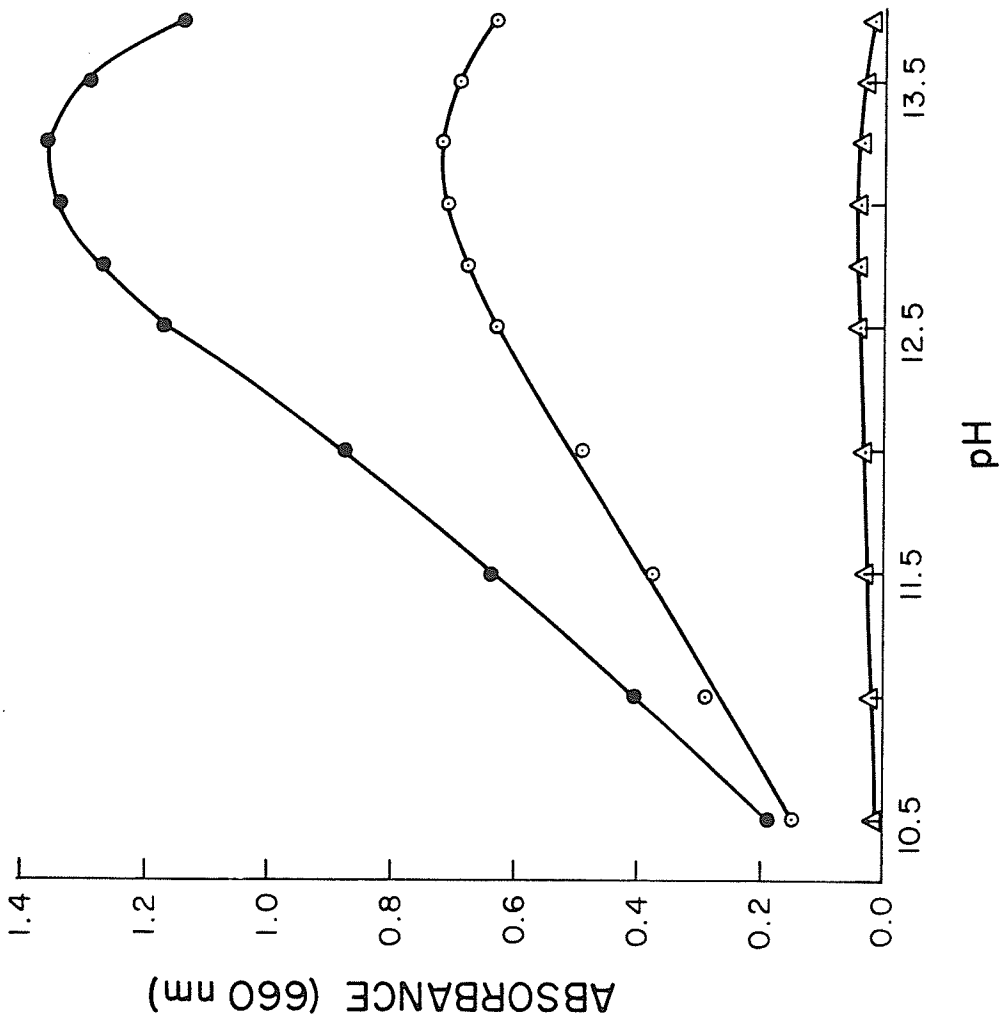
led to the re-examination of the effect of pH on the assay.

The other reason for the re-examination of the effect of pH was because of the many values that have been quoted as optimum for color formation. Most of the pH optima in the phenolic-hypochlorite methods that have been reported are below 12.5 (Bietz, 1974; Yamaguchi *et al.*, 1970; Fawcett and Scott, 1960). However an optimum pH of 12.8-13.1 in the incubation mixture for maximum color formation was reported by Pym and Milham (1976).

To determine the pH optimum for the salicylate-hypochlorite method outlined here, the pH of the incubation mixture was adjusted with phosphate and sodium hydroxide solutions. The results that were obtained (Fig. 3) indicated that maximum color development occurred when the incubation mixture had a pH of 12.9 to 13.3. When the micro-Kjeldahl digestions were done by the standard procedure reported in the Materials and Methods Section, routinely the resultant pH of the incubation mixture was 13.1. The pH-absorbance curve that was obtained differs from that reported by Pym and Milham (1976) in that no shoulder on the curve was observed in this study.

4.1.3 Effect of Available Chlorine Concentrations on the Absorbance Values. For the ammonia nitrogen estimations with nitroprusside as a catalyst, various concentrations of available chlorine have been used in the phenol-based methods. These concentrations have ranged from as low as 0.016% (Miller and Rice, Jr., 1963) to as high as 0.095% (Searcy *et al.*, 1965) on the incubation mixture basis. Preliminary results using the Wall and Gehrke (1975) modification of the salicylate-based method of Reardon *et al.* (1966) indicated that an optimum concentration

Figure 3. Color yield as a function of incubation pH for 0 µg N △—△ , 5 µg N ⊙—⊙ and 10 µg N ●—●.


ABSORBANCE (660 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10.5
11.5
12.5
13.5
pH

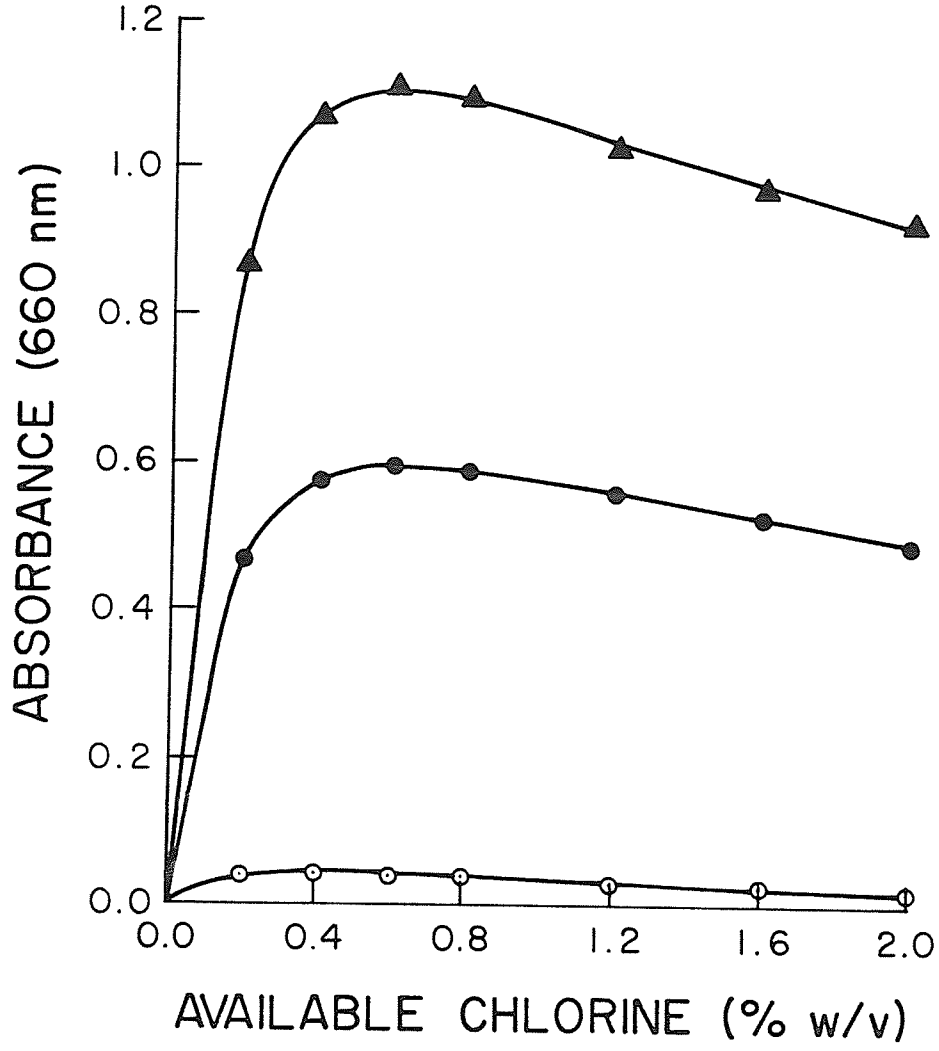
for available chlorine occurred between the above two values.

To determine the optimum level of available chlorine for maximum color formation, commercial bleach was diluted with distilled deionized water to prepare hypochlorite solutions containing available chlorine in the range 0-2.0% w/v. When these solutions were used in the standard procedure they resulted in available chlorine concentrations in the range 0-0.15% w/v on an incubation mixture basis.

The absorbance values were measured for 0, 4 and 8 µg N at the various available chlorine levels (Fig. 4). It was found that for maximum sensitivity using the proportions outlined in the standard procedure and an incubation at 25° C for 30 minutes, the hypochlorite reagent should be diluted to contain 0.60% w/v available chlorine. This corresponds to 0.046% w/v available chlorine in the incubation mixture. Incubation concentrations in the range 0.040-0.052% w/v were found to give essentially the same absorbance value.

4.1.4 Effect of Incubation Temperature on Absorbance Values. Initially in this assay incubations were done at 37° C for 15 minutes. Several other workers have incubated their assays at the same temperature (Yamaguchi *et al.*, 1970; Weatherburn, 1967; Reardon *et al.*, 1966). During the course of this study, however, it was noticed that if incubation of samples at 37° C was delayed after reagent additions, higher absorbance readings would be recorded. A similar observation had been made by Koops *et al.* (1975). Using the salicylate-dichloroisocyanurate method these workers reported that any delay between addition of reagents at room temperature and incubation at 40° C for 15 minutes resulted in an increase in the absorbance value. They recommended that

Figure 4. Effect of available chlorine concentration on color yield for 0 µg N ⊙—⊙ , 4 µg N ●—● and 8 µg N ▲—▲.


1.2
1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCE (660 nm)
0.0
0.4
0.8
1.2
1.6
2.0
AVAILABLE CHLORINE (% w/v)

not more than two samples be treated at one time to avoid the increase in absorbance readings with delay in incubation.

Preliminary investigations showed that the increase in the absorbance values with delay in the incubation was related to one of the reagents used (hypochlorite). To demonstrate this, three conditions were used and the results that were obtained are shown in Table 1. In all the three conditions 8 μg N were used.

For condition A, all reagents were added and the complete reaction mixture placed at the incubation temperature immediately upon addition of the hypochlorite. For condition B, all reagents except hypochlorite were added and the samples kept at 22° C for 10 minutes. Hypochlorite was then added and the reaction mixture immediately placed at the incubation temperature. For condition C, all reagents including hypchlorite were added and kept at 22° C for 10 minutes before incubating at one of the higher temperatures. In all cases the samples were incubated at the higher temperatures for 30 minutes. For C, the actual incubation time was 40 minutes, but since all samples should have reached maximum color development (see below, incubation time), comparisons to A and B should not be seriously in error.

It was found that regardless of the conditions used, the higher the incubation temperature the lower was the absorbance value. When values obtained in conditions A and B were compared, it was found that it was a part of the complete reaction mixture that was labile and not an intermediate involving just ammonia and salicylate in alkaline solution. Had the latter been the case, condition B would have resulted in diminished color formation as compared to condition A. However, when the values

TABLE 1. Effect of incubation temperature on the maximum absorbance of a standard nitrogen sample (8 µg N/sample)

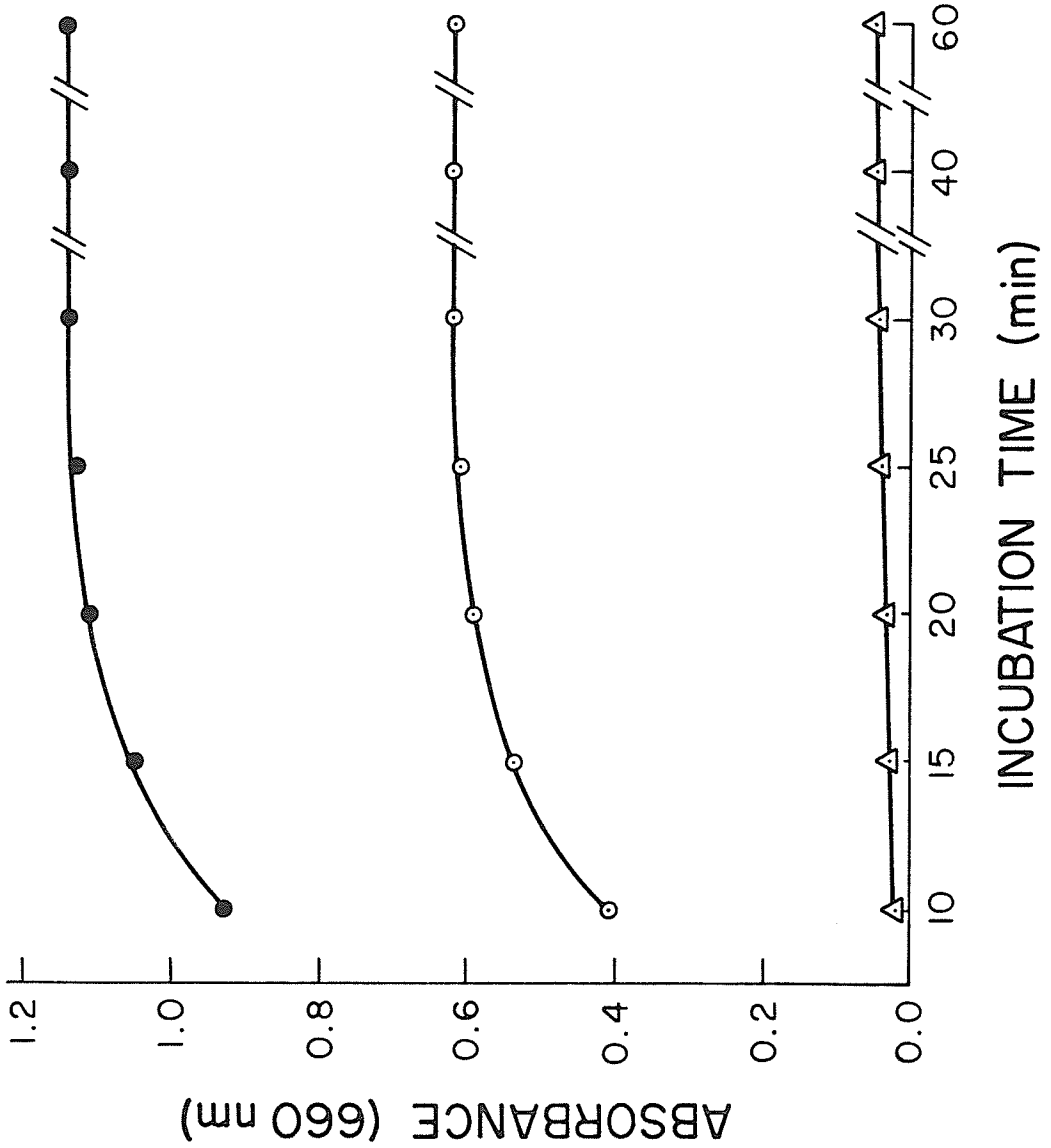
Experimental condition	Percent of Maximum Absorbance					
	Temperature °C					
	25	30	35	40	45	50
A	99	95	90	86	79	74
B	99	97	90	86	81	76
C	100	99	96	95	93	90

obtained in condition C were compared to those obtained in condition A, the effect that Koops *et al.* (1975) observed in their study using a 40° C incubation temperature was clearly demonstrated. A delay of only 10 minutes in transferring the complete reaction mixture from room temperature to a higher incubation temperature resulted in a higher color yield. This would appear to be due to a heat labile intermediate in the overall reaction (i.e., after addition of hypochlorite reagent) as opposed to the final chromophore being heat labile. The color once fully developed was found to decrease by less than 5% upon a further incubation at 50° C for 30 minutes.

For higher sensitivities (Table 1) an incubation temperature of 25° C as opposed to the higher temperatures would be recommended. The sample plus salicylate-nitroprusside and working buffer mixture should be allowed to reach this temperature prior to addition of hypochlorite reagent. This procedure also overcomes the inherent problem of having to treat only a few samples at one time (Koops *et al.*, 1975; Felker, 1977).

4.1.5 Effect of Incubation Time on Absorbance Values. The time that it took to reach maximum color development at 25° C using the optimum conditions of reagents and pH found here was measured. The results are shown in Figure 5 for 0, 4 and 8 µg N. It was found that an incubation time of 30 minutes was sufficient for full color development. With incubation times greater than 30 minutes a further, but slower increase in blank values was observed.

Figure 5. Effect of incubation time on color development for 0 µg N △—△ , 4 µg N ⊙—○ and 8 µg N ●—●.


ABSORBANCE (660 nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10
15
20
25
30
40
60
INCUBATION TIME (min)

4.2 Accuracy of the Assay

To test the accuracy of this procedure under the conditions found optimum in this study, protein values obtained by the procedure were compared to those obtained by the classical titration method. The values shown in Figure 6 were found to be in excellent agreement over the protein range that was examined.

4.3 Sensitivity of the Assay

The phenol-hypochlorite method for the nitrogen estimation was chosen in this study for its sensitivity. It was, therefore, desirable that this sensitivity be maintained or improved under the conditions that were found optimum. However the relative sensitivities of various methods are complicated by the fact that the various procedures employ different degrees of dilution. To make comparison with the other methods the data were, therefore, reduced to a common value: the absorbance obtained per microgram of nitrogen in a final dilution of 10 ml. The values are shown in Table 2 and indicate the color yield efficiencies achieved by the various procedures.

Although Patton and Crouch (1977) indicated that their procedure was superior in both sensitivity and precision over the previous methods, Table 2 shows that there were other salicylate-based procedures (Pym and Milham, 1976; Koops *et al.*, 1975; Felker, 1977) that were more sensitive. The method that is reported in this study was found to be at least 40% better in the relative sensitivity than the other reported methods (Table 2).

Figure 6. Comparison of the titration method with the colorimetric ammonia-phenol-hypochlorite method for ammonia estimation in micro-Kjeldahl digest. Duplicate digestion and analyses were carried out for each of the 17 duplicate samples and duplicates were not significantly different from each other. In the linear regression equation, X = protein value determined by titration.

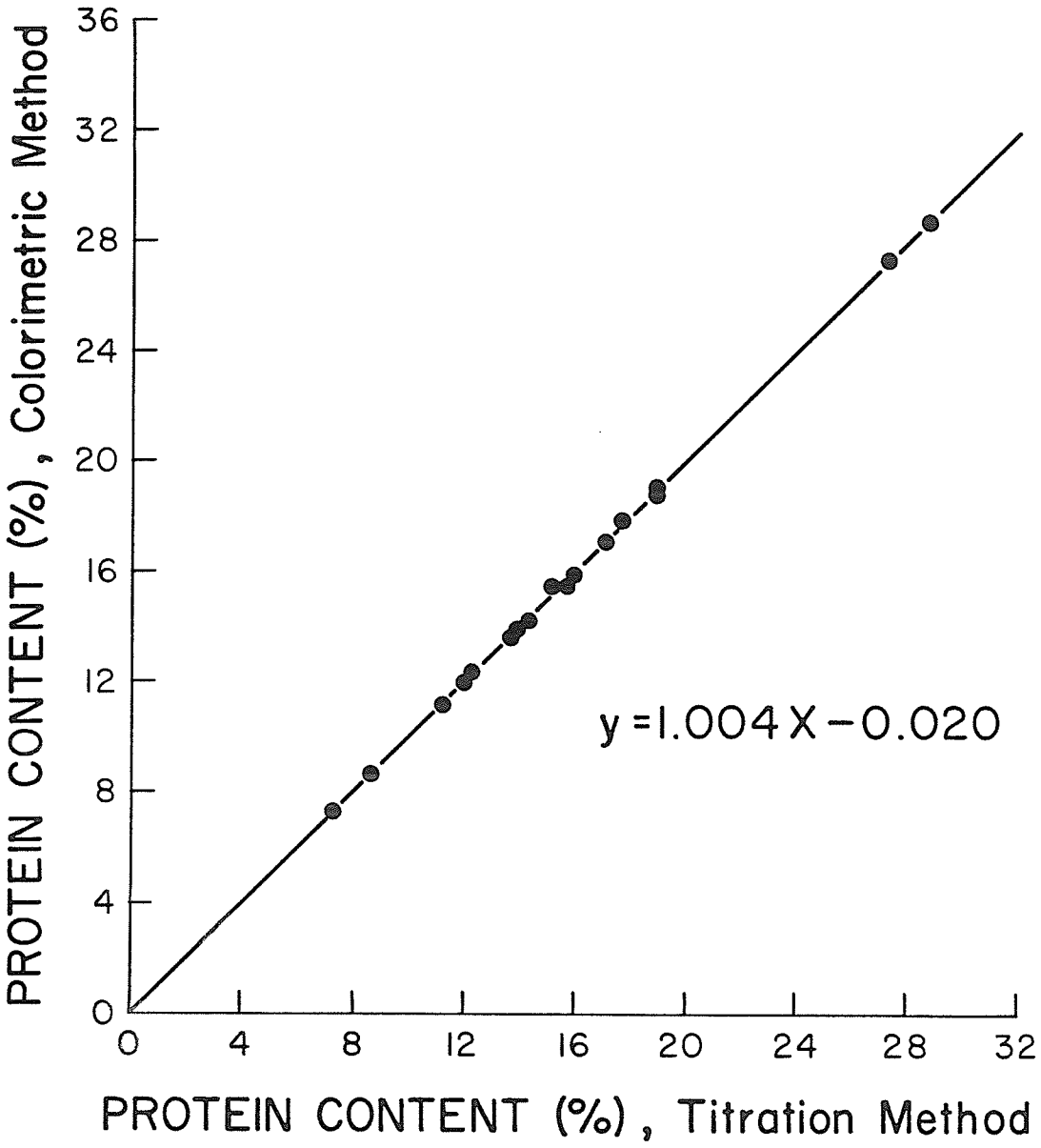

PROTEIN CONTENT (%), Colorimetric Method
36
32
28
24
20
16
12
8
4
0
0
4
8
12
16
20
24
28
32
y = 1.004 X − 0.020
PROTEIN CONTENT (%), Titration Method

TABLE 2. Comparison of relative sensitivities of previously reported ammonia-phenol-hypochlorite methods

Absorbance/ 10 µg N	Final dilution volume (ml)	Reference	Absorbance/ 1 µg N/10 ml
1.65	2.31	Bietz, 1974	0.038
1.06	10.0	Reardon *et al.*, 1966	0.106
0.430	25.0	Patton and Crouch, 1977	0.108
2.55	4.3	Felker, 1977	0.110
1.33	9.1	Koops *et al.*, 1975	0.121
0.488	25.0	Pym and Milham, 1976	0.122
1.38	12.6	This thesis	0.174

5. General Discussion

For maximum color development it was found that the reagent concentrations, incubation pH and temperature as well as incubation time are important. Felker (1977) indicated that the salicylate reagent should be made fresh everyday, while Reardon *et al.* (1966) reported that the same reagent was stable enough under usual laboratory conditions for as long as 5 months. In this study the salicylate-nitroprusside reagent stored in the dark, at 4° C and at room temperature, for up to 18 months showed that this reagent was quite stable. The only noticeable difference was that for reagent stored at room temperature the blank values increased, but the net color values obtained for a given amount of ammonia were identical to those obtained with a fresh reagent.

To minimize the effect of dissolved carbon dioxide on pH the alkaline buffer solutions were kept tightly capped. These reagents were usually prepared fresh every two weeks.

The stock hypochlorite solution (commercial bleach) was, however, found to deteriorate. Over an 18 month storage period at room temperature the available chlorine content dropped from 5.2% to 3.9% w/v. This required an adjustment to the dilution ratio over this storage period to maintain the optimum level of available chlorine in the incubation mixture.

Pym and Milham (1976) reported that the absorbance maximum of the chromophore formed in the phenol-hypochlorite-ammonia reaction is dependent on pH. When the absorption spectrum in this assay was examined it was found that the λ max occurred at 647 nm and this is in good agreement with the value reported by Pym and Milham (1976) at a pH in the

region of 13.0. For convenience and because the absorption curve was found to be broad, all subsequent measurements were made at 645 nm.

6. Summary

1) In the nitrogen standard procedure presented above the following conditions were found optimum for both maximum sensitivity and accuracy.
   a) A 100 ml of salicylate-nitroprusside should contain 20 g of sodium salicylate and 30 mg of sodium nitroprusside.
   b) The pH of the incubation mixture should be between 12.9-13.3.
   c) The available chlorine in the incubation mixture should be 0.040-0.052% w/v.
   d) The incomplete reaction mixture should be allowed to reach incubation temperature, before addition of the last reagent, the hypochlorite solution. This overcomes the inherent problem of having to treat only a few samples at a time.
   e) The absorbance readings should be taken at 645 nm.

2) When the assay conditions were as stated above, the standard procedure presented here resulted in at least 40% improvement in sensitivity over the previous methods.

3) Under the above conditions for the standard procedure, the protein values that were obtained in this assay were found to be in excellent agreement with the classical micro-Kjeldahl titration method values.

II. ENZYMIC SOLUBILIZATION OF CEREAL PROTEINS

1. Review of Literature

A number of enzymes were tested for their ability to solubilize cereal flour proteins. These enzymes included trypsin, alpha-chymotrypsin, Pronase, papain and thermolysin. For each a discussion follows that summarizes their properties which are relevant to protein sample hydrolysis or solubilization.

1.1 Trypsin

Trypsin, a low molecular weight enzyme of 24,500 daltons (Kay *et al.*, 1961), is elaborated and produced from the pancreas in the inactive form of trypsinogen. The active trypsin is obtained from trypsinogen by a limited proteolysis at pH 8.0 catalyzed by small amounts of trypsin or enterokinase (Keil, 1971).

Calcium ions stabilize the enzyme against autolysis and increase the activity of the enzyme. However calcium ions are not obligatory for tryptic activity (Green and Neurath, 1953). Sipos and Merkel (1970) observed that calcium ions caused a temperature dependent activation of trypsin. At 40° C using urea denatured hemoglobin as substrate the calcium dependent activation was found to be confined to pH 7.0-9.5 while using p-Tos-L-Arg Ome as substrate at 60° C the activation was confined in the pH range of 7.5 to 8.5.

Up to 10^{-6} M calcium ions concentration was found (Sipos and Merkel, 1970) not to have any influence on the enzymatic activity of trypsin against p-Tos-L-Arg Ome at the optimum pH 8.1 and 60° C. As the concentration of calcium ions increased from 10^{-6} to 10^{-3} M, enzyme activity increased. There was no further increase in enzymatic activity of trypsin beyond 10^{-3} M Ca^{++}.

That the enzymatic activity of trypsin is increased by not only calcium ions but by some other ions was reported by Green and Neurath (1953). In the presence of these ions both the amidase and esterase activities of trypsin increased maximally by 25%. Some buffer species have also been reported to influence the tryptic activity. In cooled mixed solvents Bennett *et al.* (1972) showed that tryptic hydrolysis of N-benzoyl-L-arginine ethyl ester (BAEE) was accelerated by phosphate buffer, an effect not observed with the other buffers that were used.

Trypsin exerts its hydrolytic activity on protein substrates in the pH range 6-11 and possesses a rather flat optimum range between pH 7.5-9.0 (Bier and Nord, 1951). At 40° C with hemoglobin as substrate Sipos and Merkel (1970) reported a maximum activity in a similar pH range of 7.0-9.5.

Trypsin is most stable around pH 3.0 (Keil, 1971). At the pH optimum for maximum enzyme activity the enzyme autolyses (Keil, 1971). The autolysis is considerably retarded by Ca^{++} ions (Bier and Nord, 1951; Sipos and Merkel, 1970). In the absence of calcium ions the enzyme was completely inactivated in 6 hours at pH 7.9 and 26° C. Under the same conditions but in the presence of 0.001 M Ca^{++} ions less than 5% of activity was lost (Bailey, 1967_a). The enzyme was found to be

fully active in urea solutions up to 6.5 M (Delaage and Lazdunski, 1968) and in ethanol (Schwert and Eisenberg, 1949).

Trypsin has a very narrow specificity catalysing the hydrolysis of peptide bonds involving the carboxyl groups of arginine and lysine (Keil, 1971). In both synthetic and polypeptide substrates, trypsin hydrolyses the bond involving arginine 25 times faster than the bond involving lysine (Wang and Carpenter, 1967). Substituents which eliminate the positive charge of the ε-amino group of lysine block the trypsin activity (Benoiton and Denault, 1966). The peptide bond of carboxyl group of arginine or lysine involving cystine is hydrolysed at a very low rate and is not hydrolysed if it involves the proline residue (Keil, 1971).

Trypsin splits terminal basic residues although at a lower rate (Kreil and Tuppy, 1961) thus behaving like an exo-peptidase. The rate of hydrolysis of smaller peptides is affected by the number of amino acid residues (Yamamoto and Izumiya, 1967).

1.2 Alpha-Chymotrypsin

Alpha-chymotrypsin, molecular weight of 25,000 daltons, is, like trypsin, produced from the pancreas in the inactive form of chymotrypsinogen (Hess, 1971). Unlike trypsinogen which is converted to the active form by a number of other enzymes, chymotrypsinogen is converted to the active form by trypsin only (Kraut, 1971).

Calcium ions also activate alpha-chymotrypsin but this action is much less important than with trypsin (Desnuelle, 1960_a).

Alpha-chymotrypsin acts optimally on proteins in a broad pH range of 7.0-9.0; with synthetic substrates the optimal pH is 7.8 (Desnuelle,

1960_a). Yamashita (1960) reported a pH optimum of 7.7 with synthetic substrates.

When alpha-chymotrypsin is heated at 100° C in M/400 hydrochloric it is rapidly inactivated. However, if the heated solution is cooled and allowed to stand at 20° C, the solution recovers its original activity (Northrop *et al.*, 1955). Like trypsin alpha-chymotrypsin is most stable at pH 3.0 (Desnuelle, 1960_b).

Chymotrypsin primarily catalyses the hydrolysis of amide bonds of proteins and peptides adjacent to the carbonyl groups of the aromatic L-amino acid residues of tryptophan, tyrosine and phenylalanine (Blow, 1971). Hydrolysis adjacent to other hydrophobic residues occurs more slowly as, for example, with histidine, leucine and methionine.

1.3 Papain

Papain, a sulfhydryl protease is isolated from the latex of the green fruit of *Carica papaya*. The molecular weight of the pure enzyme is 23,000 daltons (Drenth *et al.*, 1968), a value obtained from the amino acid composition of the molecule (Light *et al.*, 1964) as corrected on the basis of data obtained from x-ray analysis. The protein has 212 residues (Arnon, 1970).

The structural conformation of papain is stabilized by three disulphide bridges. Their complete rupture results in the disruption of the protein as indicated by the loss of biological activity, catalytic as well as immunological activities (Shapira and Arnon, 1969).

The enzyme requires a free sulfhydryl group for its catalytic activity. Activation is achieved by mild reducing agents such as cysteine, sulfide, sulphite, cyanide and reduced glutathione (Arnon, 1970; Kimmel

and Smith, 1954). Conversely, oxidizing agents inactivate the enzyme (Kimmel and Smith, 1954).

Maximum activation was found to occur upon simultaneous application of a thiol compound like cysteine and a heavy metal binding agent like ethylenediaminetetraacetic acid, EDTA (Kimmel and Smith, 1954). Addition of 2,3-dimercaptopropanol, a compound which combines the functions of both a thiol compound and a metal binder was found to cause maximum activation of papain (Stockell and Smith, 1957).

Finkle and Smith (1958) showed that papain, after activation, has a thiol content of 0.3-0.8 groups per molecule and this was proportional to the activity of the particular preparation. The proportionality between activity and thiol content has also been demonstrated by Sanner and Phil (1963).

In the presence of air and low concentration of cysteine, papain is reversibly inactivated and is inhibited by divalent ions (Sluyterman, 1967). It is inhibited by sulfhydryl compounds like p-chloromercuribenzoate (PCMB) and iodoacetamide (IAA) (Finkle and Smith, 1958) and aldehydes reagents like phenylhydrazine and hydroxylamine (Masuda, 1959).

The ionic strength as well as the buffer species have been shown to affect papain activity. Stockell and Smith (1957) using 2,3 dimercaptopropanol activated enzyme at pH 5.2 and 38° C showed that the velocity of hydrolysis of benzoyl-L-arginamide (BAA) was increased by an increase of ionic strength at values below 0.05. At ionic strengths from 0.05 to 0.3 there was no increase in velocity.

Murray (1933) demonstrated that at a given pH papain was more active in acetate than in phthalate and still more active in citrate.

Kimmel and Smith (1954) using cysteine as the activating agent found that at a given pH value, papain was considerably more active in citrate than in acetate. Furthermore, at alkaline pH values no activity was measurable in TRIS (tris (hydroxylmethyl) amino methane), but in phosphate buffers at identical pH values the enzyme was very active. Using gelatin as substrate and KCN activated papain, Hinkel, Jr. and Alford (1951) showed that in phosphate buffer the total digestion of substrate was only 80% that obtained in citrate or acetate buffers.

That the pH-activity curves of the enzyme were also dependent on the buffer species has been shown. The pH optimum of digestion of gelatin at 40° C using a KCN activated papain was found to be 5.0 with citrate buffer, but 4.0 with phosphate buffer (Hinkel, Jr. and Alford, 1951). No maximum was reached in acetate buffer as the pH curve still shifted further to lower pH values but the workers concluded that it was probably close to pH 3.0. Murray (1933) found that the pH optimum and the level of activity of papain depended on the buffer used in the reaction mixture.

The substrate is also not without influence on the pH optimum of the enzyme. As measured by the amount of trichloroacetic acid non-precipitable protein, KCN activated commercial papain at 40° C in 0.05 M citrate buffer showed that the pH optimum with casein as substrate was 5.0. With crystalline bovine serum albumin the pH optimum was 3.5-4.0 (Hinkel, Jr. and Alford, 1951). Commercial egg albumin showed maxima at both pH 4.0 and pH 7.0-8.0. Beef powder did not exhibit any clear maximum; the digestion was constant from pH 5.0-8.0.

Bondi and Birk (1954) using HCN activated papain in M/15 phosphate

buffer at an incubation temperature of 37-38° C, showed that the enzyme gave a higher degree of hydrolysis at pH 7.5 than at pH 5.0. Later Byers (1967) used KCN activated enzyme at a higher incubation temperature (60° C) in citrate phosphate buffer (pH 5.1-7.6) and borate buffer (pH > 7.6) to hydrolyze extracted maize leaf protein. Under these conditions a maximum degree of hydrolysis of extracted protein was obtained at a lower pH of 6.6 while maximum protein solubilization was obtained at a pH of 8.6. It was reported that the juice of *Carica papaya* had a better activity at 60-65° C than at other temperatures (Hwang and Ivy, 1951). It was also found that papain digested more protein at 70° C than at any lower temperatures (Hwang and Ivy, 1951).

While different activities of different preparations of the same enzyme may be explained, a fact that is difficult to explain is the differences in the pH optimum shown by different preparations of papain (Hinkel, Jr. and Alford, 1951).

Papain has very little specificity. It will degrade most protein substrates more extensively than trypsin, pepsin or alpha-chymotrypsin, in many cases giving rise to free amino acids (Hill, 1965). Smith and Kimmel (1960) also indicated that papain produces more extensive hydrolysis of protein substrates than either pepsin or trypsin. It is capable of splitting substrates with or without a free amino group, but not dipeptides (Smith and Kimmel, 1960). The enzyme exhibits stereochemical specificity toward the L-form of the substrates tested (Smith and Kimmel, 1960).

Peptide bonds formed by the carboxyl groups of α-amino substituted arginine and lysine are the most susceptible to papain (Hill, 1965).

It hydrolyses similar derivatives of glutamine, histidine, glutamic acid, leucine, glycine and tyrosine.

Peptides in which phenylalanine is three or more residues from the C-terminal end are good substrates for papain (Schechter and Berger, 1968). The bond split is always the next-but-one from phenylalanine residue in the direction of the C-terminal. Peptides containing phenylalanine as the second residue from the C-terminal are hardly attacked and they are competitive inhibitors of the enzyme (Schechter and Berger, 1968).

1.4 Pronase

Pronase, a commercial name for enzyme preparation secreted by the mold *Streptomyces griseus*, has been reported to have the broadest substrate specificity of all the protein hydrolysing enzymes presently known (Nomoto *et al.*, 1960_a). While the extent of hydrolysis of protein by many other proteinases is limited to 10-30% that with *Streptomyces griseus* protease (Pronase) reaches an upward limit of 60-90% (Nomoto *et al.*, 1960_a).

Nomoto *et al.* (1960_b) using *Streptomyces griseus* protease which was shown to be homogeneous by ultra-centrifugal, electrophoretic and enzymological analyses showed that the enzyme had a very broad specificity. It was found to possess the specificities of pepsin, trypsin and α-chymotrypsin. Unlike pepsin, however, Pronase specificity was not restricted by amidation of the C-terminal carboxyl group of the substrate; unlike pepsin, trypsin and α-chymotrypsin, Pronase activity was not prevented by the presence of free amino groups (Nomoto *et al.*, 1960_b).

While it was thought that Pronase was only one proteolytic enzyme

with a broad specificity (Nomoto and Narahashi, 1959), evidence has since been obtained for the occurrence of several kinds of exopeptidases and endopeptidases. There were two kinds of peptidases (amino- and carboxypeptidase) as well as EDTA sensitive and insensitive proteinases (Narahashi and Yanagita, 1967).

Narahashi *et al.* (1968) by use of column chromatography with CM-cellulose, DEAE-Sephadex and Amberlite CG-50 resin, fractionated eleven proteolytic enzymes from Pronase. Four were neutral proteinases, three alkaline proteinases, three aminopeptidases and one carboxypeptidase. Therefore in view of the multi-enzyme nature of Pronase reported (Narahashi *et al.*, 1968) the broad specificity of the enzyme documented by Nomoto *et al.* (1960_a) and Nomoto *et al.* (1960_b) is not surprising.

Since Pronase is a mixture of different enzyme activities, the property of the enzyme will depend on the type of activity being considered. In the presence of calcium ions aminopeptidase activity and carboxypeptidase activities showed a pH optimum of 8.3 and 7.5, respectively. Both the proteinase activity against casein and esteriolytic activity against benzoyl-L-arginineethylester had a pH optimum of 7.0-9.0 (Narahashi and Yanagita, 1967). With glycyl-L-leucine, carbobenzoxy-L-glutamyl-L-tyrosine and butyric acid methyl ester as substrates, the pH optimum was 8.5, 8.2 and 8.5-9.3, respectively (Nomoto *et al.*, 1960_a). Morihara *et al.* (1968) reported a broad pH optimum of between 7.0-8.5 for both casein and synthetic substrates.

When casein was used as substrate, the activity measured could either be due to neutral proteinases or alkaline proteinases. Narahashi and Yanagita (1967) differentiated these two activities and

showed that with casein as substrate, the neutral proteases had a pH optimum of 7.5-8.0 and the alkaline proteases a pH optimum of 9.0-10.0. The alkaline proteinase as well as the carboxypeptidase and aminopeptidase activities were inhibited by EDTA, while the alkaline proteinase was not. The alkaline proteinase was also not inhibited by diisopropylfluorophosphate (DFP) (Narahashi *et al.*, 1968).

The temperature optimum for Pronase with casein as substrate in 0.01 M calcium acetate, pH 7.4 was between 40° and 60° C (Nomoto and Narahashi, 1959). Fritz *et al.* (1973) using the amount of lysine released from barley protein as a measure of activity, reported that the enzyme was twice as active at 60° C as at 70° C and at 40° C, it was less active than at 60° C.

1.5 Thermolysin

Thermolysin is an extra-cellular, thermostable and neutral protease isolated from *Bacillus thermoproteolyticus* (Endo, 1962). One mole of the enzyme contains 1 g-atom of zinc and 4 g-atom of calcium (Feder *et al.*, 1971). Zinc is essential for the catalytic activity of the enzyme (Roche and Voordouw, 1978). Holmquist and Vallee (1974) found that when zinc was removed, the enzyme became inactive. The activity could be restored by addition of zinc or other metals. When Zn^{++}, Co^{++} and Mn^{++} were added in stoichrometric amounts, 100, 200 and 10%, respectively of the activity of the native enzyme was obtained. Zinc in excess of that required to induce activity inhibited the enzyme.

Various buffers have been shown to have an inhibitory effect on the enzyme activity. Drucker and Borchers (1971) reported that as compared to the other buffers they used, tris buffer was found to have an

inhibitory effect on the enzyme. The enzyme is inhibited by phosphate and to a lesser extent by cacodylate as compared with the acetate buffers (Feder and Schuk, 1970). Oxalate and citrate buffers have been observed to have inhibitory effects (Matsubara, 1970).

Different pH optima for the enzyme activity have been reported in literature. Feder and Schuk (1970) observed that with synthetic substrates the enzyme exhibited a pH optimum of 7.0 for activity. Morihara *et al.* (1968) reported a broad pH optimum of 7.0-8.5 for both synthetic substrates and casein.

While zinc does not contribute to the stability of the enzyme, calcium ions do. Calcium ions prevent the enzyme from autolysis as well as making the enzyme more thermostable. Removal of calcium ions from thermolysin increased the susceptibility of the enzyme to autolytic degradation (Voordouw and Roche, 1974; Drucker and Borchers, 1971). As the temperature of the enzyme solution in 20 mM Tris-HCl buffer pH 7.0 was raised from 20° C at the rate of 2 degrees per minute, the enzyme containing no calcium but ethylenediaminetetraacetate (EDTA) was inactivated at only 40° C. The enzymes in solution containing 1 mM or 10 mM $CaCl_2$ but no EDTA were inactivated at above 70° C (Fontana *et al.*, 1975). EDTA is a chelating agent and binds the metal ions. Drucker and Borchers (1971) observed that at temperatures of 66-72° C and at calcium concentrations of less than 10^{-4} M the enzyme was susceptible to autodigestion; at calcium concentrations above 10^{-4} M it was not.

The enzyme is very stable at high temperatures. At 80° C in 0.1 M tris buffer pH 8.0 with 0.002 M $CaCl_2$ activity towards casein decreased only to half after 1 hour while at 60° C, there was no significant loss

in activity in the same period of time (Matsubara, 1967).

Since thermolysin requires zinc for activity and calcium for stability, the metal chelators such as EDTA or 1,10-phenathroline (Morihara and Tsuzuki, 1966) will inhibit its activity. Mild reducing agents like cysteine and sodium cyanide, diisoprorylphosphofluoridate (DFP), soyabean trypsin inhibitor and potato inhibitor did not inhibit the enzyme activity (Morihara and Tsuzuki, 1966).

The specificity of the enzyme is well documented. Matsubara *et al.* (1965) used beef heart cytochrome c and Matsubara *et al.* (1966) used oxidized bovine insulin and tobacco mosaic virus proteins as substrates in 0.1 M Tris, pH 8.0 containing 0.002 and 0.003 M $CaCl_2$, respectively. The workers showed that thermolysin preferentially hydrolysed the peptide bonds involving the amino groups of hydrophobic amino acid residues with a bulky side chain. Isoleucine and leucine were highly susceptible. Phenylalanine, valine, alanine and tyrosine were also susceptible.

The amino acid residues that contribute carboxyl groups to peptide bonds attacked by thermolysin have varied between hydrophilic and hydrophobic, and between basic and neutral but have not involved the acidic amino residues (Matsubara *et al.*, 1966). Prolonging the hydrolysis time or increasing the enzyme to substrate ratio lowers the specificity of the enzyme. At 36° C with cytochrome c as substrate and enzyme to substrate ratio of 1/200 and after 3.5 hours incubation time, only isoleucine, leucine and phenylalanine were susceptible. After 24 hours valine, threonine, glycine, serine and alanine were also shown to be susceptible (Matsubara, 1967). Ambler and Meadway (1968) reported that bonds involving the N-terminal side of methionine, asparagine, threonine,

histidine and glycine residues are cleaved.

Thermolysin requires L-configuration at the sensitive residue (Morihara and Ebata, 1967). Absence of a free α-amino or α-carboxyl group in the immediate vicinity of the sensitive peptide bond is necessary for susceptibility (Morihara and Ebata, 1967; Ambler and Meadway, 1968). A free ω-amino or carboxyl group does not have a marked effect on the suceptibility of adjacent bonds (Ambler and Meadway, 1968). The specificity of the enzyme is affected not only by the sensitive residue but also by the nature of at least five residues in its neighbourhood (Morihara and Oka, 1968).

Thermolysin does not cleave the peptide bond at the amino site of a hydrophobic amino acid residue which has a proline residue at the carboxyl site (Ambler and Meadway, 1968) regardless of the presence or absence of the second residue attached to the carboxyl group of proline (Matsubara *et al.*, 1969). Thermolysin has no amidase or esterase activities (Matsubara, 1970). Extremes of temperatures leaves the specificity of the enzyme unchanged. Matsubara (1967) using cytochrome c as substrate in 0.1 M tris buffer pH 8.0 with 0.002 M $CaCl_2$ showed that the specificity of the enzyme at 35° C or 76° C was the same.

1.6 Comparative Enzyme Activities

The activity of an enzyme towards any one particular protein will depend on the 'population' of the peptide bonds to which the enzyme is specific (assuming each peptide is equally exposed). The enzymes that have been discussed can be arranged in the following order of broad specificity: Pronase > papain or thermolysin > α-chymotrypsin > trypsin. At their optimum conditions for activity, Pronase therefore would be

expected to give the highest degree of hydrolysis and trypsin the lowest in a model protein with an equal number and even distribution of all the peptide bonds that occur naturally in proteins. Since such ideal protein molecules are hard to come by in nature, the activity of the above enzymes has not always been in the order given.

With different substrates trypsin with a very restricted specificity has given higher activity than either α-chymotrypsin or papain, both of which have a much broader specificity. Thus Childs (1975) using cottonseed cake found that trypsin had a higher activity than papain. Trypsin was also reported to give a higher reactivity towards fish protein concentrate than either papain or α-chymotrypsin (Bhumiratana *et al.*, 1977).

Using insoluble alfalfa protein as substrate α-chymotrypsin has been reported to give significantly higher soluble protein than either papain or trypsin (Payne and Hill, Jr., 1978). Papain has been shown to give a higher hydrolysis than some of the other enzymes discussed in this thesis. Papain degrades most protein substrates more extensively than trypsin or α-chymotrypsin, in many cases giving rise to free amino acids (Hill, 1965). Papain produced a more extensive hydrolysis of casein than either trypsin or α-chymotrypsin (Nomoto *et al.*, 1960_a).

As expected from the enzyme specificity Pronase has been shown to give higher digestibilities of proteins than papain, trypsin or α-chymotrypsin. Fritz *et al.* (1973) using barley protein as substrate reported that papain gave only 80% of the proteolytic activity of Pronase. That Pronase gave a more extensive hydrolysis of casein than either trypsin, α-chymotrypsin or papain was shown by Nomoto *et al.* (1960_a).

Thus although many conflicting reports of the relative activities of these enzymes exist these differences may be due to: i) the digestion conditions, ii) the substrates used, iii) the relative amount of active enzymes used or even iv) the method of estimating the relative activities.

1.7 Some Previous Applications of Trypsin, Alpha-Chymotrypsin, Papain, Pronase and Thermolysin

Trypsin and alpha-chymotrypsin alone or in combination with other enzymes have been commonly used to predict the nutritive value (Oke and Umoh, 1974) and the *in vivo* digestibilities (Hsu *et al.*, 1977; Saunders *et al.*, 1972) of food proteins. The enzymes have been used to extract protein from crude protein samples (Childs, 1975; Childs and Forte, 1976) and to improve the functional properties of food proteins (Bhumiratana *et al.*, 1977). In all the above reports complete conversion of the sample proteins to soluble form was not obtained.

Nair *et al.* (1976) using papain in 8 M urea at 37° C solubilized from 97.0-99.0% of the total protein from 10 mixed 1-day old food samples. The urea denatured the sample proteins while papain partially hydrolysed them.

Using thioglycolic acid activated papain, Fafunso *et al.* (1976) were able to solubilize from 52.3-73.5% of the total protein nitrogen from protein samples 'extracted' (but insoluble) from both edible and inedible vegetables. The amount of protein solubilized was calculated from the difference between the total protein present and the amount of protein left in the residue.

Byers (1967) using KCN activated papain solubilized as much as 95.7% of the total nitrogen from 'extracted' maize leaf protein in 24 hours at 60° C. Villegas and Mertz (1971) used an overnight solubilization of protein from ground and defatted maize samples with papain at 65° C prior to determination of available lysine and tryptophan from the hydrolyzates.

Tryptophan values that were determined from Pronase hydrolyzate were found to be similar to those obtained from the alkaline hydrolyzate (Spies, 1967). Tsai *et al.* (1972) used Pronase to hydrolyze protein overnight at room temperature from maize flour before available lysine was estimated.

With Pronase and a number of other proteolytic enzymes Büchmann (1979) measured the digestibility of proteins of barley, oat, rye, maize, wheat and rice flours. Pronase 0.175 mg (70 PUK mg^{-1}) in 10 ml of 0.2 borate buffer of pH 7.6 was used to hydrolyze 3.75 mg N of cereal grain at 37° C for 18 hours. The extent of hydrolysis was measured as the amount of nitrogen remaining in solution after TCA addition and spinning the samples at 2000 g for 1 hour. Addition of TCA caused precipitation of 4% more nitrogen as compared to the samples which were only centrifuged. Maize and rice gave the lowest digestibilities of 42 and 47%, respectively with Pronase. Wheat and rye gave the highest degree of digestibility of 85% each. A protein digestibility of 60% was obtained from oats.

2. Introduction

The aims of the work that was to be done in this section were: i) to evaluate the potential of different proteolytic enzymes to solubilize cereal protein and ii) to select the conditions under which maximum solubilization could be achieved.

Some of the conditions that were to be used in the enzymic solubilization of the cereal proteins were to be fixed from the start. The pH of the enzyme medium was to be 8.3. All the enzymes that were going to be used have been reported to have maximum activity at or near the pH value chosen (Literature Review).

The enzymes, trypsin, alpha-chymotrypsin, Pronase and thermolysin require calcium ions for their activity or stability. To the medium containing the above enzymes 8 mM $CaCl_2 \cdot 2H_2O$ was to be added. Papain was shown to require mild reducing agents for activity; for this enzyme 1.5 mM KCN was to be included in its medium (Appendix 1).

Since the protein solubilized by enzymes was to be determined as ammonia after micro-Kjeldahl digestion of samples, it was necessary that the buffers in which the enzymes were to be dissolved contain no nitrogen. Borate buffer (0.05 M) was chosen as one of the most suitable media for the enzymes because it has a maximum buffering capacity around the pH value chosen for the enzymic solubilization of cereal proteins. The other advantages of borate over most other buffers is that it contains no nitrogen and it is bacteriostatic (Büchmann, 1979) thus making the use of bacteriocides unnecessary.

The only parameters that were to be varied were the amount of enzyme, time and temperature of incubation. It was necessary to use as

little enzyme as possible to minimize the contamination of the cereal proteins by the enzyme protein.

Initially, the temperature optimum for each enzyme was to be determined. At their temperature optimum for maximum activity the enzymes were to be used to solubilize protein from two different cereal samples. The most suitable of the 5 enzymes would then be used to test their solubilizing abilities on 22 cereal varieties representing 7 different cereal grains. From these results a final selection of the most suitable enzyme(s) could be made for use in protein solubilization from a given type of cereal grain.

3. Materials and Methods

3.1 Samples

The following cereal samples were used in this study: four barley varieties, Betzies, Herta, Karl and Risø 1508; three maize varieties, A495 fl_2 x B8 fl_2, K_{26} x K_{52} (single cross) and $W63o_2$ x $MS206o_2$; three oat varieties, Harmon, Hudson and Terra; three rye varieties, Gazelle, Prolific and UC-90; three sorghum varieties, Riosweet, sorghum X, and Winner; four triticale varieties, Carman, Rosner, Welsh and Cocorit 71 x UC-90; and two wheat varieties, Cocorit 71 and Neepawa. Sorghum X was an unidentified variety while Cocorit 71 x UC-90 was a cross between Cocorit 71 wheat and UC-90 rye. All the samples were obtained from the Department of Plant Science, University of Manitoba.

3.2 Sample Preparation

A 20 g sample of each cereal variety was ground in a Udy Cyclone mill equipped with a 0.5 mm screen and the recovered whole flour weighed.

From each of the ground samples after a thorough mixing, samples were weighed in duplicates for the determination of total crude protein and total moisture content.

The remainder of each of the samples was defatted for 8 hours in medium coarse extraction thimbles in a soxhlet apparatus using petroleum ether (bpt 40-60° C). The heating of extraction solvent was adjusted so as to produce a condensation rate of 5 drops or more per second. The defatted samples were air dried and stored before use in open petri dishes in a glass tank equilibriated at 30% relative humidity by use of saturated calcium chloride solution. All protein values reported in this work unless otherwise specified were 'on as is' basis of the samples stored as above.

3.3 Enzymes, Enzyme Conditions and Buffer Systems Used

Five enzymes were used. These were alpha-chymotrypsin (Worthington Biochemical Corporation), activity 67.8 U/mg, Lot no. CD1 2KD; trypsin, bovine type III (Sigma), activity 10,000 BAEE units/mg protein, Lot no. 118C-8050; Pronase B grade (Calbiochem), activity 45,000 PUK/g, Lot no. 53177; thermolysin (thermophillic-bacterial protease type X), activity 55 units/mg solid, Lot no. 49C-0057. The fifth enzyme was crude papain powder type II (Sigma), activity 2.1 units/mg, Lot no. 49-9004. The enzymes were dissolved in borate buffer, 0.05 M of pH 8.3 in all the enzymic studies that were done.

For alpha-chymotrypsin, trypsin, Pronase and thermolysin, the borate buffer contained 8 mM calcium chloride dihydrate. Borate buffer containing 1.5 mM KCN was used for papain (Appendix 1).

The papain preparation was only partly soluble and the activity was extracted as follows: Crude papain (16 mg/ml) was suspended in borate buffer containing 1.5 mM potassium cyanide and mixed for 5 minutes using a magnetic stirrer. The suspension was then centrifuged for 10 minutes at 27,000 g and 4° C. The supernatant obtained was recentrifuged under the same conditions and the final supernatant after requisite dilutions in borate buffer containing 1.5 mM KCN used for cereal protein solubilization.

Protein from 100 mg of sample was solubilized in 5.0 ml enzyme solution. An enzyme protein of 10 mg for alpha-chymotrypsin, 10 mg for trypsin, 10 mg for papain, 8 mg for Pronase and 3.1 mg for thermolysin were used to solubilize every 100 mg of each sample protein. For every cereal sample the amount of enzyme protein in 5.0 ml solution was adjusted so as to maintain the above ratios with the amount of protein in 100 mg of sample. The above enzyme protein to cereal protein ratios were fixed after some preliminary investigations and used in this and all other sections of this thesis where enzymic solubilization of protein was carried out.

3.4 Protein Determination

All the protein determinations in this study were done in duplicates according to the methods outlined in Section I of this thesis. A nitrogen to protein conversion factor of 5.7 was used for all the protein values that are reported in this and all the other sections.

3.5 Moisture Determination

All the moisture determinations were done according to method 14.004 of the A.O.A.C. Official Methods of Analysis, 12th ed. (1975).

About 2 gm of samples in duplicates were used.

3.6 Amino Acid Analysis

For total amino acid analysis, a ca 40 mg of sample was hydrolyzed for 24 hours in 4.0 ml of 6N HCl at 110° C. A Beckman Model 121 Automatic Amino Acid Analyzer was used to determine the amino acids in the hydrolyzate. Where cereal residues (after enzymic solubilization of protein) were analyzed, the amount obtained from a ca 200 mg of whole sample was used. Single samples were used for amino acid analyses.

3.7 Estimation of Tannin Contents

For tannin estimation of the sorghum varieties, the samples were freshly ground as in Method 3.2 above. The tannin contents were determined on a ca 200 mg samples in duplicates using the modified vanillin procedure of Price *et al.* (1978).

3.8 Standard Conditions for Enzymic Solubilization of Cereal Protein

To 100 mg of defatted (unless otherwise specified) cereal flour in polycarbonate screw cap centrifuge tube was added 5.0 ml of enzyme solution. After capping the tube, the sample was mixed to a uniform suspension in a vortex mixer and then placed in a shaking water bath held at a predetermined incubation temperature.

During the first two hours of incubation, the sample was shaken again four times in a vortex mixer and once more in the third hour of incubation. To terminate the enzymic hydrolysis the sample was removed from the bath and immediately centrifuged at 27,000 g for 10 minutes at 4° C. The supernatant was carefully decanted and immediately heated for 5 minutes in a boiling water to inactivate the enzyme. This heat

treated supernatant was stored at 4° C until used for analysis. The material that was not solubilized and was recovered as the pellet in the centrifugation step was resuspended and washed in 5.0 ml of distilled water and centrifuged as above. The washings were discarded and the washing operation repeated one more time. The final washed pellet, referred to hereafter as the residue was stored at 4° C before being analyzed. The term 'supernatant' wherever it is used here and in all other sections should be taken to mean the clear fraction of the enzyme cereal hydrolyzate.

3.9 Determination of the Amount of Protein Solubilized

A 2.0 ml aliquot of the supernatant of cereal protein hydrolyzate was used to determine the amount of protein as in Method 3.4. The value obtained from 2.0 ml 'supernatant' was multiplied by a factor of 2.5 to obtain the total amount of protein solubilized. Ideally, the amount of protein solubilized would be determined from pooled 'supernatant' and the residue washings. When the amount of protein solubilized from different cereal grains by papain after 24 hours of incubation at 65° C was determined by the two methods there was no significant difference in the values that were obtained (Appendix 2, Table A2). Therefore for all samples the amount of protein solubilized was determined from 2.0 ml of supernatant only. Values reported were an average of 'four samples' (two sets of duplicate samples analyzed on two different occasions).

3.10 Estimation of the Degree of Protein Hydrolysis

The degree of hydrolysis of the solubilized protein was determined according to the ninhydrin method of Cocking and Yemm (1954), and

Yemm and Cocking (1955). The ninhydrin reagent was made by dissolving 400 mg of $SnCl_2 \cdot 2H_2O$ in 250 ml of sodium citrate buffer of 0.2 M, pH 5.0 and mixing it with 250 ml methyl cellosolve containing 10 g of ninhydrin (Mertz *et al.*, 1974). Reagents were made about an hour before use.

To 0.5 ml of 50 times diluted sample 'supernatant' was added 1.5 ml of the ninhydrin reagent and after mixing, the solution was heated for 20 minutes in a boiling water bath. The sample was cooled in tap water and 2.0 ml of 50% v/v of n-propanol in water was added and the sample remixed. The absorbance reading was recorded at 570 nm against an enzyme blank. Glutamate was used as the standard and all values (an average of 'four samples') reported in glutamate equivalents.

3.11 Enzyme Activity -Temperature Optimum Determinations

To determine the temperature optimum for each enzyme for maximum protein solubilizing ability, a time study series of incubations was done. Five different enzymes (Method 3.3) were used and the effects of incubation temperature and time on the amount protein solubilized were investigated. Defatted samples of maize (K_{26} X K_{52}) and sorghum X flours were used as in Method 3.8.

At timed intervals of 2, 4, 8, 16 and 24 hours, respectively, the incubations were stopped and both the 'supernatants' and residues obtained as in Method 3.8. Some of each of the 'supernatants' were analyzed for total protein as in Method 3.9. Other part of each of the 'supernatants' for those samples that were incubated for a total of 24 hours were analyzed for degree of hydrolysis as in Method 3.10. The residues were analyzed for amount of residual protein and the values

used to cross check the amount of protein that was solubilized. 'Four samples' were analyzed for each determination.

3.12 Solubilization of Cereal Proteins by Enzymes at Their Respective Temperature Optima for Activity

At their temperature optima for maximum activity the enzymes were used to solubilize protein from the other ground and defatted cereal flour samples for a period of 24 hours. The conditions of solubilization were as in Method 3.8. The 'supernatants' and the residues were recovered as in Method 3.8.

The 'supernatants' were analyzed for the amount of solubilized protein as in Method 3.9 and for the degree of hydrolysis of the solubilized protein as in Method 3.10. The residues were also analyzed for residual protein as in Method 3.4 and the values used to cross check the amount of protein that was solubilized. 'Four samples' were analyzed for each determination.

3.13 Types of Protein Solubilized

To determine the amino acids that were most easily solubilized by enzymes, the following cereal samples were used: Herta barley, maize $K_{26} \times K_{52}$ (single cross), Terra oats, Prolific rye, sorghum X, Rosner triticale and Neepawa wheat. Two sets of samples of each cereal in duplicates were weighed and solubilized by each of the enzymes at their respective temperature optima for a period of 24 hours as in Method 3.8 and the residues obtained as indicated in Method 3.8. One set of residues after micro-Kjeldahl digestions was used to predict the amount of protein in the other. The other set of sample residues together with

whole samples was subjected to a total amino acid analysis as in Method 3.6.

As a relative measure of the most easily solubilized amino acid a comparison of the amount of each amino acid in the residue protein to that in the whole cereal protein was made. The relative amount of each amino acid in the residue protein was expressed as a percentage of the relative amount of that amino acid in the whole protein. The above operation was done for each cereal for each enzyme and the value obtained gave the most easily solubilized amino acid from each cereal by each enzyme. To obtain the most easily solubilized amino acid from all the cereals for each enzyme the following was done: The relative values of each amino acid in each of the whole sample proteins were summed up and divided by the number of cereals used. The same was done for the residual proteins obtained from the enzyme and the latter value expressed as a percentage of the former. To obtain the most easily solubilized amino acid from all the cereals by the three enzymes an average value of the relative amounts of each amino acid from all the enzyme residues was obtained and expressed as a percentage of the average relative values in whole cereal proteins.

4. Results and Discussion

4.1 Flour Extraction Rates and Protein Contents

The flour extraction rate in the Udy Cyclone mill ranged from 89.3% for a floury maize (A495 fl_2 x B8 fl_2) to 98.6% for sorghum X (Table 3). Fifteen cereal grains out of a total of 22 used (and at least one variety from each of the cereal types) gave a flour extraction rate of 95% or better. The protein composition of such cereal flours would there-

TABLE 3. Extraction rates[a] and protein contents[b] of cereal flours

Cereal grain	Extraction rate (%)	Protein content (%)
barley		
Betzies	96.5	13.5
Herta	93.6	13.1
Karl	96.0	11.7
Risø 1508	94.1	14.0
maize		
A495 fl_2 x B8 fl_2	89.3	11.4
K_{26} x K_{52} single cross	97.8	14.2
$W63o_2$ x $MS206o_2$	90.3	10.4
oats		
Harmon	93.0	9.96
Hudson	95.0	10.2
Terra	91.7	13.6
rye		
Gazelle	95.4	14.0
Prolific	95.2	14.1
UC-90	96.0	15.3
sorghum		
Riosweet	96.5	12.4
X	98.6	10.9
Winner	96.7	12.3
triticale		
Carman	95.6	14.6
Rosner	92.5	17.9
Welsh	97.5	16.1
Cocorit 71 x UC-90	97.2	19.7
wheat		
Cocorit 71	97.0	13.9
Neepawa	95.0	12.9

[a] 'on as is' sample basis

[b] on a dry sample basis

fore be considered to closely approximate that of the whole cereal grains. The lowest flour extraction rates were obtained with the floury maize varieties A495 fl_2 x B8 fl_2 and $W63o_2$ x $M206o_2$.

The protein contents of the flours so obtained ranged on 'dry matter basis' from 9.96% for Harmon oats to 19.7% in a triticale obtained by crossing Cocorit 71 wheat to UC-90 rye (Table 3).

4.2 Temperature Optima for Maximum Protein Solubilizing Ability

4.2.1 Alpha-Chymotrypsin and Trypsin. These two enzymes (Figs. 7 and 8) in a period of 24 hours at an incubation temperature of 37° C solubilized 57.4% and 44.0%, respectively of maize (K_{26} x K_{52}) flour protein. Alpha-chymotrypsin solubilized 56.0% of the protein in the first 8 hours of incubation, while a further incubation of 16 hours resulted in an addition of less than 2.0% more soluble protein (Fig. 7); trypsin solubilized a total of 44.0% of the protein in the first 4 hours of incubation and any further incubation for up to 24 hours did not give any more soluble protein (Fig. 8). A value of 15.4% of the total maize (K_{26} x K_{52}) protein was soluble in borate incubation buffer after shaking for 5 minutes and centrifuging the suspension at 27,000 g for 10 minutes at 4° C. For sorghum X flour 4.9% of the total protein was soluble under the same conditions.

Since the two enzymes solubilized only about half of the total protein and most of it in only the first few hours of a 24 hour incubation period, it seemed unlikely that the values could be much improved by change of temperature condition only. The two enzymes were, therefore, considered not suitable for this work and were not used for any further cereal protein hydrolysis.

Figure 7. Alpha-chymotrypsin solubilizing ability of maize protein at 37° C. Protein (%) solubilized ⊙ — ⊙ , protein in residue ● — ● .

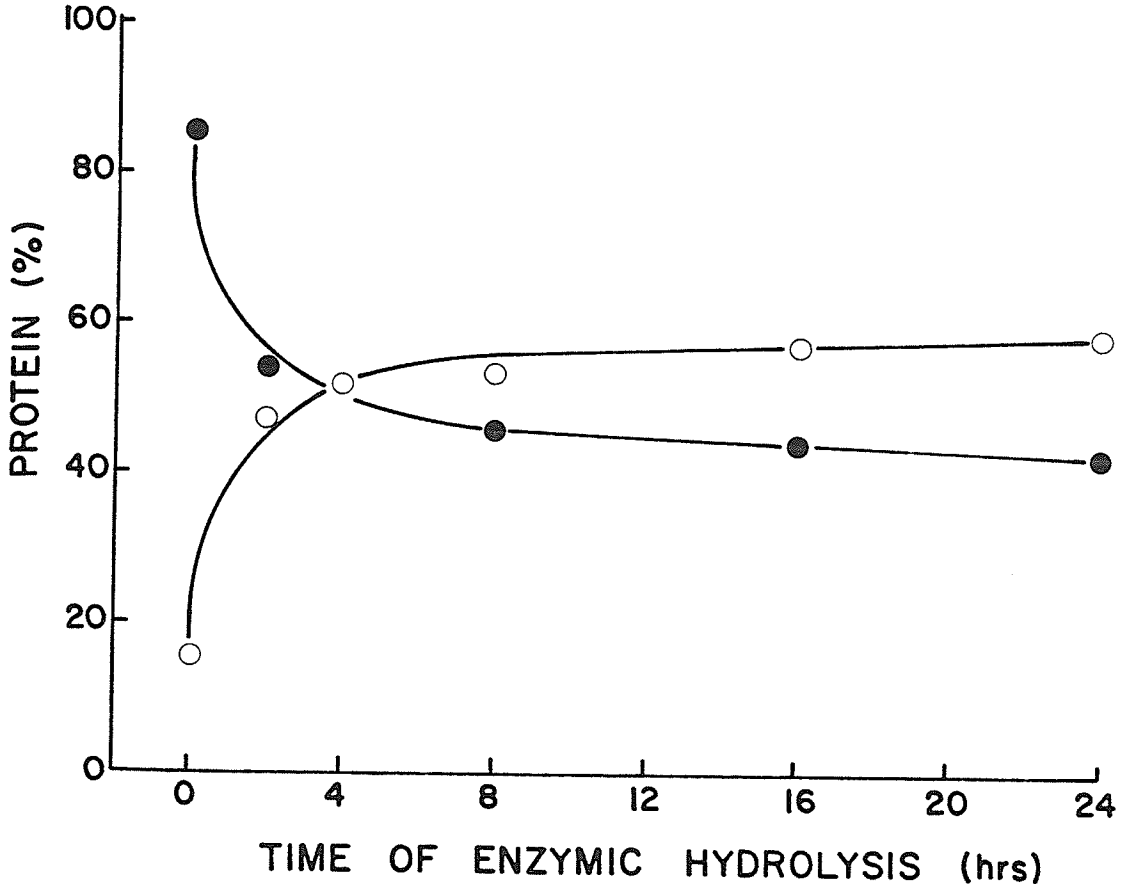
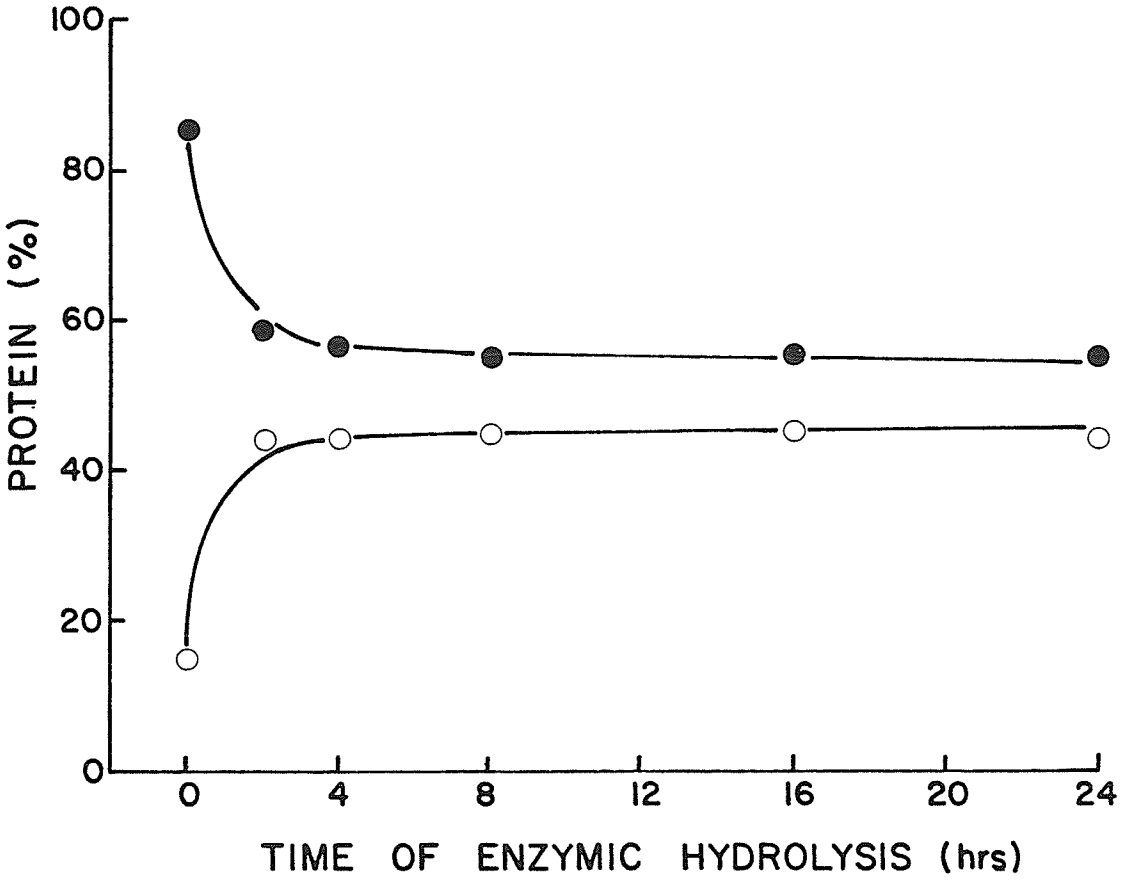

100
80
60
40
20
0
PROTEIN (%)
0
4
8
12
16
20
24
TIME OF ENZYMIC HYDROLYSIS (hrs)

Figure 8. Trypsin solubilizing ability of maize protein at 37° C. Symbols represent the same values as in Fig. 7.

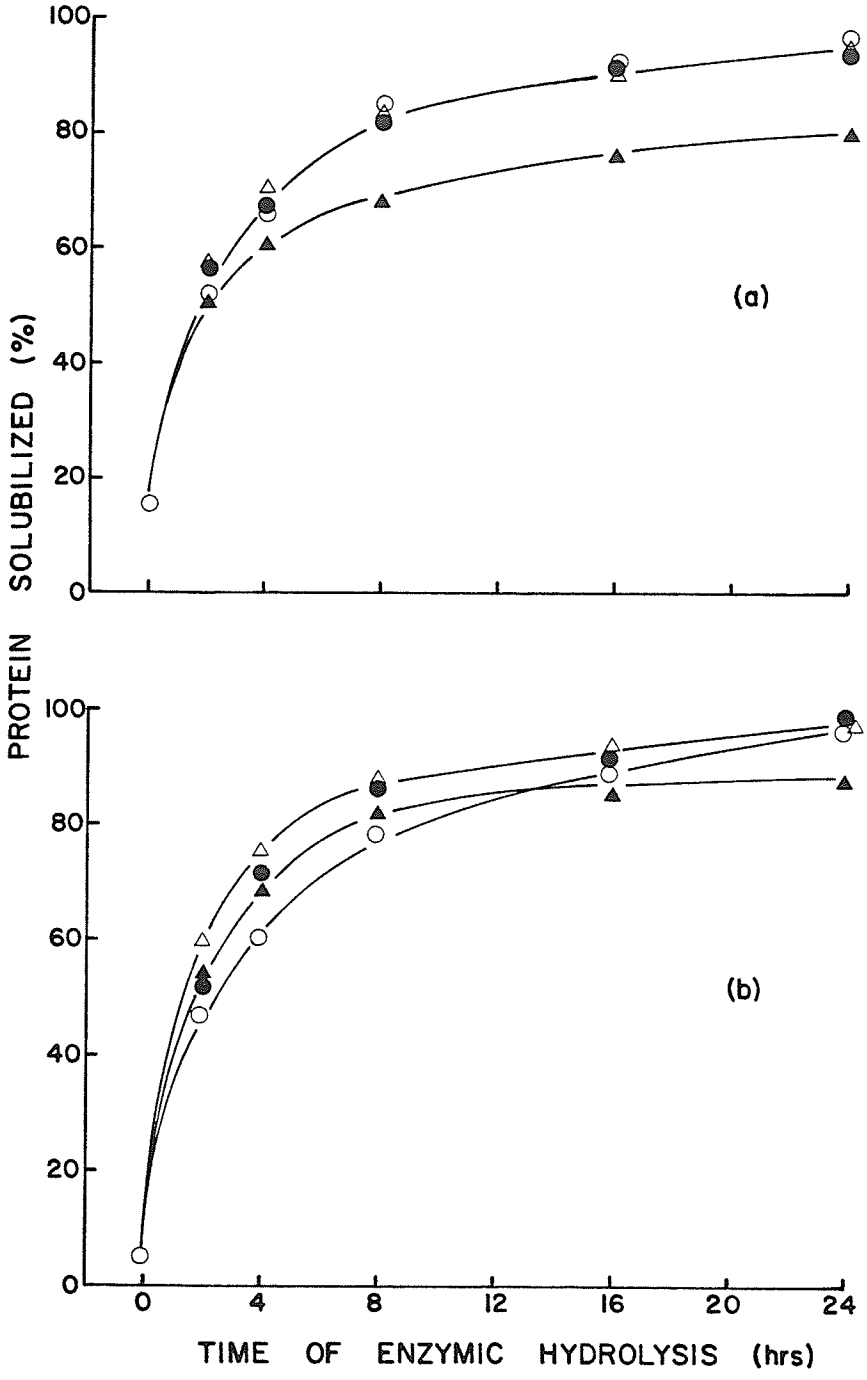

100
80
60
40
20
0
PROTEIN (%)
0
4
8
12
16
20
24
TIME OF ENZYMIC HYDROLYSIS (hrs)

4.2.2 Pronase. With maize (K_{26} x K_{52}) protein as substrate, the Pronase enzyme activity curve in the period of up to 24 hours did not change significantly by varying the incubation temperature from 37-50° C (Fig. 9a). At 37° C, 45° C and 50° C, 95.1%, 94.0% and 94.5%, respectively of total protein were solubilized. Increasing the incubation temperature to 57° C decreased both the initial rate in the first 8 hours of incubation and the final amount of protein solubilized in 24 hours to only 79.9%.

When sorghum X protein was used as substrate, an increase of temperature from 37-50° C had a more marked change in the temperature-activity curve (Fig. 9b) than with maize protein. While the activity of the enzyme in the first 8 hours was found to be lowest at 37° C, similar amounts of protein as at 45° C and 50° C were solubilized after 24 hours of incubation (Fig. 9b). The final amounts of protein solubilized at 37° C, 45° C, 50° C and 57° C were 96.7%, 96.4%, 96.1% and 86.3%, respectively. From these results it appeared that the sorghum protein was slightly more susceptible to Pronase enzyme activity than the maize protein. Increasing the incubation temperature to 57° C affected the final activity of Pronase towards maize protein more adversely than towards the sorghum protein.

At the enzyme protein to cereal protein ratios used, the optimum temperature for maximum protein solubilizing activity in 24 hours of incubation was found to be a wide temperature range of approximately 37-50° C, for both maize and sorghum proteins as substrates (Figs. 9a and 9b). At shorter incubation periods the temperature optimum was the same with maize protein while it was 45-50° C with sorghum protein as substrate. The term approximate temperature optimum has

Figure 9. Temperature optimum of Pronase for maximum protein solubilizing ability of (a) maize (b) sorghum.
37° C ⊙—⊙ ; 45° C ●—● ; 50° C △—△ ;
57° C ▲—▲ .


PROTEIN SOLUBILIZED (%)
100
80
60
40
20
0
(a)
(b)
0
4
8
12
16
20
24
TIME OF ENZYMIC HYDROLYSIS (hrs)

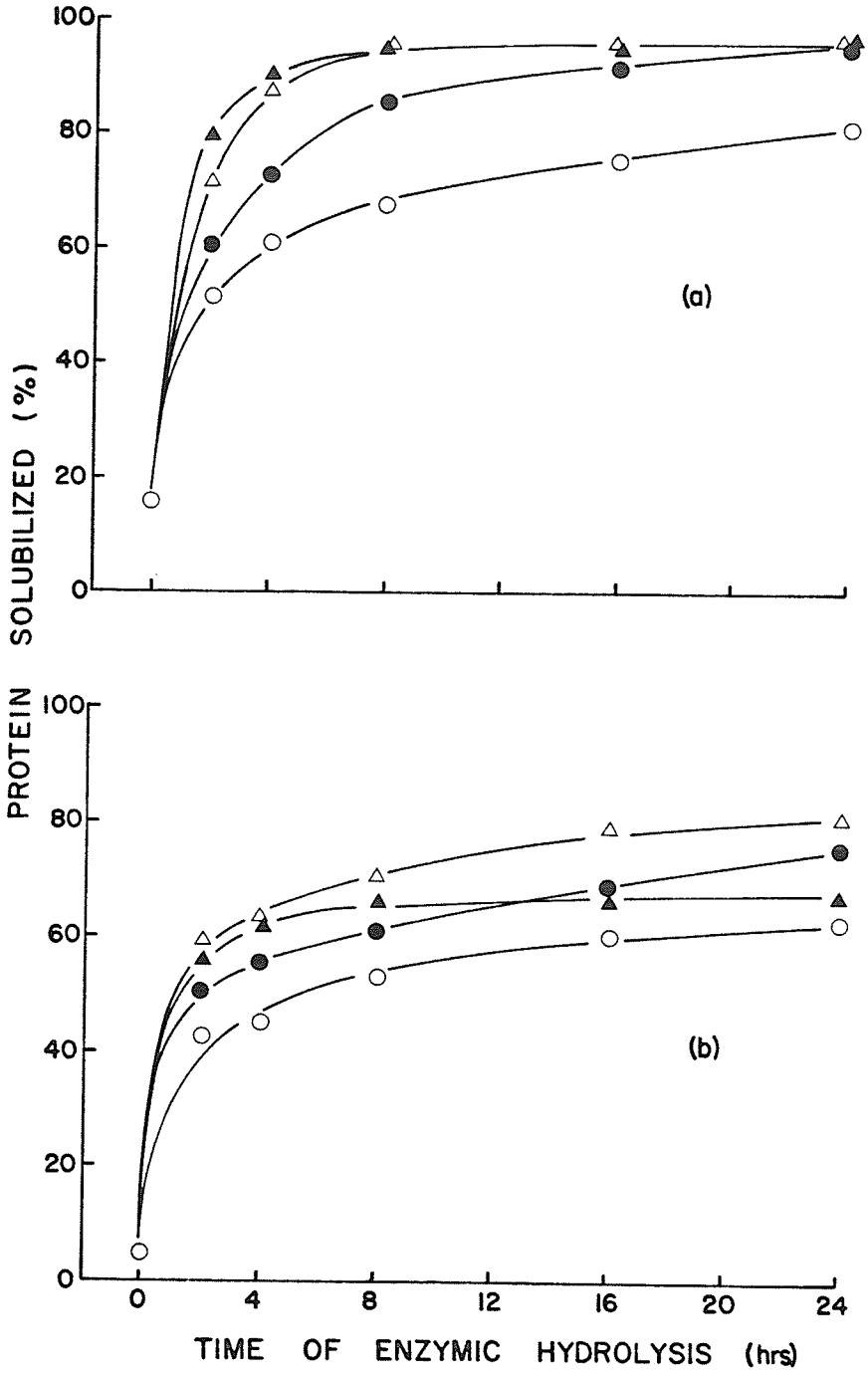
been used because temperatures between those reported here were not tested. Significant difference wherever it has been used in this thesis was reported at 95% level of probability.

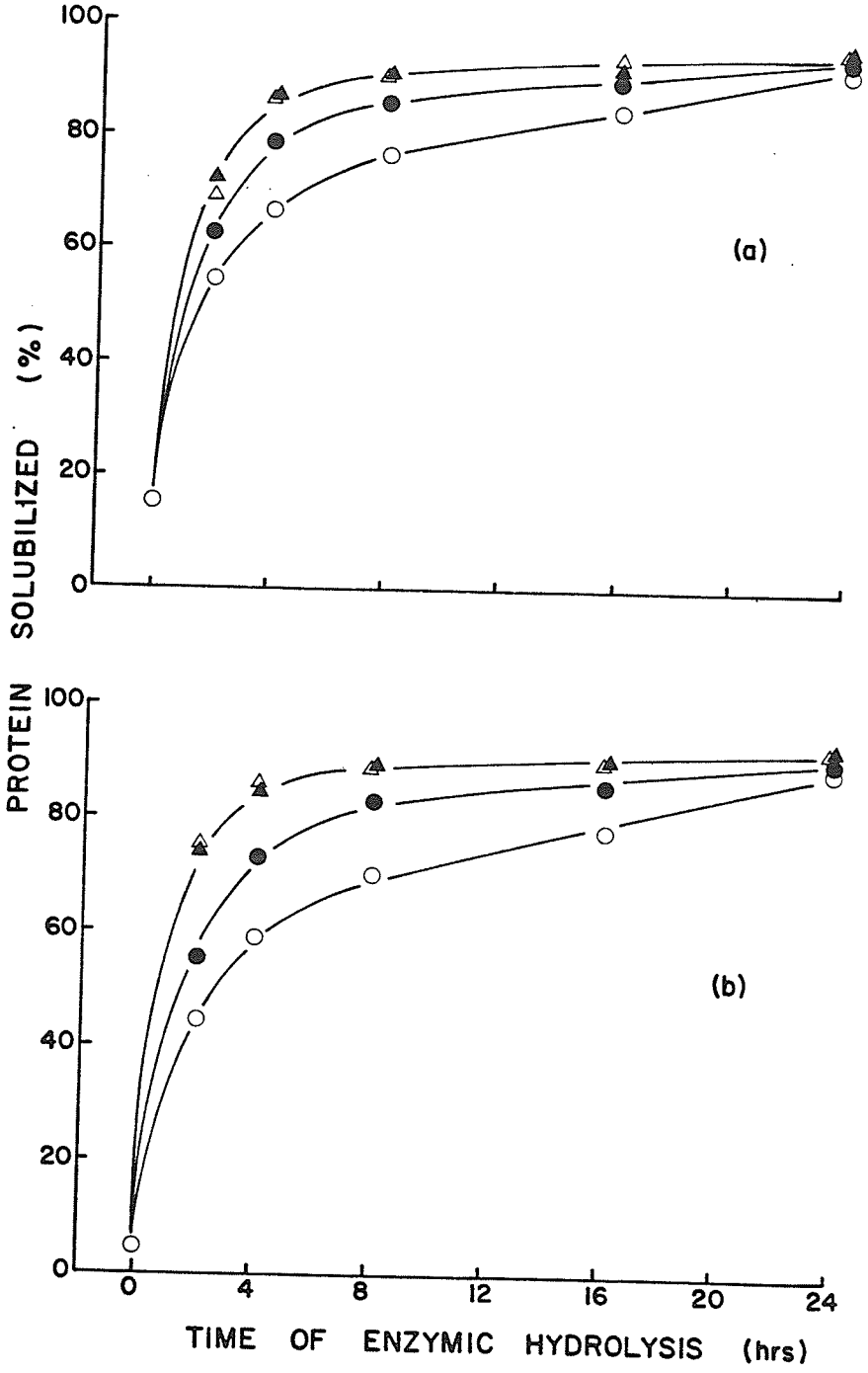
Nomoto and Narahashi (1959) using casein as substrate in 0.01 calcium acetate, pH 7.4 reported that the temperature optimum for Pronase was between 40° C and 60° C. Short incubation times of up to 45 minutes were investigated by these workers. Fritz *et al.* (1973) using barley protein as substrate in phosphate buffer of pH 7.4 and incubation times of up to 48 hours reported that Pronase enzyme had a higher activity at 60° C than at 40° C. Thus, the findings obtained in this study differ somewhat with what has been previously reported in the literature. While in this study it was found that there are some differences due to the substrates used (Figs. 9a and 9b), the activity of the enzyme towards the two substrates used in an incubation period of 24 hours was lower at 57° C than at any of the other lower temperatures tested. It is possible that the different temperature optima observed might be attributed to the different pH and buffer systems used. However, the wide temperature range for maximum activity found in this study and also earlier reported by Nomoto and Narahashi (1959) is in close agreement with the multi-enzymic nature of the Pronase preparation (Narahashi *et al.*, 1968).

All subsequent cereal protein solubilization using Pronase enzyme was carried out at 45° C.

4.2.3 Papain and Thermolysin. Increasing the temperature from 45-65° C for papain with maize (K_{26} x K_{52}) (Fig. 10a) and for thermolysin with both maize (K_{26} x K_{52}) (Fig. 11a) and sorghum X (Fig. 11b) proteins as

Figure 10. Temperature optimum of papain for maximum protein solubilizing ability of (a) maize (b) sorghum.
45° C ⊙—⊙ ; 57° C ●—● ; 65° C △—△ ;
70° C ▲—▲ .


100
80
60
40
20
0
SOLUBILIZED (%)
(a)
PROTEIN
100
80
60
40
20
0
(b)
0
4
8
12
16
20
24
TIME OF ENZYMIC HYDROLYSIS (hrs)


100
80
60
40
20
0
SOLUBILIZED (%)
(a)
PROTEIN
100
80
60
40
20
0
(b)
0
4
8
12
16
20
24
TIME OF ENZYMIC HYDROLYSIS (hrs)

substrates increased the rate of enzyme activities for incubation times less than 24 hours. The initial rate for papain was slightly higher at 70° C than at 65° C with maize protein as substrate. For thermolysin there was no significant difference in activity at either 65° C or 70° C for either substrates over the incubation times tested. After a 24 hour incubation time, the amount of maize protein solubilized by papain at 45° C, 57° C, 65° C and 70° C was 81.2%, 95.3%, 95.5% and 95.6%, respectively. For thermolysin with maize protein (Fig. 11a) as substrate, 91.8%, 93.5%, 94.0%, and 93.1% of total protein was solubilized under the same incubation temperatures. With sorghum protein (Fig. 11b) as substrate 88.6%, 91.0%, 91.6% and 90.1% were solubilized at these same temperatures in 24 hours of incubation.

The temperature optimum for thermolysin activity was found to be 65-70° C at the shorter incubation times. However, when incubation times of 24 hours were considered, raising temperatures from 45-70° C had no significant effect on the amount of protein solubilized. The amount of protein solubilized by papain after 24 hours at 45° C was, however, found to be significantly different from the amount solubilized at the higher temperatures that were tested.

It was found that papain (Fig. 10a) and thermolysin (Figs. 11a and 11b) have maximum activities at relatively high temperatures (65-70° C). Similar temperatures to the ones found in this study optimum for the activities of the two enzymes have been reported for papain (Hwang and Ivy, 1951; Byers, 1967); at these temperatures thermolysin was reported to be quite stable (Matsubara, 1967; Drucker and Borchers, 1971).

With sorghum protein as substrate (Fig. 10b) papain was found to have maximum activity at 65° C. At this temperature 81.3% of the sorghum protein was solubilized in 24 hours. The slightly different temperature optimum and the lower protein solubilization obtained by papain on sorghum may be accounted for by the fact that a high concentration of tannin (5.4%) was present in this sorghum (Table 4). Possibly the tannins may have affected the papain activity.

Based on the results obtained in this study, Pronase, papain and thermolysin enzymes were found suitable for cereal protein solubilization. At their respective temperature optima, these enzymes solubilized most of the proteins from both sorghum and maize samples under the conditions tested. The incubation temperatures of 45° C for Pronase and 65° C for both papain and thermolysin were hereafter referred to as the optimum temperatures for the respective enzymes.

4.3 Solubilization of Cereal Flour Proteins by Enzymes at Their Respective Temperature Optima

4.3.1 Amount of Protein Solubilized. The amounts of protein that were solubilized from each sample by Pronase, papain and thermolysin after 24 hours at their temperature optima for maximum activities are shown in Table 5. The most susceptible proteins to enzymic solubilization were those from triticale and wheat in which at least 95% of the total protein was solubilized by each of the three enzymes. Maize and rye proteins were found to be the second most susceptible cereal proteins that were used. Oats, in which a maximum of 87.2% of the total protein was solubilized by any one of the enzymes, were found to be the most resis-

TABLE 4. Tannin content[a] of sorghum samples

Sorghum variety	Tannin content
Winner	0.10
Riosweet	3.2
Variety X	5.4

[a]percent 'on as is' sample basis

TABLE 5. Amount of cereal flour protein solubilized by enzymes at their respective temperature optima in 24 hours

Sample	Protein solubilized (%)		
	Pronase	Papain	Thermolysin
barley			
Betzies	96.9	96.9	93.4
Herta	94.4	93.9	91.3
Karl	94.6	94.7	90.8
Risø 1508	92.3	95.6	88.7
maize			
A495 fl_2 x B8 fl_2	96.6	97.9	93.5
K_{26} x K_{52}	96.2	95.5	93.9
$W63o_2$ x $M206o_2$	96.7	98.1	92.8
oats			
Harmon	76.4	75.6	71.8
Hudson	75.9	75.2	71.8
Terra	87.2	82.2	75.6
rye			
Gazelle	97.0	96.5	95.0
Prolific	95.9	94.0	93.5
UC-90	97.0	96.2	94.6
sorghum			
Riosweet	94.1	83.6	90.7
X	95.7	81.3	91.0
Winner	97.3	96.9	94.2
triticale			
Carman	97.7	96.6	96.3
Cocorit 71 x UC-90	98.2	97.2	96.4
Rosner	97.3	96.0	95.1
Welsh	97.8	97.0	96.0
wheat			
Cocorit 71	98.7	98.5	97.2
Neepawa	98.0	96.6	96.3

tant cereal proteins to the enzymic activities.

Two sorghum varieties, Riosweet and sorghum X, were very resistant to papain solubilization with only 83.6% and 81.3% of the total protein solubilized respectively. A third variety, Winner, had 96.9% of its total protein solubilized by papain. Winner sorghum protein was solubilized to the highest extent by any of the three enzymes as compared to the other two varieties. However, the activities of both Pronase and thermolysin were not as adversely affected by Riosweet and sorghum X as was papain activity.

On average both papain and Pronase gave similar percentages of protein solubilization for all the cereals tested except for the two sorghum varieties, Riosweet and sorghum X, and Terra oats. Thermolysin solubilized only marginally less protein than papain and Pronase for most of the cereals. For Riosweet and sorghum X, however, it proved more effective than papain although it was slightly less effective than Pronase.

In all the cereal grain varieties used, with the exception of oats and Karl barley, at least 95.0% of the total protein was solubilized by at least one of the enzymes. Such soluble protein fractions can be considered to closely approximate both the total amount and composition of the whole cereal proteins for the cereals tested. Determination of total protein or amino acid composition from these enzymic hydrolyzates would therefore give values very close to the ones that would be obtained from whole sample proteins.

The reasons for the resistance of the oat proteins to solubilization by any one of the three enzymes and of Riosweet and sorghum X to

solubilization by papain as compared to other cereals were not apparent. It was therefore found necessary to do further studies to find out what it was about these cereal samples that was affecting the enzyme activities.

The presence of tannins, polyphenolic compounds have been reported in sorghum. The tannins depressed growth in chicks (Featherston and Rogler, 1975; Rostago *et al.*, 1973) and in rats (Featherston and Rogler, 1975) due to their effect on proteolytic enzymes. The tannins also lowered the *in vitro* digestibility of proteins (Ramachandra *et al.*, 1977). The tannins got associated with the proteins (Ramachandra *et al.*, 1977); this probably altered the enzyme specificities. Sorghum varieties vary considerably in their tannin contents (McMillan *et al.*, 1972). There is a positive correlation between sorghum tannin content and seed color (McMillan *et al.*, 1972), with high tannin sorghums being darker.

Since sorghum X was darker than the other two varieties, it was thought that tannin at least in this one variety might have affected the papain activity. The tannin contents of the sorghum varieties were therefore determined. The results that were obtained are shown in Table 4. Winner sorghum which was found to be the most susceptible to the enzyme activities contained 0.1% of tannins on 'as is basis'. Riosweet and sorghum X which were more resistant to enzyme activities contained 3.2% and 5.4%, respectively. It is therefore very likely that tannins were responsible for the much reduced papain activity towards the two sorghum varieties observed in this study. Hence, papain would only be suitable for protein solubilization from the low tannin sorghums. McMilliam *et al.* (1972) in a study of 142 domestic

and exotic lines of sorghums reported tannin contents of 0.1-8.0%. Riosweet and sorghum X are, henceforth, referred to in this study as the high tannin sorghums.

All the cereal flour samples that were used in this study had been defatted. When the undefatted oat samples were treated with the three enzymes only papain gave a different degree of solubilization as compared to the defatted oat flour (Table 6). In this case almost complete solubilization of protein was obtained with the undefatted oat flour samples.

Of the three oat varieties tested, much more of the Terra oat protein was solubilized by each of the enzymes (Table 6). Hence, there seems to be a slight varietal difference in the protein susceptibility to enzyme activities.

With KCN activated papain, Byers (1967) reported that defatting of an extracted maize leaf protein with a neutral organic solvent slightly increased the degree of protein digestion. Tsai *et al.* (1972) reported a similar observation when using either Pronase or a mixture of alcalase and pancreatic trypsin to hydrolyze defatted maize protein. Defatting of samples in this case (Tsai *et al.*, 1972) with n-hexane, prior to enzymatic hydrolysis gave higher values of lysine in the solubilized protein. Tsai *et al.* (1972) postulated that defatting allowed the enzymatic hydrolysis of a relatively lysine-rich protein (or proteins) otherwise protected by association with the lipid fraction. If defatting at the same time did not protect other protein from the enzymatic hydrolysis, then defatting would be expected to have increased the total amount of protein hydrolyzed or solubilized.

TABLE 6. The effect of defatting oat flour samples on the amount of protein solubilized by enzymes

Enzyme	Oat Variety	Protein Solubilized (%)	
		Defatted	Undefatted
Papain	Harmon	75.6	94.7
	Hudson	75.2	94.1
	Terra	82.2	98.2
Pronase	Harmon	76.4	76.2
	Hudson	75.9	76.0
	Terra	87.2	87.9
Thermolysin	Harmon	71.8	72.6
	Hudson	71.8	70.7
	Terra	75.6	75.3

The decrease in papain activity towards protein of the petroleum ether defatted oat samples is not in agreement with what has been reported in the other cereals (Byers, 1967; Tsai *et al.*, 1972). It is possible that defatting of the oat flour samples might have changed the conformation of protein molecules so as to be less susceptible to papain, while such a change in conformation left the specificities of the other two enzymes not much changed.

4.3.2 Degree of Hydrolysis of Solubilized Protein. The extent of protein hydrolysis obtained by each of the three enzymes from the samples that were used is shown in Table 7. The degree of hydrolysis obtained with Pronase was approximately 2½ and 3 times that obtained with thermolysin and papain, respectively. In every case the degree of hydrolysis was highest for Pronase and least for papain.

With each of the three enzymes on average the highest degrees of hydrolysis were obtained for sorghum, maize and oat flour protein. The exceptions were for papain with the high tannin sorghums and the oats. Although Pronase gave three times more hydrolysis of protein than papain (Table 7), the two enzymes solubilized similar amounts of protein (Table 5) from all the samples except for the high tannin sorghums.

Thermolysin which gave a slightly higher degree of hydrolysis than papain, solubilized less protein than papain from all cereals except for the high tannin sorghums. Comparison of Tables 5 and 7 shows that the amount of protein solubilization and the degree of protein hydolysis are not correlated.

TABLE 7. The degree of hydrolysis of soluble protein obtained by enzymes after 24 hours at their respective temperature optima

Sample	Degree of Hydrolysis (glutamate equivalents x 10^{-2})		
	Pronase	Papain	Thermolysin
barley			
Betzies	506	163	201
Herta	512	164	211
Karl	527	151	213
Risø 1508	574	180	259
maize			
A495 fl_2 x B8 fl_2	618	248	277
K_{26} x K_{52}	648	207	255
$W63o_2$ x $M206o_2$	610	230	283
oats			
Harmon	558	179	250
Hudson	588	172	273
Terra	584	176	199
rye			
Gazelle	488	187	219
Prolific	516	186	222
UC-90	516	160	199
sorghum			
Riosweet	679	181	277
X	636	173	258
Winner	671	213	321
triticale			
Carman	517	188	214
Cocorit 71 x UC-90	507	190	180
Rosner	524	171	208
Welsh	533	172	219
wheat			
Cocorit 71	564	185	222
Neepawa	530	163	166

4.3.3 Susceptibility of Different Proteins to Enzymic Activities. Table 8 shows the amino acid composition of the cereal protein and of the residue proteins obtained from each of the 7 cereal grains after each enzymic hydrolysis. Values for 16 amino acids are reported. The purpose of this analysis was to determine what types of the cereal proteins were more susceptible to the enzymic solubilization. This would help in the interpretations of some of the results obtained in this study. However, from Table 8 alone, it is difficult to draw conclusions. This is because each of the different cereal proteins were not equally susceptible to all the enzymes and neither were all the cereal grain proteins equally susceptible to each of the enzymes used. General trends would be more apparent.

If all the amino acids in each of the cereal grain proteins were equally solubilized by the enzymes, one would expect to find similar amino acid patterns on a protein basis in both whole and residue proteins. This is a highly theoretical supposition because it disregards among other factors the enzyme specificities as well as the effect each cereal protein may have on the enzymes due to their differences in amino acid composition as well as conformation. However it is a logical starting point. Any deviations from similar amounts of each of the amino acids in both residue and whole protein would mean that the particular amino acid had an altered degree of solubilization as compared to the other amino acids. The most easily solubilized amino acid would have the least proportion in the residue protein as compared to the whole cereal protein, while the least solubilized would have the highest proportion.

Table 8. Amino acid composition[a,b] of whole cereal and residue[c] proteins

Amino acids	Wheat				Maize				Triticale				Barley				Rye				Sorghum				Oat				
		Residues				Residues				Residues				Residues				Residues				Residues				Residues			
	Whole	Pa	Pr	Thr	Whole	Pa	Pr	Thr	Whole	Pa	Pr	Thr	Whole	Pa	Pr	Thr	Whole	Pa	Pr	Thr	Whole	Pa	Pr	Thr	Whole	Pa	Pr	Thr	
Protein	11.2	0.50	0.42	0.61	13.2	0.55	0.74	0.90	15.8	0.78	0.69	0.77	11.5	0.91	0.85	1.10	12.6	0.86	0.53	0.94	9.90	1.66	0.58	1.05	12.6	undet	2.50	1.65	2.04
Lysine	3.16	5.01	3.14	3.95	3.01	6.05	5.21	4.76	3.27	4.66	2.71	4.38	3.92	4.32	3.32	3.88	3.83	5.26	5.15	4.17	2.27	1.31	3.02	2.89	4.48	3.62	4.39	3.20	3.85
Histidine	2.55	1.29	1.64	2.13	2.77	1.54	1.95	4.15	2.40	1.27	1.26	2.22	2.33	1.66	1.63	2.07	2.31	1.46	2.34	1.93	2.35	1.28	3.21	3.83	1.35	1.66	2.94	2.42	2.85
Ammonia	3.93	1.93	2.12	1.59	3.13	1.76	1.70	1.49	3.69	2.01	1.64	1.78	3.41	2.01	1.65	1.67	3.46	2.18	2.49	1.66	3.51	3.43	1.93	1.56	2.99	2.19	3.03	2.64	2.82
Arginine	5.06	4.43	3.75	5.90	5.07	3.88	3.63	4.32	5.62	4.84	3.65	8.03	5.34	4.73	4.31	6.02	5.41	5.05	5.60	6.60	3.68	2.06	4.18	5.72	7.34	5.28	9.07	6.88	9.29
Asparagine	5.64	5.89	5.72	6.05	7.20	5.64	4.95	4.24	6.05	5.16	4.69	7.63	6.36	6.06	6.28	7.48	7.38	5.98	8.46	7.04	7.69	5.97	5.99	5.49	9.16	6.85	11.2	9.52	11.1
Threonine	3.30	2.97	2.98	3.18	3.83	4.50	4.80	4.38	3.11	2.66	2.43	3.77	3.57	3.14	3.21	3.66	3.52	3.18	4.64	3.96	3.49	2.48	3.94	3.71	3.75	2.38	3.31	2.90	3.44
Serine	5.16	2.46	3.39	3.46	5.65	2.98	3.75	3.42	4.95	3.31	3.06	4.82	4.59	3.66	3.69	4.41	4.85	3.33	4.93	4.45	4.97	3.71	4.11	4.37	5.37	2.83	3.73	3.80	4.65
Glutamic acid	34.2	7.40	6.61	8.59	24.4	7.99	10.4	9.89	32.5	7.13	5.23	10.6	29.6	8.52	8.04	10.1	29.4	8.39	9.22	9.38	25.5	22.0	9.29	11.9	24.2	10.8	19.9	16.5	20.2
Proline	10.7	5.17	5.57	4.65	9.30	6.37	8.92	11.4	10.5	4.53	4.18	5.02	12.1	3.64	3.96	3.97	10.9	3.75	6.31	4.24	9.33	6.35	9.35	7.54	6.17	3.99	4.31	4.15	4.30
Glycine	4.60	5.36	6.48	5.95	3.73	5.19	5.60	5.34	4.41	4.95	4.65	6.49	4.46	4.73	5.07	5.57	4.52	5.86	8.65	6.45	3.26	2.26	6.95	6.04	5.59	4.14	5.30	4.54	5.20
Alanine	4.02	4.77	4.55	4.85	9.08	4.42	5.55	4.84	3.99	3.80	3.64	5.69	4.42	4.69	4.91	5.69	4.40	4.20	6.13	5.34	10.8	9.43	5.56	5.53	5.30	3.36	5.02	4.31	5.09
Valine	4.99	4.30	3.95	4.28	5.20	4.69	4.45	5.28	4.56	4.30	3.28	5.34	5.43	4.74	4.33	5.07	5.10	4.80	5.66	4.99	5.62	4.50	5.10	5.24	5.88	3.92	6.18	5.02	5.98
Methionine	1.63	1.91	0.51	1.27	1.85	1.74	1.38	1.49	1.35	0.66	0.54	0.80	1.52	0.44	0.36	0.83	1.42	1.30	0.77	0.91	1.54	1.00	0.99	0.94	1.44	0.79	1.88	0.62	0.91
Isoleucine	3.93	2.73	2.33	2.60	4.01	2.84	2.68	2.39	3.76	2.71	1.96	3.25	3.83	3.03	2.62	3.10	3.70	3.12	3.50	3.16	4.48	3.58	3.07	2.96	4.11	3.20	5.39	4.34	5.30
Leucine	7.89	5.34	5.96	6.21	16.6	6.05	8.37	7.98	7.44	5.15	4.65	7.40	8.04	6.40	6.00	7.00	7.10	5.85	7.83	6.87	16.1	15.0	7.58	7.81	8.55	5.53	9.37	7.73	9.37
Tyrosine	3.05	2.20	1.42	1.87	4.50	2.85	2.30	2.46	2.88	2.01	1.23	2.46	3.16	1.92	1.46	1.94	2.57	2.24	2.11	1.34	4.16	2.53	1.66	1.88	3.87	1.89	2.77	2.09	3.48
Phenylalanine	5.25	2.71	2.90	3.41	6.19	3.05	3.67	3.13	5.47	2.58	2.45	4.16	5.89	3.52	3.51	4.19	5.23	3.10	4.47	4.06	5.75	4.69	3.60	3.61	5.62	3.75	7.09	5.67	6.79
% N Recovery	94.4	60.8	59.3	64.3	96.5	63.5	68.4	71.2	92.1	58.6	48.3	76.9	92.3	62.0	58.3	68.8	91.1	64.8	81.5	70.5	95.3	76.8	71.2	72.2	93.1	61.4	95.1	78.1	93.7

[a]Amino acids are reported as mg per 100 mg protein.

[b]Both the amount of glutamine and glutamic acid are reported as glutamic acid while asparagine and aspartic acid are reported as aspartic acid.

[c]Pa, Pr and Thr represent residues after protein solubilization with papain, Pronase and thermolysin, respectively.

The relative degrees of solubilization of the different amino acids were obtained as in Materials and Methods 3.13. Table 9, column 1, shows the most easily solubilized amino acids by the enzymes. Glutamic acid, proline and tyrosine were found to be the most easily solubilized amino acids. Of all the amino acids, glycine and lysine were found to be the most resistant to enzymic solubilization. Other amino acids that were resistant to the enzymic solubilization were threonine, arginine, alanine and aspartic acid in that order of decreasing resistance. The most striking feature about these results is that most of those amino acids that are found in largest amounts in cereal storage proteins (Kasarda *et al.*, 1971) were found to be the most easily solubilized by the enzymes. Of the basic amino acids histidine was found to be the most easily solubilized. What these results suggested was that the storage proteins, prolamines and glutelins, were more susceptible to the enzymic solubilization than the albumins and globulins. However, there was not enough data to prove this was the case.

The author in the process of obtaining the most easily solubilized amino acid (Table 9, column 1) averaged the amino acid composition of different whole cereal proteins as well as the amino acid composition of residues obtained from 3 different enzymes. This type of analysis would seem questionable. However, when each cereal protein and each enzyme was separately considered (Table 9, columns 3-9), it was still found that the most easily solubilized amino acid was glutamic acid. Glycine and lysine were solubilized to the lowest extent in most cases. The only exceptions were for those cereal proteins that were most resistant to enzymic solubilization (i.e., oat, sorghum and barley) (Table 5).

Table 9. Susceptibility[a,b] of different amino acids to enzymic solubilization

Column	1	2			3			4			5			6			7			8			9			
		Average for 7 cereals per enzyme			Each Cereal Type per Enzyme																					
	Average for 7 cereals and 3 enzymes				Wheat			Maize			Triticale			Barley			Rye			Sorghum			Oat			
Amino acids		Pa	Pr	Th	Pa	Pr	Th	Pa	Pr	Th	Pa	Pr	Th	Pa	Pr	Th	Pa	Pr	Th	Pa	Pr	Th	Pa[a]	Pa	Pr	Th
Glutamic acid	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	2	1	1	1	14	1	2	1	4	4	3
Proline	2	2	4	2	2	4	2	8	13	13	2	2	2	3	2	1	2	3	2	6	11	9	7	2	3	2
Tyrosine	3	4	3	4	8	3	3	7	3	5	8	4	5	5	4	4	9	4	7	4	2	1	2	3	2	6
Methionine	4	9	2	3	14	2	7	13	11	9	4	3	3	1	3	3	11	2	3	5	6	5	4	15	1	1
Phenylalanine	5	5	5	6	5	5	4	4	4	3	3	5	4	4	5	5	3	5	4	13	5	6	9	14	13	12
Serine	6	3	8	8	3	8	6	5	6	8	6	8	9	9	10	10	5	8	8	9	9	10	3	1	5	5
Leucine	7	8	6	6	6	10	8	2	2	2	7	9	8	8	8	7	7	10	10	16	3	3	7	10	11	11
Isoleucine	8	10	7	7	7	6	5	9	7	7	9	6	6	7	6	6	8	6	6	11	7	7	15	16	16	16
Histidine	9	6	9	13	4	7	9	6	9	15	5	7	7	6	7	8	4	7	5	1	15	15	11	13	14	14
Valine	10	12	11	9	9	11	10	12	12	11	13	11	10	10	9	9	13	11	11	12	10	11	9	10	10	10
Aspartic acid	11	14	12	10	12	14	12	11	8	6	10	12	12	13	14	14	6	12	9	10	8	8	14	11	15	13
Alanine	12	13	14	11	15	15	14	3	5	4	14	15	14	15	15	16	14	15	14	15	4	4	5	6	9	9
Arginine	13	7	10	15	10	9	13	10	10	10	12	10	16	12	11	13	12	9	15	2	13	14	12	12	12	15
Threonine	14	11	13	12	11	12	11	14	14	12	11	13	11	11	13	12	10	13	12	8	12	12	5	5	7	7
Lysine	15	16	15	14	16	13	15	16	16	16	16	14	13	16	12	11	16	14	13	3	14	13	16	8	6	4
Glycine	16	15	16	16	13	16	16	15	15	14	15	16	15	14	16	15	15	16	16	7	16	16	13	7	8	8

[a]The figure 1 indicates the most easily solubilized amino acid while 16 the least easily solubilized. Where two amino acids have the same degree of solubilization, they are given the same ranking figure and the following figure is not used.

[b]Pa, Pr and Th are short for papain, Pronase and thermolysin, respectively.

Pa[a] - papain on an undefatted oats sample.

When the average for 7 cereals for each enzyme was considered (Table 9, column 2), it was again found glutamic acid was the most labile while glycine and lysine were the most resistant to enzymic solubilization.

Thus, the data analysis that was done here was not seriously in error. When Table 9 was examined more closely, however, it was found that most other amino acids were solubilized to different extents by different enzymes. This was expected because of the different enzyme specificities. However, at the top half of Table 9, the most easily solubilized amino acids occurred in greater frequency for all enzymes and cereal grains. At the bottom half of the table most resistant amino acids likewise occurred in greater frequency for all enzymes.

In this context, that an amino acid was said to be more easily solubilized should not be taken to mean that the peptide bond involving that particular amino acid was more labile to enzyme attack. Cleavage of peptide bonds at other points may solubilize polypeptides containing a large proportion of that amino acid.

The recoveries of total nitrogen as amino acids (Table 8) in the residues were much lower than the whole samples. These low recoveries may be explained by the assumption that as the amino acid and protein nitrogen in the residues were removed the non-protein and non-amino acid nitrogen contributed more to the residue nitrogen. Some of the amino acids are also destroyed in the standard acid hydrolysis of proteins and this would also lower the recoveries of all samples. However, the low nitrogen recoveries would be expected to affect the values of the amino acids in the residues in a similar manner. Hence, the ranking of the amino acids (Table 9) stays basically the same.

4.3.4 Amount of Lysine (%) Solubilized after 24 Hours by Enzymes at Their Respective Temperature Optima for Activity. The total amounts of lysine solubilized from the cereal flour proteins by each of the three enzymes in 24 hours at their temperature optima are shown in Table 10. Beside each lysine value is the amount of glutamic acid that was solubilized under the same conditions. Table 5 shows the total amount of protein that was solubilized from the whole cereal proteins under the same conditions.

Since from Table 9, it was found that lysine compared to the other amino acids was one of the most resistant to the enzymic solubilization, the percentage of this amino acid solubilized should be less than the total amount of protein solubilized. Likewise glutamic acid which was found to be the most labile amino acid to the enzymic solubilization should give a higher percent solubilization than the total protein that was solubilized. Comparisons of the values in Tables 10 and 5 shows that in fact this was the case.

With the exception of the maize $K_{26} \times K_{52}$ for which papain was most effective, Pronase solubilized more lysine from each of the other cereals than the other two enzymes. The maximum lysine solubilized ranged from 91.5% for maize to 96.4% for triticale.

5. Comparison of Protein Solubilization to Literature Values

All the literature values that have been used for comparison in this section have been reviewed in subsection 1.7 of Literature Review on enzymic solubilization of cereal proteins. Any reference, therefore, cited here should be referred to this subsection for the respective

TABLE 10. Relative amount of lysine (and glutamic acid) solubilized after 24 hours by enzymes at their respective temperature optima for activity

Sample	Amino Acid Solubilization (%)					
	Papain		Pronase		Thermolysin	
	Lys	Glu	Lys	Glu	Lys	Glu
Herta barley	91.3	97.7	93.7	98.0	90.5	96.7
Maize, $K_{26} \times K_{52}$	91.5	98.6	90.2	97.6	89.1	97.2
Terra oats (undefatted)	96.1	97.9	-	-	-	-
Prolific rye	90.6	98.0	94.3	98.7	91.6	97.6
Sorghum X	-	-	92.2	97.9	86.5	95.0
Rosner triticale	92.8	98.9	96.4	99.3	93.5	98.4
Neepawa wheat	92.9	99.0	96.3	99.3	93.2	98.6

values.

Under the conditions that were used in this study (Materials and Methods Section) a higher degree of protein solubilization was obtained with Pronase than the values that were reported by Büchmann (1979). It was found that the most resistant cereal protein to Pronase enzymic activity in this study (Table 5) was that from oats while Büchmann (1979) reported maize protein was the most resistant of the cereals he used. However, rice and oat proteins (Büchmann, 1979) were the next most resistant to Pronase activity. Although rice was not used in the study reported in this thesis, the results agreed to some extent with Büchmann's (1979) as far as oats were concerned. Wheat and rye, reported as the most susceptible proteins by Büchmann (1979), were found to be some of the most susceptible in the results obtained in this study (Table 5).

Comparison of the protein solubility obtained with papain (Table 5) to values that have been reported in the literature was made difficult due to the differences in conditions used. When protein samples are purer it would be expected that they would be more accessible to the enzymic activities. All other conditions remaining the same, higher degrees of hydrolysis or solubilization would therefore be expected.

Unlike Byers (1967) or Fafunso *et al.* (1976) no attempt was made to extract the cereal proteins in this study before they were subjected to enzymic hydrolysis. Under Byers' (1967) conditions it is unlikely that all the protein was extracted from the leaves before it was subjected to enzymic hydrolysis. Using similar methods of extractions (Pirie, 1971), 35-50% of the total protein was extracted from cereal

leaves such as maize. The amount of protein that was extractable under the conditions of Fafunso *et al.* (1976) before these proteins were subjected to enzymic hydrolysis was at most 73.5%.

In this study proteins were solubilized from whole cereal flours. Since the flour extraction rate for most of the samples used was at least 95% (Table 3), the proteins so tested closely approximated the composition of the whole cereal proteins. Except for the defatted oats the high tannin sorghums, Prolific rye, Betzies and Karl barleys, the amount of protein solubilized from different cereals by papain (Table 5) were similar or slightly higher than the values obtained by Byers (1967) on extracted maize leaf protein. The values obtained in this study (Table 5) were much higher than those reported by Fafunso *et al.* (1976) on extracted vegetable proteins.

Using Pronase and trypsin, Saunders and Kohler (1972) determined the *in vitro* protein digestibility in wheat millfeeds. The workers found that the enzymically indigestible protein was divided into two types: i) the protein that was of limited digestibility because it was tightly bound in the cellulosic matrix of the aleurone cells and ii) the protein that remained indigestible even after a severe cellulase treatment of aleurone cell wall. The protein that is enclosed in the cell walls of the outer seed coverings is rich in basic amino acids (Kasarda *et al.*, 1971). In the study reported in this thesis the basic amino acids were found to be less solubilized by enzymes than most of the other amino acids. This would appear to be due to cell wall enclosed proteins.

The resistance of the cell wall enclosed proteins to enzyme diges-

tion, however, fails to explain the much reduced enzymic protein solubilization of the oats as compared to the other cereal grains (Table 5). In the view of the author of this thesis, it would appear that those protein fractions rich in basic amino acids are also resistant to enzymic hydrolysis. This would explain the fact that oat proteins with a very high content of globulins and albumins of about 80% as compared to values of less than 20% (Johnson and Lay, 1974) for most other cereal types used in this study were found to be more resistant to enzymic solubilization. Rice, also with much higher glutelin contents than other cereals (Johnson and Lay, 1974), was found by Büchmann (1979) to contain some of the most resistant cereal proteins to enzymic solubilization. It would seem therefore that the second type of protein found by Saunders and Kohler (1972) to be enzymically indigestible were really the basic-amino-acid-rich protein fractions.

6. Summary

1) At least 95% of the total protein of the samples that were used in this study can be solubilized by at least one of the enzymes Pronase, papain or thermolysin.

2) Of the 5 enzymes tested for the cereal protein solubilizing ability, trypsin and α-chymotrypsin were the least effective with neither solubilizing more than 60% of the total protein.

3) Pronase and papain gave similar amounts of protein solubilization while thermolysin gave slightly less for most of the samples that were used.

4) Pronase gave the highest degree of protein hydrolysis while papain gave the least for all the samples that were tested with the three enzymes (1).

5) The enzyme papain was found unsuitable for the solubilization of protein from the high tannin sorghums.

6) Of the three enzymes, Pronase, papain and thermolysin, it is only papain that was found suitable for essentially complete solubilization of protein from oat samples.

7) The amino acids that occur in higher frequency in the storage proteins were found to be more susceptible to enzymic solubilization than the other amino acids.

8) Unlike the amounts of protein solubilized, Pronase on average solubilized higher amounts of lysine than either papain or thermolysin from all the samples that were tested.

III. AVAILABLE LYSINE ESTIMATION

1. Review of Literature

The methods used for the estimation of available lysine are based on the reactivity of the free ε-amino group of lysine with specific colorimetric reagents. The amount of the reagent that reacts is taken to be a direct measure of available lysine. The reaction may be allowed to take place in intact proteins followed by hydrolysis of the protein. Alternatively, protein could be hydrolysed first and then reacted with the reagent. In both procedures the available lysine which is labelled must be separated and quantitated.

The term available lysine in amino acid analysis is almost always applied to the lysine molecule whether in bound or free form that has a free ε-amino group. When the ε-amino group is bound in any form then that bond is not susceptible to enzymatic hydrolysis in monogastric animals (Roach *et al.*, 1967) and such lysine has no nutritive value.

The reagents that have been commonly used for the determination of the ε-amino groups are the Sanger's (1945) reagent 1-fluoro-2,4-dinitrobenzene (FDNB), 2,4,6-trinitrobenzenesulfonic acid (TNBS) and 2-chloro-3,5-dinitropyridine (DNPyr). Besides reacting with the epsilon amino groups, the above reagents will react with α-amino groups. Other functional groups like the imidazole of histidine and the guanidine of arginine have been reported to react with FDNB and DNPyr (Selim, 1965;

Tsai *et al.*, 1972). Each of the above methods has been applied to estimation of available lysine and each method has various advantages and disadvantages. These will be discussed with relation to each other as they are applied to intact proteins followed by hydrolysis of the labelled samples or as they are applied to previously hydrolysed samples. The FDNB and TNBS will be discussed as they are used in intact proteins while the FDNB and DNPyr will be discussed as they are used in previously hydrolysed protein samples. All the problems discussed in FDNB and TNBS in intact proteins methods except those directly associated with acid hydrolysis of samples also apply to FDNB and DNPyr when they are applied to previously hydrolysed samples. These problems will therefore not be rediscussed for those methods in which these reagents are allowed to react with previously hydrolysed samples.

1.1 Reaction of Epsilon-Amino Group with Colorimetric Reagent in Intact Proteins

1.1.1 The 1-Fluoro-2,4-Dinitrobenzene Methods. Sanger (1945) first used this reagent for the determination of the N-terminal amino groups of proteins and peptides. The reagent also reacts with the ε-amino groups of lysine and hence under certain conditions can be used to determine the amount of available lysine. The use of this reagent for determinations of ε-amino groups was based on two observations. Those were: i) the bond formed between ε-amino group of lysine and FDNB was stable under the conditions used for the acid hydrolysis of proteins; ii) washing the hydrolysed dinitrophenylated protein with ether presumably left ε-DNP-lysine as the only aqueous soluble amino acid derivative.

Carpenter and Ellinger (1955) used FDNB reagent to estimate the available lysine in feeding stuffs. The results for 15 feed samples of animal by-products showed a highly significant correlation of 0.97 with results obtained in a biological assay of protein quality with chicks.

However, the Carpenter and Ellinger (1955) method has some interferences. Although most of the colored DNP-amino acids that might be present in the hydrolyzate are removed by ether extraction, in addition to ε-DNP-lysine, the aqueous phase still contains α-DNP-arginine, δ-DNP-ornithine and ε-DNP-hydroxylysine (Carpenter, 1960) which may give apparent available lysine values. Although δ-DNP-ornithine and ε-DNP-hydroxylysine would be measured in full, their levels are quite low in food products (Carpenter, 1960). Interferences due to α-DNP-arginine can be eliminated by making use of methoxycarbonyl chloride (MC-Cl) (Bruno and Carpenter, 1957). Use of MC-Cl made ε-DNP-lysine ether soluble. The ether was evaporated and ε-DNP-lysine redissolved in the aqueous phase before the absorbance readings were recorded. FDNB has also been reported to react with the phenolic hydroxy group of tyrosine (Kotaki and Satake, 1964). Interferences due to o-DNP-tryrosine are eliminated by recording the absorbance of ε-DNP-lysine at wavelengths in which o-DNP-tyrosine does not absorb (Selim, 1965).

During the acid hydrolysis of dinitrophenylated proteins, the recoveries of ε-DNP-lysine are much reduced. In the case of the animal proteins, Carpenter (1960) showed that these recoveries were as low as 90%. Carpenter (1960) therefore recommended a correction factor. In plant proteins where the carbohydrate concentrations are higher, Booth (1971) showed that the recoveries of ε-DNP-lysine were even lower

(60-85%).

The presence of carbohydrates during acid hydrolysis of DNP-proteins causes various effects. The NO_2 groups of DNP-amino acids may be converted by the reducing sugars to NH_2 groups which have a lower color intensity (Blom *et al.*, 1967). The dinitrofluorobenzene may be degraded to dinitrophenol and this is especially so in the presence of high concentrations of carbohydrates. Using ethyl ether as the solvent Conkerton and Frampton (1959) found that it was difficult to separate the dinitrophenol so produced from the ε-DNP-lysine. However, the absorption of the former compound was dependent on pH while that of the latter was not. Thus the workers by taking the absorbance readings of a mixture of dinitrophenol and ε-DNP-lysine at two different pH values were able to correct for error caused by the dinitrophenol. Unlike Conkerton and Frampton (1959), Selim (1965) reported that the dinitrophenol was completely soluble in ethyl ether while the ε-DNP-lysine was not soluble at all and hence the two could easily be separated.

The colored humins arising from carbohydrates during acid hydrolysis of dinitrophenylated proteins may contribute to too high available lysine values (Holm, 1971). Use of methoxycarbonyl chloride, besides removing interferences due to α-DNP-arginine eliminated the interference due to the yellow colored humins (Carpenter, 1960).

Other methods have been used to correct for the low recoveries of ε-DNP-lysine and the variable values in the presence of carbohydrates. The ratio of the weight of sample to the volume of acid used was shown to be critical to the available lysine values obtained. As long as this ratio was kept low, the destruction of amino acids during the acid

digestion was minimized. This was demonstrated to be true by Booth (1971) using various materials, by Matheson (1968) on groundnut and by Rao *et al.* (1963) on cottonseed. Various chromatographic methods (Datta, 1976; Holm, 1971; Rao *et al.*, 1963) have also been used to isolate ε-DNP-lysine from interfering humins and other compounds present in the acid hydrolyzate of dinitrophenylated samples.

Roach *et al.* (1967) used a difference method to determine the available lysine. Total lysine in acid hydrolyzate was determined in an automatic analyzer. Free lysine in solution after a separate hydrolysis of the protein treated with FDNB according to the Carpenter (1960) method was also determined in the automatic analyzer. The difference between the above two values was taken as a measure of available lysine. Later this difference technique was modified by Ostrowski *et al.* (1970) to make use of automatic analyzer unnecessary. The free lysine was separated in column chromatography before quantitation by ninhydrin reagent. The absorbance readings were taken at 570 mn in a spectrophotometer.

The methods for available lysine determination using FDNB have been reported to be laborious and time consuming and are therefore not suitable for the routine analyses of proteins (Kakade and Liener, 1969). Besides FDNB is a vesicant agent, and, hence, needs careful handling. For these reasons use of TNBS for available lysine determinations has been recommended (Hall *et al.*, 1973).

1.1.2 The 2,4,6-Trinitrobenzenesulfonic Acid Methods. The TNBS procedure used only a 1-hour hydrolysis time as compared to an overnight hydrolysis with FDNB methods (Kakade and Liener, 1969). The short

hydrolysis time obviated the need for the correction factors encountered with FDNB procedures when applied to foods rich in carbohydrates.

TNBS is more selective in its reactivity than FDNB. It will react with α-amino groups and will form ω-substituted derivatives such as ε-TNP-lysine. However, unlike FDNB, TNBS will not react with the phenolic hydroxy group of tyrosine nor with the imidazole imino-groups (Kotaki and Satake, 1964). Reaction of TNBS with amino groups occurred at far milder conditions than reactions with FDNB (Okuyama and Satake, 1960).

The ε-TNP-lysine is, however, much less stable than ε-DNP-lysine. Under alkaline or acid conditions the ε-TNP-lysine breaks down to picric acid and the free amino acid (Kotaki and Satake, 1964). After heating ε-TNP-lysine at 110° C for 10 hours, 26% of ε-TNP-lysine broke down to free lysine (Kotaki and Satake, 1964). Hence, under prolonged heating in acid conditions the TNBS methods like the FDNB methods would require factors to correct for the amount of ε-TNP-lysine destroyed. Prolonged hydrolysis was however shown to be unnecessary to obtaining accurate available lysine values. This is because both free and peptide form lysine will be equally detected (Kakade and Liener, 1969).

Like FDNB, the TNBS methods are subject to the same type of interferences. With the TNBS methods, the yellow products obtained following acid hydrolysis except lysine, hydroxylysine, cadavarine, ornithine and the amino sugars are extracted into the ether (Hall *et al.*, 1973). Hence, ether extraction alone does not eliminate all the interferences. In their use of 2,4,6-trinitrobenzensulfonic acid as the protein coupling reagent, Hall *et al.* (1975) reported that the presence of carbohy-

drate during the hydrolysis caused production of a dark brown compound. Depending on the amount of carbohydrate present, a heavy black precipitate was formed. The black precipitate affected the measurement of ε-TNP-lysine by both contributing to the color intensity at the wavelength at which ε-TNP-lysine was measured as well as adsorbing some of the ε-TNP-lysine in solution (Hall *et al.*, 1973). Treating the hydrolysed samples with sodium hypochlorite had a decoloring action and reduced interference due to carbohydrates by 70-90% or more. Hence, the determination of available lysine in carbohydrate-rich material was made more accurate (Hall and Henderson, 1979). TNBS can be used to determine available lysine in cereals and in feeds provided that the ratio of sample to acid volume is kept low (Hall *et al.*, 1973).

1.1.3 Effect of Free and N-terminal Lysine on FDNB and TNBS Methods. Both free and N-terminal lysine upon treatment with FDNB and TNBS form α,ε-bis-DNP-lysine and α,ε-bis-TNP-lysine respectively, and these are soluble in ether used to wash the labelled proteins (Okuyama and Latake, 1960). The α,ε-bis-DNP-lysine is relatively stable to prolonged heating with 6 N HCl. The α,ε-bis-TNP-lysine upon heating in 6 N HCl for 10 hours at 110° C decomposed to give 81% of ε-TNP-lysine and 17% free lysine (Kotaki and Satake, 1964). Thus both the N-terminal and free lysine cannot be detected in procedures using FDNB followed by ether extraction nor in procedures using TNBS in which acid hydrolysis does not precede the ether extraction.

1.1.4 The Terminology Available Lysine as Applied to Available Lysine Estimation in Intact Proteins. When the FDNB and TNBS have been used

for the determination of available lysine in intact proteins from protein or feed samples it has been questionable whether all the free ε-amino groups have been accessible to these reagents. This led to the proposition of the term accessible lysine by Booth (1971) to distinguish it from the lysine which although has a free ε-amino group did not in some way or other react with the colorimetric reagents.

1.2 Reaction of Epsilon Amino Group with Colorimetric Reagents After Protein Hydrolysis

If the protein is first hydrolysed before it is allowed to react with the colorimetric reagents the problems of inaccessible lysine would be largely eliminated. However, this would generate more N-terminal and/or free lysine which would be lost during the organic solvent extraction stage. There are reagents that will react selectively with the α-amino but not with the ε-amino groups (Selim, 1965). Use of these reagents before dinitrophenylation reactions are allowed to take place will produce only ε-DNP-lysine because the α-amino groups are already blocked. This has made it possible for the partial or complete hydrolysis of proteins before reaction with colorimetric reagents.

Determination of the ε-amino group after the hydrolysis of protein into free amino acids was done by Selim (1965). In his procedure (Selim, 1965), protein samples were first hydrolysed in 6 N HCl. The α-amino groups were blocked with copper ions at an alkaline pH of 9.0 followed by treatment with FDNB at the same pH value and ether extraction at an acid pH. However, since this method made use of acid hydrolysis prior to reaction with FDNB, total lysine and not available lysine was determined.

Determination of available lysine after hydrolysis of proteins is made possible by the application of enzymes. Use of enzymes does not release the bound ε-amino group of lysine. However, there is no known single enzyme to date that will completely hydrolyse all the peptide bonds in a protein molecule. Complete hydrolysis of protein requires use of more than one enzyme (Bennett *et al.*, 1972; Hill and Schmidt, 1962).

When use of TNBS was made in the determination of available lysine complete hydrolysis of protein was not necessary (Kakade and Liener, 1969). Small TNP-peptides possessed essentially the same solubility (Kotaki and Satake, 1964) and spectrophotometric (Okuyama and Satake, 1960) properties as the free TNP-amino acids. Similarly, Tsai *et al.* (1972), using 2-chloro-3,5-dinitropyridine have shown the color yield of free lysine was the same as that of lysine dipeptides. Thus it is possible to determine available lysine from enzymic hydrolyzates in which there is not a complete hydrolysis of all the peptide bonds.

Following the enzymic hydrolysis of proteins, the α-amino groups are selectively blocked with copper ions at an alkaline pH while the free ε-amino groups remain unreactive. After removing excess copper the supernatant is treated with the colorimetric reagent followed by washing with an organic solvent. Selim (1965) used FDNB as the colorimetric reagent, followed by ether washing. Villegas and Mertz (1971) and Tsai *et al.* (1972) used DNPyr as the colorimetric reagent and washed the reacted samples with ethyl acetate. Use of enzymes to hydrolyse the proteins also avoids the problems encountered with the acid hydrolysis of carbohydrate rich samples. Although both FDNB (Selim,

1965) and DNPyr (Tsai *et al.*, 1972) have been used quite successfully for estimation of lysine in previously hydrolysed samples, DNPyr would be more preferred because FDNB is vesicant.

1.3 Choice of Wavelength for Absorbance Recording of the Lysine Chromophore

The maximum absorption of ε-DNP-lysine has been reported to occur at 364 nm (Blom *et al.*, 1967), 350 nm (Selim, 1965), of ε-DNPyr-lysine at 340 nm (Tsai *et al.*, 1972) and that of ε-TNP-lysine at 346 nm (Kakade and Liener, 1969). However at these wavelengths o-DNP-tyrosine, o-DNPyr-tyrosine and o-TNP-tyrosine do strongly absorb. Mainly to avoid this interference, measurement of ε-lysine chromophore at higher wavelengths of 435 nm for ε-DNP-lysine (Rao *et al.*, 1963), 390 nm for ε-DNP-lysine (Selim, 1965), 400 nm for ε-DNPyr lysine (Tsai *et al.*, 1972) and 415 nm for ε-TNP-lysine (Hall and Henderson, 1979) have been recommended.

1.4 Application of Methods Used for Available Lysine Estimation to Cereal Proteins

In the cereal proteins most of the protein is insoluble. Therefore the methods that are used for the estimation of available lysine by allowing the colorimetric reagents to react before sample hydrolysis, will not be applicable as all the free ε-amino groups may not be accessible to reagents (Booth, 1971). The cereal proteins have to be solubilized prior to available lysine estimation. Acid hydrolysis of samples prior to available lysine estimation cannot be done because this will at the same time release the blocked ε-amino groups. Even if all the ε-amino groups in the intact cereal proteins were able to react with the

colorimetric reagents, later acid hydrolysis of the reacted samples would cause other problems (discussed above) due to the high carbohydrate contents of samples. Hence in cereal grains, acid hydrolysis before or after the samples have reacted with colorimetric reagents will introduce errors in the available lysine estimations.

Mainly to overcome the problems of cereal protein insolubility and to avoid the problems associated with acid hydrolysis of cereal samples, enzymes have been found to be an attractive alternative (Villegas and Mertz, 1971; Tsai *et al.*, 1972; Fritz *et al.*, 1973). Another advantage of using enzymes is that the bound ε-amino group is not released in the course of protein solubilization. Enzymes usually do not give a complete hydrolysis of protein samples. However, available lysine in peptide form (Tsai *et al.*, 1972; Kakade and Liener, 1969) was found to be equally detectable as compared to free available lysine. Hence, enzymes have been successfully used in sample hydrolysis before estimation of available lysine.

At 400 nm it has been shown that only three amino acids would slightly interfere with the absorbance readings of ε-DNPyr-lysine (Tsai *et al.*, 1972). It has therefore not been found necessary to use the methods discussed above to isolate the lysine chromophores after the organic solvent extraction step. Readings can directly be taken after washing of the samples with the organic solvent.

2. Introduction

Of those methods outlined for available lysine estimation, the method presented by Tsai *et al.* (1972) was selected for use. Preliminary use of this assay confirmed two observations in the literature.

Firstly, the buffers which have been used can affect the values obtained. Two buffers, sodium carbonate-bicarbonate and sodium borate have been used. Selim (1965) found that the presence of carbonate during the dinitrophenylation stage of lysine with 1-fluoro-2,4-dinitrobenzene in borate buffer interfered with the assay. With the available lysine assay described by Tsai *et al.* (1972), no such interference was noted. Tsai *et al.* (1972) therefore indicated that the two buffers could be used interchangeably without any effect on the values obtained.

Secondly, the presence of amino acids other than lysine in the assay can have a significant effect on the available lysine values estimated to be present. Tsai *et al.* (1972) pointed out the slope of standard lysine curves obtained when no enzymatic hydrolyzate supernatants were included were lower than those in which the supernatants were included. The fact that the available lysine method was going to be applied to enzymatically solubilized protein with variable levels of free amino acids made a knowledge of the effect of other amino acids on this assay particularly pertinent. Since these factors were related to both the sensitivity and accuracy of this assay the optimum conditions as related to these two factors were reinvestigated. The optimized assay conditions were then applied to estimation of available lysine from cereal protein hydrolyzates.

3. Materials and Methods

3.1 Materials

The samples that were used for the available lysine estimation are shown in Materials and Methods 3.1 of Section II. The 19 barley samples

that were used for the determination of the correlation between available and total lysine values were selected from a collection of samples obtained from Dr. B. Rossnagel of the University of Saskatchewan.

3.2 Sample Preparation

All the cereal samples were ground and defatted as in Materials and Methods 3.2 of Section II. For the estimation of available lysine the samples were solubilized by the enzymes for a period of 24 hours as in Method 3.8 of Section II. The hydrolysed samples were centrifuged at 700 g for 5 minutes and the clear supernatants so obtained were used for the estimation of available lysine. All samples were in 0.05 M borate buffer of pH 8.3.

3.3 Reagents

Two buffers, namely sodium carbonate-bicarbonate (0.6 M) of pH 9.0 and sodium borate (0.05 M) of pH 9.0, were prepared and used for up to two weeks. Copper phosphate suspension was prepared according to Tsai *et al.* (1972) and used for up to 1 week. The reagent 2-chloro-3,5-dinitropyridine (DNPyr) was also prepared according to Tsai *et al.* (1972) in methanol immediately before it was used. Hydrochloric acid (1.0 N) was used to acidify samples after the incubations. The buffers and copper phosphate suspension were stored at 4° C when not in use.

3.4 Reagent Proportions

In this study the reagent proportions of Villegas and Mertz (1972) and Tsai *et al.* (1972) as listed below were used:

Reagent	Proportion by Volume	
	Villegas and Mertz (1971)	Tsai *et al.* (1972)
Sample	1.0 ml	0.5 ml
Buffer	0.5 ml	1.0 ml
Copper phosphate suspension	0.5 ml	0.5 ml

3.5 Procedures Used to Test the Effect of Buffer Species on the Absorbance Values

Procedure 1: To 0.5 ml of sample in a 12 x 40 mm test tube a volume of 1.0 ml borate buffer was added followed by 0.5 ml of copper phosphate suspension. The mixture was shaken occasionally in a vortex mixer for 5 minutes. The sample was centrifuged at 700 g for 5 minutes to precipitate the excess copper phosphate suspension. From the clear supernatant fraction 1.0 ml of sample was pipetted into a 20 x 125 mm Kimax screw cap test tube and 0.1 ml of DNPyr reagent was added. The sample was shaken in a vortex mixer. The reaction was allowed to proceed at room temperature (22-24° C) in a covered (dark) shaking water bath for 2 hours. The sample was then acidified with 4.0 ml of 1.0 N HCl. The ε-DNPyr-lysine was freed from excess reagent and other DNPyr-amino acids by washing with ethyl acetate (3X 4.0 ml aliquots). Each time the sample was inverted about 10 times and the organic layer removed by suction provided by a water aspirator. The absorbance of the aqueous phase was recorded at 400 nm relative to sample free blank.

Procedure 2: Was as in Procedure 1 except that 1.0 ml of carbonate buffer was substituted for the borate buffer.

Procedure 3: Was as in Procedure 2 except the sample was in carbonate buffer.

In going from Procedure 1 to Procedure 3 above the concentration of carbonate buffer increased at the expense of borate buffer.

3.6 Effect of Glycine on the Absorbance Values

The effect glycine had on the absorbance values of a 200 µg lysine·HCl was studied under the conditions of Procedures 1-3 above. Various amounts of glycine (0-0.08 mMoles) were added to the 1.0 ml buffer aliquot of each procedure.

3.7 Effect of Other Amino Acids on the Absorbance Values

The effect that other naturally occurring amino acids have on the absorbance readings was investigated under the conditions of Procedure 1. A 200 lysine·HCl sample was used in each case. Each of the amino acids tested was used at the concentration of 0.06 mMoles/sample (0.5 ml or 1.0 ml of sample diluted to a total of 2.0 ml, see Method 3.4 above).

3.8 Effect of Glycine in the Presence of Enzymic 'Supernatants' on the Absorbance Values

Using a fixed amount of lysine·HCl (200 µg) the combined effect of added glycine plus the 'supernatants' from both papain and Pronase digests were examined. The effect was followed in both Procedures 1 and 2. The lysine·HCl was added either in 0.5 ml of the enzyme 'supernatant' or in borate buffer alone. The glycine was dissolved in the assay buffer.

The papain and Pronase 'supernatants' fractions used in this study

were obtained by pooling the individual 'supernatants' from all the samples listed in Method 3.13 of Section II (with the exception of sorghum) for each enzyme. The 'supernatants' were obtained as in Method 3.2 above and were preadjusted to a pH of 9.0 before they were used.

3.9 Effect of pH on the Absorbance Values

The effect that pH has on the absorbance values of a 200 µg lysine·HCl sample in the presence of 0.06 mMoles glycine was investigated under the conditions of Procedures 1 and 2. Initially, buffers at the lowest pH values investigated were prepared. The reagents were mixed as in Procedure 1 and before centrifugation was done the pH of the mixture was adjusted with sodium hydroxide solution. At each pH value tested an aliquot was pipetted out, centrifuged and subsequent operations were carried out as in Procedure 1. Corrections were made for the dilution during the pH adjustments.

3.10 Standard Procedure

To 1.0 ml of sample a volume of 0.5 ml borate buffer, pH 9.6 containing 5 mg glycine was added. A volume of 0.5 ml copper phosphate suspension prepared in 0.05 M borate buffer of pH 9.6 was then added. The rest of the operations were carried out as in Procedure 1. Quantitation was carried by comparison to standard lysine values (100 µg lysine·HCl ≡ 80 µg lysine).

3.11 Total Lysine Determination

For total lysine determination a ca 40 mg of sample was hydrolysed in 4.0 ml of 6 N HCl at 110° C for 24 hours. Lysine in the acid hydrolyzate was then determined in Beckman Model 121 Automatic Amino Acid

Analyzer.

3.12 Estimation of Available Lysine from Cereal Proteins

For estimation of available lysine in cereal samples the enzymic 'supernatants' were obtained as in Method 3.2 above. The available lysine values were determined under the conditions of the Standard Procedure 3.10 and reported as a percent on total protein basis.

3.13 Correlation Between Available and Total Lysine Values

To check the correlation between available and total lysine values barley samples ranging in total lysine content from 3.31-5.72 mg per 100 mg protein were used. The available lysine contents of the samples were determined as in Method 3.12. Total lysine values were determined as in Method 3.11.

3.14 Available Lysine Recovery Tests

The potential effect of various carbohydrates in the enzyme digests on the recovery of available lysine was examined with one enzyme, thermolysin. Under the standard digestion conditions that have been used for thermolysin (Materials and Methods 3.2 above) 200 µg lysine·HCl plus thermolysin were incubated alone or with one of the following carbohydrates: i) 100 mg starch, ii) 100 mg sucrose, iii) 100 mg glucose. A second experiment was carried out in the same manner but with the following combinations of carbohydrates: i) 2 mg glucose + 90 mg starch, ii) 5 mg glucose + 90 mg starch, iii) 7 mg glucose + 90 mg starch. At the end of the incubation period aliquots were removed for lysine estimation by the Standard Procedure 3.10.

3.15 Estimation of Reducing Sugars

The Nelson-Somogyi method as outlined by Robyt and Whelan (1968) was used in the determination of reducing sugars. Glucose was used as the standard.

4. Results and Discussion

4.1 Optimization of Available Lysine Assay Conditions

4.1.1 Effect of Buffer Species on Absorbance Values. The effect of the buffer species on this assay was tested under three conditions: i) standard lysine sample alone, ii) standard lysine sample in papain 'supernatant' and iii) standard lysine sample in Pronase 'supernatant'. The absorbance values that were obtained from standard lysine samples under these conditions are shown in Table 11. When no enzymatic 'supernatants' were included, the values obtained in Procedure 1 were much lower than those obtained in Procedure 2. The only difference between these procedures is that in Procedure 2, 1.0 ml of carbonate buffer was used instead of 1.0 ml of borate buffer. This suggested that the buffer species used affect the absorbance values obtained.

If papain 'supernatant' was included, the absorbance readings increased to similar values under both Procedures 1 and 2 (Table 11). The Pronase 'supernatant' resulted in only slightly higher values than those from the papain 'supernatant' with Procedure 2. However in Procedure 1, the Pronase 'supernatant' gave much higher values than the papain 'supernatant'. This was despite the fact that the absorbance values due to the enzymic 'supernatants' had been accounted for.

Thus besides the differences due to the buffers themselves that

TABLE 11. The effect of buffer species under different sample conditions on the relative absorbance values of standard lysine samples[a,b]

Buffer system	Lys·HCl per sample	Absorbance (400 nm)		
		No enzyme 'supernatant'	Papain 'supernatant'	Pronase 'supernatant'
Procedure 1	100	0.016	0.105	0.160
	200	0.044	0.242	0.328
Procedure 2	100	0.094	0.107	0.117
	200	0.184	0.225	0.240

[a]Where the enzyme 'supernatants' were used, the standard lysine samples were dissolved in the 'supernatants'.

[b]The values were an average of duplicate samples.

were observed here, there seems to be differences due to the types of the enzyme 'supernatants' that were used and these were more pronounced in Procedure 1.

Both papain and Pronase solubilized similar amounts of cereal protein at their temperature optima for activity, except for the high tannin sorghum varieties (Table 5). However, Pronase gave three times more hydrolysis of soluble protein than papain (Table 7).

Earlier Finley *et al.* (1972) used FDNB to determine added lysine in fortified wheat and bulgur. At the low reagent concentrations and high temperatures used, these workers reported that they were unable to obtain complete reaction with lysine·HCl. When they increased the total amino acid concentration through the addition of the neutral amino acids, alanine or glycine, quantitative reaction was achieved. The workers found that even where the reaction was incomplete the response to increasing lysine·HCl concentrations was nearly linear.

From Finley *et al.'s* (1972) work and from the observations that had already been made in the work reported in this thesis, it seemed the differences in values caused by different enzyme 'supernatants' were probably related to the levels of 'free amino acids' (and/or small peptides). If this was so, the values obtained in this assay would be expected to increase with the amount of 'free amino acids' present in the 'supernatant' possibly to a certain limit. As long as the level of 'free amino acids' was not high enough, even the use of one enzyme to solubilize samples containing the same amount of available lysine but different percent protein or degree of hydrolysis would be expected to give different values.

Selim (1965) used hydrochloric acid to hydrolyse protein samples under conditions expected to give complete hydrolysis of all peptide bonds. Lysine in the hydrolyzate was then determined with 1-fluoro-2,4-dinitrobenzene as the colorimetric reagent. Tsai *et al.* (1972) used either Pronase or a mixture of alcalase and pancreatic trypsin to hydrolyse the samples before determination of available lysine with 2-chloro-3,5-dinitropyridine. Less free amino acids would be expected in this instance than under Selim's (1965) conditions.

Use of either borate or carbonate buffers by Tsai *et al.* (1972) (equivalent of Procedures 1 and 2, respectively, in this study) in the available lysine determinations gave similar values. The same observation was made in this study (Table 11) when a papain 'supernatant' was included. In the determination of lysine with the borate buffer, if carbonate was present (Selim, 1965), it interfered with the dinitrophenylation of lysine. Thus, it would be reasonable to expect lower values in the presence of carbonate. The same observation was made in this study (Table 11) where Pronase 'supernatant' was used.

It seems the observations that were made by Selim (1965) and Tsai *et al.* (1972) under the conditions that they worked, were not in error. However, what the two different workers might have failed to notice was the interaction in this assay between effects on absorbance values caused by the buffer species and by the level of 'free amino acids'. It was found necessary in the study reported in this thesis to delineate between these effects. At this stage it was therefore decided to test the effect the naturally occurring amino acids have on the absorbance values of standard lysine samples.

4.1.2 Effect of Different Concentrations of Glycine on the Absorbance Values of a Standard Lysine Sample. Fig. 12 summarizes the results that were obtained. Initially when no glycine was added, Procedure 1, with the highest amount of borate buffer, gave the lowest absorbance values. Procedures 2 and 3 containing carbonate buffer gave higher values. However as the concentration of glycine was increased, there was a faster increase in absorbance values in Procedure 1 than in either Procedures 2 or 3. Above 0.06 mMoles/sample there was only a slight or no further increase in absorbance values.

At 0.06 mMoles glycine concentration the highest absorbance values were obtained in Procedure 1 while the lowest in Procedure 3. At this point the effect the other amino acids have on the lysine values was reinvestigated.

4.1.3 Effect of Other Naturally Occurring Amino Acids on the Absorbance Values of a Standard Lysine Sample. The effect the other amino acids had on the absorbance values of a standard lysine sample are shown in Table 12. The concentration (0.06 mMoles/sample) for each amino acid used was based on the results obtained with glycine above.

Preliminary tests and the results of Finley *et al.* (1972) showed that the presence of free amino acids other than lysine increased the absorbance readings of standard lysine samples. Further investigations showed that the specific amino acids used fell into four groups according to their effect on absorbance values of standard lysine samples (Table 12). The amino acids in group I caused the highest increase in absorbance values, while the amino acids in group IV caused the lowest increase in absorbance values as compared to the samples that did not

Figure 12. The effect of various glycine concentrations in different buffer species on the absorbance values of a standard lysine sample. Procedures 1 to 3 (Method 3.5) were used and are indicated in the figure as Procedure 1 ⊙ — ⊙ , Procedure 2 ● — ● and Procedure 3 ▣ — ▣ . In all the three procedures 200 µg lysine·HCl was used and values were an average of two sets of duplicate samples.

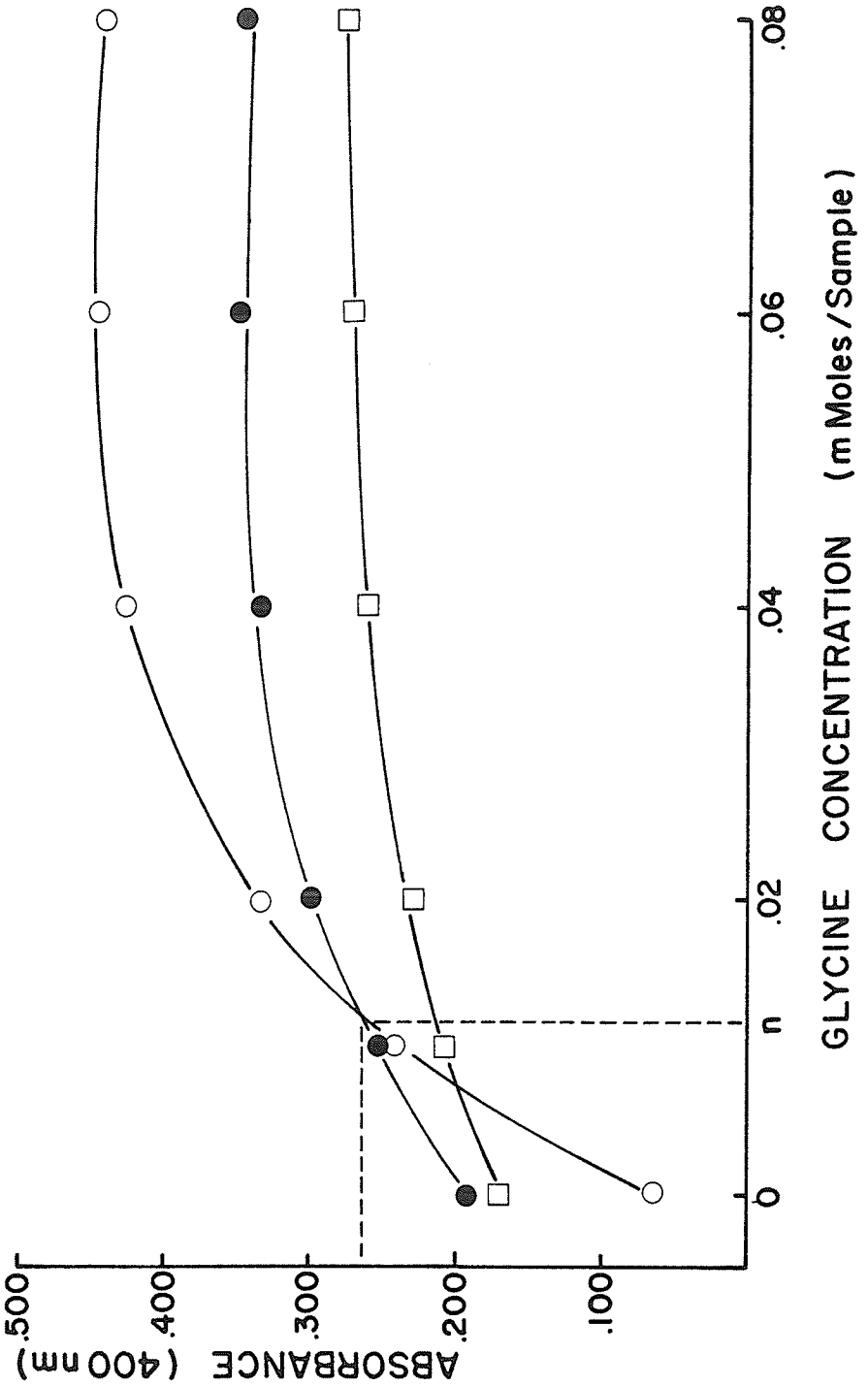

ABSORBANCE (400 nm)
.500
.400
.300
.200
.100
0
n
.02
.04
.06
.08
GLYCINE CONCENTRATION (m Moles / Sample)

TABLE 12. The effect of various amino acids on absorbance values of a 200 µg lysine·HCl sample in borate buffer (Procedure 1)

Amino acid added (0.06 mMoles/sample)	A_{400}
-	0.065
Group I	
1. Glycine	0.458
2. Alanine	0.442
3. Valine	0.455
4. Serine	0.440
5. Glutamine	0.432
6. Threonine	0.436
Group II	
7. Aspartic acid	0.364
8. Glutamic acid	0.368
9. Asparagine	0.327
10. Isoleucine	0.310
Group III	
11. Proline	0.209
Group IV	
12. Leucine	0.117
13. Phenylalanine	0.117
14. Methionine	0.102

contain 'free amino acids'.

4.1.4 Effect of Glycine in the Presence of Enzyme 'Supernatants' on the Absorbance Values. The next question that arose was whether a group I (Table 12) amino acid (glycine) at a total concentration of 0.6 m Moles per sample but in the presence of other amino acids will cause the same absorbance values as when it is alone. To test this, Procedures 1 and 2 and supernatants from enzymic hydrolyzates were used.

The results obtained are shown in Table 13. The absorbance values that were obtained from standard lysine samples in the presence of 0.06 m Moles of glycine with or without the inclusion of enzymic 'supernatants' were practically the same in any one Procedure (buffer system). Comparison of Tables 11 and 13 showed that not only did the presence of 0.06 m Moles glycine eliminate the differences in absorbance readings due to enzyme 'supernatants', but raised the absorbance readings under any one buffer system to similar values whether or not the enzyme 'supernatants' were included. Thus once a minimum concentration of glycine (and possibly any other of the amino acids in group I in Table 12) has been included the enzyme 'supernatants' will not significantly change the absorbance values of a standard lysine sample. However in terms of sensitivity the values obtained in Procedure 1 were higher than those obtained in Procedure 2.

It would be useful at this stage to cite the observations of Selim (1965) and Tsai *et al.* (1972) as concerns this assay. In Fig. 12, if one was working at a total glycine concentration corresponding to point n, one would indicate that there is no difference in values obtained in either borate buffer (Procedure 1) or carbonate buffer (Procedure 2)

TABLE 13. The effect of glycine[a] (0.06 mMoles) in the presence of enzyme 'supernatants' on the relative absorbance values of standard lysine samples[b]

Buffer system	Lys·HCl per sample	Absorbance (400 nm)		
		No 'supernatant'	Papain 'supernatant'	Pronase 'supernatant'
Procedure 1	100	0.226	0.232	0.240
	200	0.460	0.454	0.470
Procedure 2	100	0.174	0.175	0.183
	200	0.358	0.350	0.357

[a]Glycine was added as in Method 3.6.

[b]Standard lysine samples were added as in Table 11 and the values reported were an average of duplicate samples.

in this assay. At lower glycine concentrations, borate would be thought to be interfering while at higher glycine concentrations carbonate would be said to be interfering.

Since Tsai *et al.* (1972) noted no difference in absorbance values in either borate or carbonate buffers, it would be reasonable to believe they worked at a total amino acid concentration having an equivalent effect to n mMoles of glycine. This could be expected because they used enzymes to solubilize the protein. However, Selim (1965) noted that the presence of carbonate interfered. These workers used acid to hydrolyse protein samples thus generating more free amino acids than under Tsai *et al.* (1972) conditions. Most probably Selim (1965) worked at a total amino acid concentration greater than n. Borate buffer (Procedure 1) would then be more preferred in this assay because at higher free amino acid concentrations higher sensitivities would be obtained (Fig. 12) than in carbonate buffers (Procedures 2 and 3).

4.1.5 Effect of pH on Absorbance Values. Selim (1965) noted that when the pH of the reaction mixture was less than 9.0 lower absorbance values for ε-DNP-lysine were obtained. Tsai *et al.* (1972) examined the effect of pH on the absorbance values of standard lysine samples in both borate and carbonate buffers (equivalent of Procedures 1 and 2, respectively, in this text). In both buffers maximum absorbance readings were recorded at pH 9.0. For borate buffer lower pH values resulted in diminished readings. For carbonate buffer higher pH values lowered the absorbance readings much more than lower pH values.

In this study the effect pH has on the absorbance readings is shown in Table 14. The absorbance values in either borate or carbonate

TABLE 14. Effect of pH on absorbance values[a] of a 200 μg lysine·HCl sample in the presence of 0.06 mMoles glycine

pH	Absorbance (400 nm)	
	Borate buffer (Procedure 1)	Carbonate buffer (Procedure 2)
8.6	0.449	0.346
8.8	0.467	0.346
9.0	0.479	0.353
9.2	0.484	0.327
9.4	0.476	0.321

[a]The absorbance values were an average of two sets of duplicate samples.

buffer were found to be affected by pH in a similar manner as reported by Tsai *et al.* (1972). From these results (Table 14), it would be advisable to work at a pH of 9.0 or slightly higher in borate buffer (Procedure 1), but at a pH of 9.0 or slightly lower in carbonate buffer (Procedure 2). Working at the above pH values would maximize on the sensitivity of the assay.

4.1.6 Reagent Proportions. Villegas and Mertz (1971) used slightly different reagent proportions than those reported by Tsai *et al.* (1972) (Materials and Methods 3.4). From the above two reagent proportions, it is evident that under Tsai *et al.* (1972) conditions there is a much more dilution of samples than in the Villegas and Mertz (1971) reagent proportions. Although up to this stage the reagent proportions of Tsai *et al.* (1972) were used, it was realized that the sensitivity would not be high enough when the assay is applied to 'non-destructive' single kernel analyses. It was therefore found more advantageous to use the reagent proportions Villegas and Mertz (1971) to make up for the low sensitivity.

Under the conditions of this study, the cereal grain proteins were solubilized with enzymes in borate buffer, 0.05 M at pH 8.3. Thus under Villegas and Mertz (1971) conditions the pH would be shifted lower to more unfavourable values.

To make use of the more desirable reagent proportions of Villegas and Mertz (1971) and to maximize on sensitivity at the more favourable pH values, the pH of buffer and copper phosphate suspension had to be raised to values such that when they were mixed with the sample (pH ~ 8.3), the resulting pH would be 9.0. When borate buffer was adjusted

to pH 9.6 and the copper phosphate suspension was prepared in borate buffer of pH 9.6, the resulting reaction mixture had a pH of about 9.0. At the above pH values no loss of stability was observed in the reagents. For all subsequent work lysine estimation was carried according to the Standard Procedure as outlined in 3.10. This procedure is based on the reagent proportions of Villegas and Mertz (1971).

4.2 Other Reaction Conditions

In the preparation of lysine standard curves, Villegas and Mertz (1971) mixed free amino acids in different proportions. A 100 mg of the amino acid mixture was dissolved in 10.0 ml of buffer and 0.5 ml of the resulting solution added to 1.0 ml of standard lysine samples. Under Villegas and Mertz (1971) conditions, protein was solubilized from 90-100 mg of ground maize flour samples in 5.0 ml of enzyme solution. From the enzyme hydrolyzate supernatant, 1.0 ml was used for available lysine estimation. In samples 0.5 ml of buffer solution substituted the 0.5 ml of the amino acid mixture that was used in the standard samples.

Possibly Villegas and Mertz (1971) like Tsai *et al.* (1972) noted that failing to include the amino acid mixture in the standard lysine samples resulted in a lower slope. Alternatively, the amino acid mixture could have been a simulated amino acid pattern of the protein of the samples the workers were studying. Which ever of the above two reasons it was, obviously 1.0 ml of enzyme digest would contain much less 'free amino acids' than the amount of amino acids in 0.5 ml of the amino acid mixture. In the study reported in this thesis it has been shown that the absorbance of a standard lysine sample will depend on

the level of free amino acids (Fig. 12). If under Villegas and Mertz (1971) conditions the level of free amino acids was not high enough, then equal concentrations of lysine in standard and hydrolyzate samples would be expected to give different absorbance values.

Table 15 shows the absorbance values obtained from a standard lysine sample under Villegas and Mertz (1971) conditions and other conditions. Only use of 1.0 ml of Pronase enzyme 'supernatant' gave similar values to the use of 0.5 ml of amino acid mixture. Possibly papain under Villegas and Mertz (1971) conditions produced as much 'free amino acids' as did Pronase in the study reported here.

Use of glycine (Table 15) gave higher but similar values both in the presence of papain or Pronase enzyme 'supernatants'. For both higher sensitivity and to nullify the effect of the variable levels of amino acids on the determination of available lysine, glycine or probably another amino acid in group I (Table 12) should be included in both sample and standard lysine assays. Up to this stage 0.06 mMoles glycine per sample was used. On weight basis this works out to be 4.5 mg glycine per sample. For further work this value was raised to 5.0 mg glycine per sample (Fig. 12).

4.3 Precautions

One precaution that should be taken in this assay is that the amount of free amino acids should not be in excess of the copper ions present in the copper phosphate suspension. When this is the case all the free α-amino groups will not be totally blocked and will form new reactive sites during the dinitrophenylation stage. Although the other dinitrophenylated amino acids are washed into the organic layer (Tsai

TABLE 15. Absorbance values[a] obtained from a standard lysine sample (200 µg lysine·HCl) under various conditions

Conditions tested		A_{400}
0.5 ml amino acid mix)	Villegas and Mertz (1971)	0.400
1.0 ml papain 'supernatant')		0.299
1.0 ml Pronase 'supernatant'		0.404
0.06 mMoles gly. + 1.0 ml papain 'supernatant'		0.460
0.06 mMoles gly. + 1.0 ml Pronase 'supernatant'		0.453

[a]The values were an average of duplicate samples and represent the net absorbance after subtraction of the values due to blanks or the enzyme 'supernatants'.

et al., 1972) and therefore would not interfere with the available lysine measurements, two complications might arise. The first is that lysine might contribute to some of these free α-amino groups. When this is the case such lysine molecules will form α,ε-bis-DNPyr-lysine with the colorimetric reagent and since these are soluble in the organic solvent, will escape detection. The second is that if there are more reactive (α-amino groups) sites than the amount of the colorimetric reagent DNPyr, some of the ε-amino groups may fail to react.

4.4 Possible Mechanism Leading to the Increase in the Absorbance of Standard Lysine Samples in the Presence of Other Amino Acids

Finley *et al.* (1972) made some observations that suggested that the copper-lysine complex had limited solubility. In the presence of alanine a more soluble lysine copper complex was formed which resulted in higher recoveries of lysine. In the study reported in this thesis no attempts were made to find out the mechanism that led to the increase of absorbance values in the presence of free amino acids. However, a few observations were made.

When no free amino acids were included in the available lysine estimation, the supernatants obtained from Procedures 2 and 3 after the α-amino groups blocking step, were more blue colored than the supernatants obtained in Procedure 1. This suggested that there were more copper ions in solution in Procedures 2 and 3. The more blue supernatants were also found to give higher absorbance values from standard lysine samples.

As the level of free amino acids was increased in the above three procedures, the supernatants that were obtained were more blue colored

and similarly higher absorbance readings were obtained from standard lysine samples. Those amino acids (Table 12) that resulted in the lowest absorbance values from standard lysine samples were also observed to give less blue colored supernatants.

From these observations, it would seem that in the absence of other amino acids the lysine copper complex would be more soluble in carbonate (Procedures 2 and 3), than in borate (Procedure 1). However, when high levels of free amino acids were included, the lysine copper complex would be more soluble in borate buffer than in carbonate (Fig. 12). Alternatively, it might mean that when high levels of amino acids were included, the lysine-copper complex was equally soluble in borate and carbonate buffers but the presence of carbonate may cause other interferences. The differences in absorbance values obtained when different amino acids were included (Table 12) would therefore probably depend on the effectiveness of these amino acids in solubilizing the lysine-copper complex. In the study that is reported here the amino acids that were tested were shown to fall into four groups (Table 12). Those amino acids in group I would be the most effective while those in group IV would be the least effective in solubilizing the lysine-copper complex.

4.5 Estimation of Available Lysine from Cereal Proteins

Using the Standard Procedure 3.10 above, available lysine values were determined from the enzymically solubilized cereal proteins obtained in Method 3.2. The values that were obtained are presented in Table 16 together with the total lysine values determined as in Method 3.11.

TABLE 16. Comparison of available lysine values[a] obtained from different enzyme 'supernatants' to total lysine values[b]

Cereal grain	Total Lysine Values (mg/100 mg Protein)	Available Lysine Values (mg/100 mg Protein)		
		Thermolysin 'supernatant'	Papain 'supernatant'	Pronase 'supernatant'
barley				
Herta	3.92	3.93	3.57	6.57
Karl	3.91	3.82	3.50	6.47
Risø 1508	5.44	4.65	4.97	7.01
maize				
$K_{26} \times K_{52}$	2.92	2.68	2.55	3.85
A495 fl_2 x B8 fl_2	3.99	3.51	3.46	4.92
$63o_2$ x $MS206o_2$	4.63	4.26	4.22	5.89
rye				
Prolific	3.83	3.75	3.81	7.08
Gazelle	3.71	3.94	3.65	7.12
UC-90	3.70	4.37	4.00	7.38
sorghum				
X	2.27	2.05	-	2.53
Winner	2.64	2.43	2.66	3.67
Riosweet	2.05	2.02	-	3.03

TABLE 16 Continued.

Cereal grain	Total Lysine Values (mg/100 mg Protein)	Available Lysine Values (mg/100 mg Protein)		
		Thermolysin 'supernatant'	Papain 'supernatant'	Pronase 'supernatant'
triticale				
Rosner	3.39	3.68	3.14	6.77
Welsh	3.30	3.26	3.13	6.22
Carman	3.40	3.52	3.43	6.29
GA_{530} x UC-90	2.93	3.34	2.97	6.20
wheat				
Neepawa	3.16	3.34	3.07	6.54
Cocorit 71	2.96	3.32	2.97	5.84

[a]Available lysine values were an average of two sets of duplicate samples.

[b]Total lysine were values from single samples.

The reason for comparing the available lysine values obtained in this study with the total lysine values was to find out how well the method under the study is performing. However the method under study will detect only those lysine molecules with free ε-amino groups.

The ε-amino group is easily blocked by reaction with other groups even under very mild conditions (Blom *et al.*, 1967). Any discrepancies therefore between the total moles of ε-amino groups and the total moles of lysine present in any one sample will be reflected in the values obtained by the two methods. Caution should therefore be used in the interpretation of these results.

From Table 16 the available lysine values determined from the Pronase 'supernatants' were too high compared to total lysine values. Available lysine values determined from thermolysin and papain 'supernatants' agreed more closely among themselves and with the total lysine values. Available lysine values from thermolysin 'supernatants' were generally slightly higher than from papain 'supernatants'.

For wheat, triticale, one sorghum variety and two rye varieties the available lysine values from the papain 'supernatants' agreed quite closely with the total lysine values. For both barley and maize available lysine values obtained from papain 'supernatants' were lower than the total lysine values.

For the thermolysin 'supernatants' the available lysine in two barley varieties, two rye varieties, sorghum, triticale and one wheat variety agreed quite well with the total lysine values, but were on average slightly higher than total lysine values. With maize and Risø 1508 the available lysine from thermolysin 'supernatants' were lower than total lysine values.

Of the cereal types that were used (Method 3.1 in Section II) it was found that available lysine cannot be determined from oats using the methods presented in this thesis. Defatted oats were found to resist enzymic solubilization of protein (Table 5). Although this problem was overcome by using papain on the undefatted sample (Table 6) the 'supernatant' when used became cloudy after ethyl acetate washing stage giving too high an absorbance value (data unrecorded). The much higher fat content (Shukla, 1975) of the undefatted oats as compared to other cereals that were used in this study was thought to be the source of this interference.

From Table 16 three trends are apparent as relates to the available lysine values. These are: i) Different enzymes even if they solubilize similar amounts of protein do not give similar available lysine values from the same samples. ii) Even a one enzyme 'supernatants' of protein from different cereals does not give available lysine values of equal proportions to total lysine values. iii) Furthermore, use of one enzyme on different varieties of the same cereal gave available lysine values not equal in proportions to total lysine values.

4.5.1 Correlation Between Available and Total Lysine Values. If the method under study is to be of use, it should be able to rank samples treated under similar conditions in the same order for available lysine as for total lysine values. Of the cereals that were used in this study, barley and maize have wide ranges of lysine contents. Barley samples were more readily available and so they were used for the correlation study.

In Table 17 the available lysine values obtained from the enzymic

'supernatants' of barley samples were compared to the total lysine values. Generally for the samples that were used, the available lysine values from both papain and thermolysin 'supernatants' ranked the samples in the same order as the total lysine values. However, with papain, the available lysine values were lower than total lysine values and this difference became exaggerated at the higher total lysine values. With thermolysin, the available lysine values were higher than the total lysine values at the lower total lysine values and were lower at the higher total lysine values.

From linear regression analysis, the correlation coefficient between available lysine values from papain and thermolysin 'supernatants' and the total lysine values was 0.92 and 0.79, respectively. Thus available lysine values from papain 'supernatants' were better than those from thermolysin 'supernatants' in predicting total lysine values in the barley samples.

4.5.2 Available Lysine Recoveries. It was not apparent why the available lysine values as determined from the enzymic 'supernatants' did not agree more closely with the total lysine values. Blom *et al.* (1967) pointed out that the free ε-amino group of lysine is very reactive. If the relative moisture content in the atmosphere was rather high (70%), or the moisture content of the sample exceeded certain limits, the ε-amino group might react with free sugars at temperatures as low as 30-40° C (Blom *et al.*, 1967). The conditions under which the samples were solubilized by the enzymes in this study were such that these reactions may have taken place.

The recoveries of available lysine from 200 μg of lysine·HCl incu-

TABLE 17. Comparison between available[a] and total[b] lysine values

Sample No.	Total Lysine Values (mg/100 mg protein)	Available Lysine Values (mg/100 mg protein)	
		Papain 'supernatant'	Thermolysin 'supernatant'
1	3.31	3.10	3.77
2	3.52	3.19	4.02
3	3.56	3.28	3.94
4	3.60	3.33	3.92
5	3.64	3.32	4.02
6	3.72	3.40	4.24
7	3.77	3.14	3.58
8	3.98	3.52	4.11
9	4.10	3.97	4.48
10	4.10	3.98	4.28
11	4.43	3.75	4.50
12	4.44	3.66	4.25
13	4.48	3.90	4.30
14	4.60	3.73	4.26
15	4.63	3.73	4.21
16	4.74	3.85	4.48
17	5.45	4.18	5.34
18	5.50	4.35	4.50
19	5.72	4.76	4.60

[a,b] as in Table 16.

bated with thermolysin in the presence of 100 µg of different carbohydrates is shown in Table 18a. Recoveries of only 74.8% were obtained in the presence of the reducing sugar glucose. In the presence of either sucrose or starch the recoveries were not significantly affected (Table 18a).

While the reducing sugar glucose seriously lowered the available lysine recoveries from standard samples, the amount of reducing sugars used was much in excess of what would be present in cereal samples. For a two-row barley variety Betzies, the amount of total reducing sugars at maturity was found to be 0.2% of kernel dry matter (Laberge *et al.*, 1973). If the barley varieties used in the present study contained similar amounts of reducing sugars (0.2 mg/100 mg sample) would they lower the recovery of available lysine during the solubilization of cereal proteins? When incubated with thermolysin and standard lysine (200 µg lysine·HCl), 0.5 mg glucose in the presence of 90 mg starch did not cause any detectable reduction in available lysine recoveries.

However, after the incubation of cereal flour samples with the enzyme the amount of reducing sugars might not necessarily be the same as initially. Under the conditions of incubation temperature (65° C) and pH (8.3) used in this study any residual amylases in the flour might be active (Reed and Thorn, 1971). In this case more reducing sugars than initially present may be found. When the thermolysin barley hydrolyzates were analyzed for reducing sugars (Method 3.15 above) more reducing sugars than reported for mature kernels (Laberge *et al.*, 1973) were found. At the end of incubation time the amount of

TABLE 18. Recoveries of available lysine[a] in the presence of different carbohydrates

Condition	Recovery (% of total)
(a)	
lys. + 0 mg carbohydrate	100.2
lys. + 100 mg starch	99.2
lys. + 100 mg sucrose	97.3
lys. + 100 mg glucose	74.8
(b)	
lys. + 0 mg carbohydrate	100.8
lys. + 2 mg glucose + 90 mg starch	97.9
lys. + 5 mg glucose + 90 mg starch	93.4
lys. + 7 mg glucose + 90 mg starch	93.5

[a]The values represent an average from a 200 μg lysine·HCl samples in duplicates.

reducing sugars was found to range from 5.3-7.0% glucose equivalents on a dry sample basis in the 19 barley samples that were used. These values of reducing sugars were used to check the recoveries of available lysine from 200 μg lysine·HCl in the presence of 90 mg pure starch plus glucose at values equivalent to the reducing sugars found. The results are presented in Table 18b.

In the presence of 5 mg or 7 mg of glucose the recovery of available lysine was found to be 93.5%. It is therefore quite possible that some of the free ε-amino groups were reacting with the reducing sugars and becoming unavailable during protein solubilization and/or available lysine estimation. Under the conditions used in the estimation of lysine in cereal proteins (Method 3.12), it was not exactly known how much of the free ε-amino groups in the samples would get blocked. The other reducing sugars present in cereal samples may not react with lysine at the same rate as glucose. Neither is the reactivity of the ε-amino group of free lysine necessarily the same as that of lysine in peptide form.

It was expected that the papain hydrolyzate might have more reducing sugars than the thermolysin hydrolyzate for several reasons. First, where the thermolysin might inactivate the amylases, the papain enzyme is known to activate β-amylases (Bendelow and Meridith, 1955). Secondly, comparison of values in Table 17 showed that papain 'supernatant' gave lower available lysine values than thermolysin 'supernatant'. After incubation with papain, the samples were however found to contain less than 0.5% glucose equivalents of reducing sugars.

For papain, potassium cyanide was used for its activation. Cyanide

is known to react with the aldehyde groups of reducing sugars to form cyanohydrins (Buchanan and Byers, 1969). In this case less or no reducing sugars would be detected. Under the conditions used in the study reported in this thesis, it was not known how much of the free sugars might have reacted with the free ε-amino groups or with the cyanide.

All high-lysine mutants in barley and maize have been reported to have the same characteristics of accumulating more sugars (both reducing and non-reducing) than the normal varieties (Kreis, 1977). This possibly may explain to some extent the observations made in Subsection 4.5.1 above, that as the total lysine values in samples increased the available lysine values got comparatively lower. Reducing sugars were shown (Tables 18a and 18b) to lower the available lysine recoveries.

If free ε-amino groups of lysine react with reducing sugars at such mild conditions as used in this study, then estimation of available lysine as opposed to total lysine would be a better predictor of the nutritional quality of cereal grains.

4.5.3 Estimation of Available Lysine from Pronase 'Supernatants'. From the results obtained (Table 16) Pronase would not be a suitable enzyme to hydrolyse samples before the estimation of available lysine. Some of the available lysine values obtained from Pronase 'supernatants' were twice the total lysine values. The cause of these high values observed was not apparent. Preliminary investigations however showed that when Pronase was used alongside papain and thermolysin to hydrolyse pure standard proteins (bovine serum albumin, casein, egg white lysozyme), higher available lysine values were still obtained from Pronase 'super-

natants'. This suggested that the other cereal components in the Pronase enzyme 'supernatants' were not the cause of the higher values recorded.

Inclusion of Pronase enzyme 'supernatant' gave a higher absorbance reading from standard lysine samples than did papain 'supernatant' (Table 15) although both hydrolyzates were shown to contain the same amount of protein (Table 5). However, this was demonstrated to be related to the amount of free amino acids present in the assay (Fig. 12); Pronase enzyme generated three times the degree of protein hydrolysis as did papain (Table 7). In the presence of 0.06 mMoles of glycine similar absorbance values were obtained from standard lysine samples in the presence of either papain or Pronase 'supernatants' (Table 13); thus glycine was added to all samples analyzed for available lysine.

At 400 nm, the only other amino acids that interfered with the lysine determination when 0.6 M carbonate buffer was used were tryptophan, histidine, and arginine (Tsai *et al.*, 1972). In these amino acids the indole ring of tryptophan, the imidazole group of histidine and the guanidine group of arginine react with the colorimetric reagent to give apparent lysine values. Since these groups are equally present in intact or hydrolysed proteins, they should cause an equal interference in all the enzyme hydrolyzates. Whatever the cause of the much higher absorbance values recorded for Pronase enzyme was, most probably it was related to the degree of hydrolysis obtained or to the enzyme specificity. Thermolysin which gave a slightly higher degree of hydrolysis of protein than papain (Table 7) also gave higher available lysine values than papain (Tables 16 and 17).

5. Previous Available Lysine Methods

From the results obtained in this study, it seemed that enzymes are not equally suitable for the solubilization of cereal proteins before estimation of available lysine in the 'supernatants' (Table 16). In the previous methods for the estimation of available lysine, Pronase (Tsai *et al.*, 1972; Fritz *et al.*, 1973), mixture of alcalase and pancreatic trypsin (Tsai *et al.*, 1972) and papain (Villegas and Mertz, 1971; Fritz *et al.*, 1973) have been used to solubilize cereal proteins. None of the above workers except Fritz *et al.* (1973) have commented on the suitability of the enzymes they used for the cereal protein solubilization before estimation of available lysine in the hydrolyzates.

Using barley as substrate, Fritz *et al.* (1973) found that when papain was used to solubilize samples, it gave only 80% of the available lysine values obtained when Pronase was used. Even if papain and Pronase solubilized the same amounts of lysine, Pronase would have been expected to give higher absorbance values under Fritz *et al.* (1973) conditions for two reasons: i) Pronase gives a higher amount of 'free amino acids' than papain and hence a higher absorbance reading from a standard lysine sample (Table 15). ii) Pronase 'supernatants' were found to have an 'inherent' property of giving much higher apparent available lysine values than total lysine values (Table 16).

Under the conditions of Villegas and Mertz (1971), Tsai *et al.* (1972) and Fritz *et al.* (1973), the amounts of 'supernatants' that were used for available lysine determination most likely did not contain high enough levels of 'free amino acids'. This deduction has been made in view of the results obtained in this study (Fig. 12; Table 15). In this

study it has been shown that under Villegas and Mertz (1971) and Tsai *et al*. (1972) assay conditions, an equivalent of at least 0.06 m Moles of glycine should be included in both the standard (Fig. 12) and 'supernatants' (Table 15) to obtain similar absorbance values from similar amounts of lysine.

6. Summary

1) The absorbance values of lysine samples were found to be affected by the level of free amino acids other than lysine. The free amino acids fell into four groups according to their effect on absorbance values.

2) The effect of free amino acids on absorbance values of lysine samples were found to be more pronounced in borate than in carbonate buffer.

3) When a total of 0.06 mMoles of glycine was included, in either borate or carbonate buffers, the absorbance readings of lysine samples did not change with any further increase in the level of free amino acids.

4) Under the modified conditions of the standard procedure presented here, an improvement of 50% in relative sensitivity over the Tsai *et al.* (1972) assay procedure for the same concentration of sample was realized.

5) The different enzymes, used for cereal protein solubilization, were found to be not equally suitable in available lysine estimation even if they solubilized similar amounts of protein. Of the three enzymes that were used, papain was found to give available lysine values more closely correlated to the total lysine values.

6) The effect that different enzymes had on the apparent available lysine values in the hydrolyzate was divided into two: i) the effect related to the level of 'free amino acids' in the hydrolyzate and

this could be controlled by addition of glycine and ii) a second 'inherent' effect probably related to the specificity of the enzymes. The second effect was not able to be controlled in the work reported here.

7) Using the procedures presented in this thesis, it was found that the apparent available lysine values estimated from undefatted oat samples were too high because of 'cloudiness' probably related to the high fat content of oat samples.

8) The available lysine values as determined from sample 'supernatants' were found to get comparatively lower than total lysine values at the higher lysine values. This was possibly related to the level of free reducing sugars in the high lysine samples. The reducing sugar glucose was found to lower available lysine recoveries.

IV. TRYPTOPHAN ESTIMATION

1. Review of Literature

Methods that are commonly used for the determination of tryptophan employ an initial hydrolysis of the sample followed by detection of the amino acid in the hydrolyzates. The procedure used for the detection predetermines the type of hydrolysis to be performed on the samples. Complete hydrolysis of the protein sample to free amino acids is required if an amino acid analyzer is to be used to quantitate the tryptophan (Robel, 1967). Use of colorimetric, spectrophotometric or fluorescent methods for quantitation may or may not require complete hydrolysis. The review that follows centers mainly on the methods of hydrolysis of samples and estimation of tryptophan.

1.1 Hydrolysis of Samples

1.1.1 Acid Hydrolysis. Use of the standard acid hydrolysis conditions employed for the determination of other amino acids leads to an extensive destruction of tryptophan. Acid causes oxidative destruction of tryptophan (Basha and Roberts, 1977). To surmount this problem many modifications of the acid hydrolytic conditions have been introduced.

When mercaptans were added to 6 N HCl in the absence of oxygen and carbohydrates, the recovery of tryptophan was improved (Matsubara and Sasaki, 1969); the recoveries ranged from 80-90%. By use of 3 N

p-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole in evacuated sealed tubes at 110° C, Liu and Chang (1971) obtained recoveries of 90 ± 3% in purified proteins after 24 hours of hydrolysis. Penke *et al.* (1974) showed that free tryptophan with mercaptoethanesulfonic acid and with p-toluenesulfonic acid in the presence of tryptamine or thioglycolic acid gave recoveries of about 95% after 24 hours at 110° C. Use of Matsubara and Sasaki (1969) conditions of 6 N HCl containing 4% thioglycolic acid gave lower recoveries of about 80%.

In the presence of high concentrations of carbohydrates, however, low recoveries of tryptophan were obtained even with the modified acid procedures (Concon, 1975). Thus such methods are not applicable for tryptophan analysis in cereal seed tissues. As an alternative basic hydrolysis has been sought.

1.1.2 Basic Hydrolysis. Unlike acid hydrolysis where starch causes oxidative destruction of tryptophan, during the basic hydrolysis of proteins the presence of starch protects tryptophan from destruction (Hugli and Moore, 1972). Basic hydrolysis in either $Ba(OH)_2$ or NaOH have mostly been used. When $Ba(OH)_2$ was used, some losses occurred due to adsorption of tryptophan in the precipitate during the precipitation of Ba^{++} by $SO_4^{=}$ or $CO_3^{=}$ (Robel, 1967). For this reason use of NaOH has usually been favoured. The tubes have to be evacuated in basic hydrolysis of protein even in the presence of starch, otherwise losses of tryptophan become extensive (Hugli and Moore, 1972).

Destruction of tryptophan in alkaline hydrolysis of proteins was found to be caused by cystine, cysteine, serine and threonine (Spies and Chambers, 1949). Later Spies (1967) found that during the alkaline

hydrolysis of protein, histidine was an effective protective agent of tryptophan against destruction by both cystine and serine; basic lead acetate mainly protected against destruction by cystine. Hence, the worker used 5 N NaOH saturated with lead acetate and containing histidine to hydrolyse proteins. Under these conditions the recoveries of pure tryptophan were not reduced. Alkaline hydrolysis is more likely to obtain quantitative recoveries of tryptophan than acid hydrolysis (Hugli and Moore, 1972). However, most methods of alkaline hydrolysis are either time consuming or inaccurate (Oelshlegel, Jr. *et al.*, 1970). To avoid silicate formation during alkaline hydrolysis special polypropylene coated pyrex tubes have to be used (Oelshlegel *et al.*, 1970). Just as acid hydrolysis results in extensive destruction of tryptophan (Friedman and Finley, 1971), so does basic hydrolysis result in partial destruction of lysine (Davies and Thomas, 1973).

1.1.3 Enzymatic Hydrolysis. To avoid the problems encountered during either acidic or basic hydrolysis of samples, enzymatic hydrolysis has been an attractive procedure. However, there is no one enzyme to date that can hydrolyse all the peptide bonds that occur in proteins. Complete hydrolysis requires application of more than one enzyme (Hill and Schmidt, 1962; Bennett *et al.*, 1972) and this takes a long time.

Partial hydrolysis of proteins places a restriction on the methods that can be used in estimating tryptophan. Only methods that equally detect tryptophan in free or peptide forms could be used. All the same many workers have used a partial enzymic hydrolysis of the protein before estimation of tryptophan by various methods.

Spies (1967) showed that when proteins were partially hydrolysed

with Pronase, before tryptophan estimation with Ehlich's reagent, the values were similar to those obtained from alkaline hydrolyzates. Villegas and Mertz (1971) have used a partial hydrolysis of maize flour proteins with papain before estimation of tryptophan by the modified glyoxylic acid method of Opienska-Blauth *et al.* (1963).

1.2 Estimation of Tryptophan

Once the protein is in solution several methods can be used for the estimation of the tryptophan content. Hydrolysis of the protein by enzymes before estimation of tryptophan was selected on this study (see Literature Review on enzymic solubilization of cereal proteins). A consequence of the decision to use enzymes for protein solubilization was that the protein was partially hydrolysed and thus the tryptophan could occur in both free and peptide bound forms. For this reason the discussion that follows has been limited to those methods which can estimate tryptophan when it occurs in both free and peptide forms.

1.2.1 Direct Ultra-Violet Methods. These methods are based on the fact that in the ultra-violet region, i.e., in the region 250-320 nm, most of the amino acids in a protein molecule are transparent. In this region it is only the amino acids tryptophan, tyrosine and phenylalanine that absorb light. Absorption due to phenylalanine is small and can therefore be disregarded (Goodwin and Morton, 1946). Using this method Goodwin and Morton (1946) recorded the absorbance due to the two amino acids at two wavelengths, one at which their extinction was the same and at another wavelength. The tryptophan and tyrosine were then computed by comparison with standard mixtures of tyrosine and tryptophan. Later,

Bencze and Schmid (1957) modified this technique by using the slope of a line drawn tangent to two characteristic maxima of the absorption curve in the range between 278 nm and 293 nm to measure the amount of tryptophan and tyrosine. This procedure diminished the error due to bathochromic shift in the absorption of tyrosine and tryptophan.

However, one major problem that has been encountered with the application of the absorption spectroscopy (uv methods) is the effect of compact, globular proteins on the absorption due to tryptophan and tyrosine (Edelhoch, 1967). By dissolving the samples in guanidine hydrochloride the solubility of proteins was improved (Edelhoch, 1967), but many proteins were not soluble under these conditions. Edelhoch's (1967) method, like those of Bencze and Schmid (1957) and Goodwin and Morton (1946), made use of tedious calculations and comparison to either free amino acids or model proteins to obtain the tryptophan and tyrosine values.

Although in enzymic protein hydrolyzates the problems of compact and globular proteins as well as insoluble proteins are less likely to be encountered some other problems arise. These methods are indirect and involve two determinations as well as long calculations and would not be suitable for routine use.

1.2.2 Fluorescence Spectroscopy Methods. Fluorescence methods are based on the observation that apart from tryptophan and tyrosine, the other amino acids do not fluoresce or their fluorescence is negligible. Although tyrosine is strongly fluorescent, the choice of wavelength of excitation and emission is such that the fluorescence contribution of this amino acid can be eliminated (Sasaki *et al.*, 1975).

The problems that are encountered in these methods are due to the fact that the fluorescence of tryptophan is strongly influenced by its micro environment. Even in denatured proteins, the fluorescence may differ substantially from that of free tryptophan (Sasaki *et al.*, 1975). To overcome this difficulty Duggan and Udenfriend (1956) employed alkaline hydrolysis; this however led to losses of tryptophan as well as production of other fluorescent products. Shelton and Rogers (1971) measured the fluorescence of proteins dissolved in sodium dodecyl sulfate and mercaptoethanol but obtained low quantum yield. Recently, Sasaki *et al.* (1975) used a mixture of chymotrypsin and Pronase to partially hydrolyse the samples followed by dilution in 6M urea to eliminate the quenching effects of native or denatured proteins. However, since reducing the digestion volume to less than 50 µl resulted in significant losses (Sasaki *et al.*, 1975), this method cannot be successfully applied under the conditions of the study reported in this thesis.

1.2.3 Colorimetric Methods. The colorimetric estimation of tryptophan makes use of specific chromophores. The amount of chromophore that reacts with tryptophan is taken as a direct measure of the amino acid.

There are many colorimetric methods that are used for tryptophan estimation. Only some of the most common ones will be briefly discussed here.

Gaitonde and Dovey (1970) presented a method for determination of free tryptophan or tryptophan in intact protein by treating the protein with ninhydrin in a mixture of formic acid and hydrochloric acid for 10 minutes at 100° C. The absorbance readings were recorded at 390 nm.

In this method, however, the extinction coefficient of the chromophore varied somewhat with the nature of the model compound that was used.

Koshland's reagent, 2-hydroxy-5-nitrobenzylbromide, although widely used for soluble proteins has been reported to be not always sufficiently specific and accurate (Friedman and Finley, 1971). The methods based on sulfenyl halides involve unnecessarily long separation procedures (Boccu *et al.*, 1970) which makes these methods unsuitable for routine analysis.

The majority of the colorimetric methods used for quantitative determination of tryptophan are based on the color reaction between the indole of tryptophan and the aldehydes in acid medium (Opienska-Blauth *et al.*, 1963). Of the aldehydes p-dimethylaminobenzaldehyde (DAB) (Spies and Chambers, 1949) and the glyoxylic acid (Opienska-Blauth *et al.*, 1963) methods have been most commonly used. Use of DAB does not give the same color yield for both free and peptide bound tryptophan (Spies, 1967). This method therefore when applied to enzymic hydrolyzates may give variable results.

The glyoxylic acid has been shown to be one of the most accurate methods (Fischl, 1960). It can be used for determination of both free and bound tryptophan (Opienska-Blauth *et al.*, 1963). However, because of the lability of glyoxylic acid, methods were developed to generate glyoxylic acid *in situ* from glacial acetic acid by oxidizing agents like potassium persulfate or hydrogen peroxide (Fischl, 1960). Later it was shown that ferric chloride could also be used as an oxidizing agent (Opienska-Blauth *et al.*, 1963).

In this study the modified glyoxylic acid method of Opienska-

Blauth *et al.* (1963) as reported by Villegas and Mertz (1971) was considered most suitable for tryptophan estimation from enzyme hydrolyzates.

2. Introduction

Two reagents were used in the Opienska-Blauth *et al.* (1963) method for the estimation of tryptophan as reported by Villegas and Mertz (1971). These were glacial acetic acid containing ferric chloride (Reagent A) and 30 N sulphuric acid (Reagent B). These two reagents were mixed to make Reagent C.

Villegas and Mertz (1971) recommended that each bottle of acetic acid used for making Reagent A be tested in Reagent C for color-forming ability in the presence of tryptophan. This would imply that different batches of acetic acid were not equally suitable for use in tryptophan estimation. Later, it was shown that when acetic anhydride was included in the formulation of Reagent A, the color forming ability of Reagent C with tryptophan improved (Dalby and Tsai, 1975). Dalby and Tsai (1975) found that 0-4% v/v of acetic anhydride in Reagent A depending on the glacial acetic acid batches gave maximum color forming ability; higher acetic anhydride levels depressed color formation. Makinde *et al.* (1976), however, reported that an acetic anhydride value of 8-10% v/v resulted in maximum color formation.

In view of the variable conditions of Reagent A reported by the different workers, it was found necessary to ascertain the right reagent conditions before this assay could be applied to estimation of tryptophan in cereal protein hydrolyzates. What follows, therefore, is a presentation of the factors that were investigated and the results that

were obtained.

3. Materials and Methods

3.1 Reagent Preparations

The reagents were prepared according to Dalby and Tsai (1975) as follows: Reagent A was 270 mg of $FeCl_3 \cdot 6H_2O$ dissolved in 0.5 ml of distilled deionized water and made up to a liter with glacial acetic acid. Reagent B was 30 N H_2SO_4. Reagent C was made by mixing Reagents A and B in equal volumes and used after 1-2 hours as recommended by Villegas and Mertz (1971).

3.2 Standard Procedure

For all analyses 4.0 ml of Reagent C was added to 1.0 ml of test solution containing 0-50 µg tryptophan and the mixture vigorously mixed. Incubation at 65° C for 15 minutes in a shaking water bath immediately followed. After incubation, the samples were cooled for 5 minutes in cold tap water and the absorbance recorded at 545 nm in 1 cm path length cuvettes.

4. Results and Discussion

4.1 Optimization of Tryptophan Assay Conditions

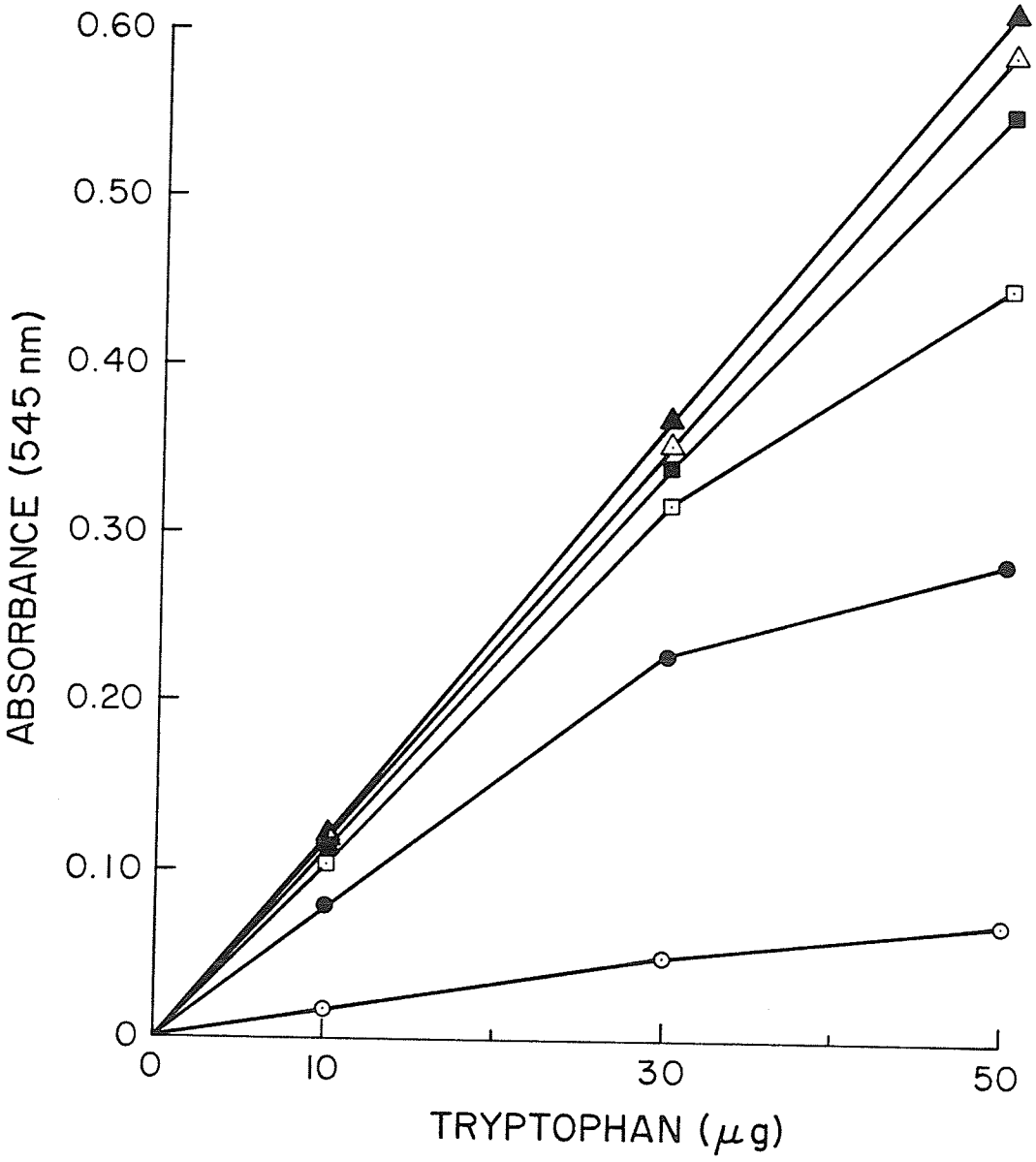
4.1.1 Acetic Anhydride Requirements. When Reagent A containing no acetic anhydride was used immediately after preparation, little color formation was obtained between Reagent C and standard tryptophan samples. Addition of acetic anhydride to Reagent A at the same concentrations recommended by either Dalby and Tsai (1975) or by Makinde *et al.* (1976)

improved the color forming ability of Reagent C. Higher concentrations of acetic anhydride in Reagent A than the ones recommended by the above workers improved the color forming ability even more. In addition, storage of Reagent A containing no or suboptimum levels of acetic anhydride led to improvement in color forming ability. When Reagent A, containing higher levels of acetic anhydride, was similarly stored there was no improvement in color forming ability.

From these observations it appeared that the levels of acetic anhydride recommended by Dalby and Tsai (1975) and by Makinde *et al.* (1976) were suboptimum. Furthermore, the assessment of acetic anhydride requirements for the Reagents A not equally stored would not be accurate as this reagent was found to change with storage time.

To assess the acetic anhydride requirements, a series of Reagent A solutions containing increasing levels of acetic anhydride was used soon after it was prepared. As the level of acetic anhydride was increased from 0-32% v/v in Reagent A, the color forming ability of Reagent C in the 0-50 µg tryptophan range, improved (Fig. 13). Color forming ability increased only slightly by increasing the concentration of acetic anhydride from 16-32% v/v. At the concentrations of 16 and 32% v/v a linear color response was obtained in the 0-50 µg tryptophan range. Lower acetic anhydride values did not give linear responses. At least 16% v/v acetic anhydride was required in Reagent A for both near maximum color yield and linearity. Values reported by either Dalby and Tsai (1975) or by Makinde *et al.* (1976) were found not to give maximum absorbance values or linear responses.

Figure 13. Comparison of color intensity and curve linearity in response to incorporation of acetic anhydride into the Reagent A formulation. Each point represents an average of duplicate samples. Acetic anhydride was present at 0% ⊙—⊙ ; 4% ●—● ; 8% □—□ ; 12% ■—■ ; 16% △—△ ; and 32% ▲—▲.

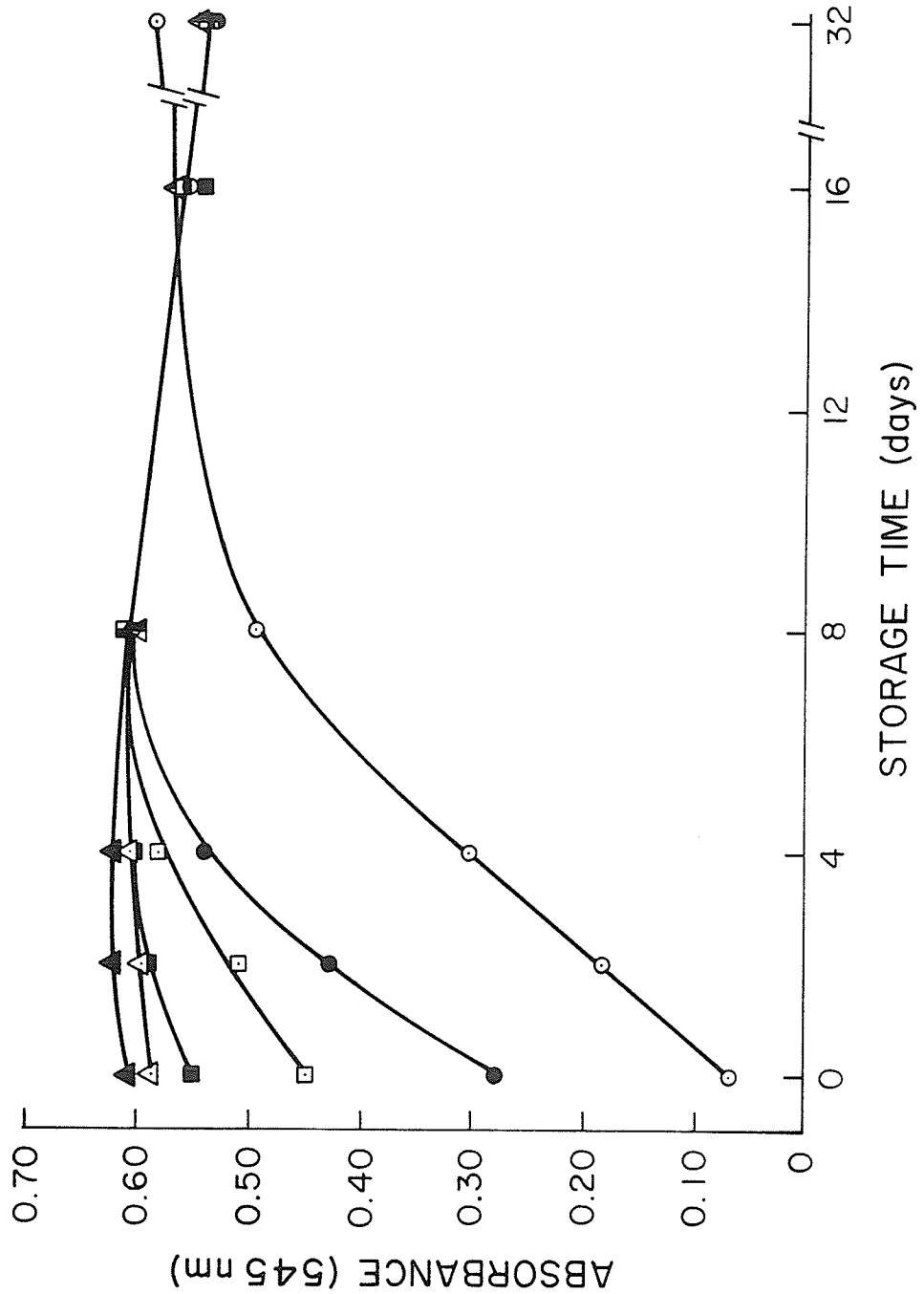

0.60
0.50
0.40
0.30
0.20
0.10
0
ABSORBANCE (545 nm)
0
10
30
50
TRYPTOPHAN (μg)

4.1.2 Effect of Storage of Reagent A on Absorbance Values. To reinvestigate the effects of storage on this assay, Reagent A solutions containing 0-32% v/v acetic anhydride were prepared. These reagents were stored at room temperature (~23° C) in tightly capped transparent bottles for up to 32 days on benches unprotected from light, and were used for tryptophan estimation at various intervals over this time period.

The results obtained showed that maximum sensitivity at any level of acetic anhydride varied with the age of the reagent during the period tested (Fig. 14). There was an initial increase in sensitivity to a maximum value that was common for all Reagent A solutions containing acetic anhydride. The time required to reach maximum sensitivity was less for those Reagents A containing higher levels of acetic anhydride (Fig. 14). After maximum sensitivity was attained further storage resulted in a slow loss in sensitivity. Loss in sensitivity started to be noticed after 8 days of storage. For those Reagents A containing acetic anhydride the color forming ability decreased to a common value after 32 days. Where no acetic anhydride was added to Reagent A, only an increase towards the maximum value was observed in the 32 days storage period. Longer storage periods were not tested.

4.1.3 Effect of Ultra-Violet Light on Reagent A. The changes that were observed during storage occurred to those reagents stored under light in transparent bottles. When these reagents were stored in the dark, the same effects were not apparent. It was therefore thought the changes in Reagent A that were observed were related to light. At this point it was decided to test whether uv light would produce the same

Figure 14. Changes in color-forming ability with storage of Reagent A solutions containing 0-32% v/v acetic anhydride. Symbols correspond to the acetic anhydride concentrations denoted in Fig. 13. Each point represents the average absorbance of a duplicate sample containing 50 μg tryptophan.


ABSORBANCE (545 nm)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
0
4
8
12
16
32
STORAGE TIME (days)

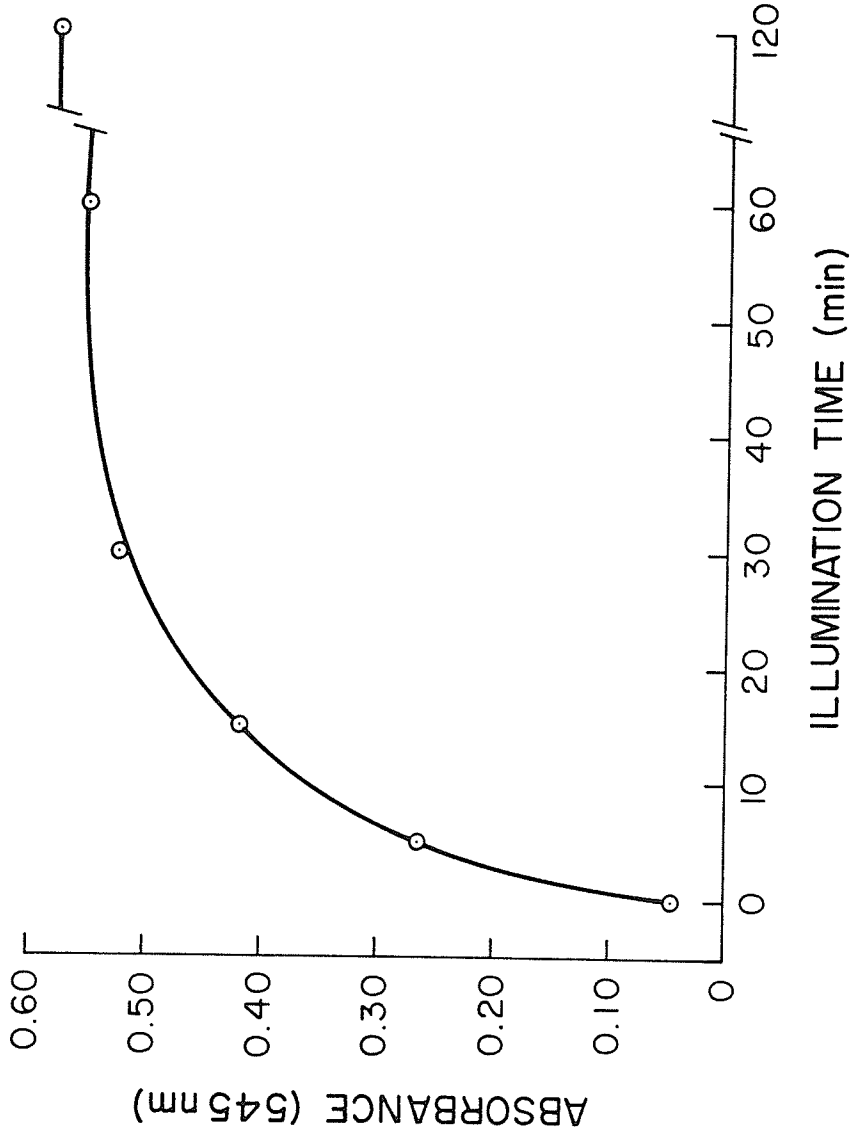
effect. For this, Reagent A containing no acetic anhydride was illuminated with ultra-violet light from a Blak-Ray B.100 A Long Wave Uv Lamp (Ultra-violet, Products, Inc., San Gabriel, California, U.S.A.). At timed intervals, portions of the illuminated Reagent A solutions were removed and used as in the standard procedure for tryptophan estimation.

After a 2-hour illumination period in the clear storage bottle the values that were obtained (Fig. 15) were similar to those obtained after 32 days of storage on the bench in transparent bottles (Fig. 14). When Reagent A in a brown bottle was illuminated for as long as 30 minutes no changes were observed. Storage of Reagent A containing 0, 4 and 16% acetic anhydride in brown bottles or in foil covered transparent bottles for as long as 32 days did not change the color forming ability of the reagents. The results obtained in this study as concerns Reagent A showed that the effects observed were related to exposure to light conditions.

4.1.4 Effect of Delayed Incubations on the Absorbance Values. In the course of this study, it was found that any delay after reagent additions but before incubation at 65° C for 15 minutes caused a decrease in the color forming ability of Reagent C. The depressed color values were found to be higher for those Reagents A containing higher acetic anhydride percent or those Reagents A stored longer in transparent bottles. A further investigation was therefore done to find the relationship between the above stated parameters.

Reagent A containing 8, 16 and 32% v/v acetic anhydride, respectively, were prepared and stored in transparent bottles for up to 32

Figure 15. Increase in color-forming ability with prolonged exposure to uv light of Reagent A containing no acetic anhydride. Each point represents the average absorbance of a duplicate sample containing 50 μg tryptophan.


ABSORBANCE (545nm)
0.60
0.50
0.40
0.30
0.20
0.10
0
0
10
20
30
40
50
60
120
ILLUMINATION TIME (min)

days under similar conditions to the study shown in Fig. 14. At intervals portions of these reagents were used for estimation of tryptophan from standard samples. In one series, samples were incubated soon after addition of Reagent C and in the other series, a delay of 15 minutes at room temperature preceded incubation at 65° C. The results obtained are shown in Table 19 and represent the percent color intensity obtained from samples whose incubation at 65° C was delayed for 15 minutes as compared to the samples immediately incubated. It was found that the color depression with delay in incubation was greater for those reagents that contained higher acetic anhydride concentrations. The color depression with delay in incubation was also found to be higher for those Reagents A stored longer regardless of the concentration of acetic anhydride.

4.2 Adopted Standard Procedure

In accordance with the results that were obtained, the Standard Procedure 3.2 above was slightly modified as follows: i) Reagent A containing 16% v/v acetic anhydride was used in the preparation of Reagent C for near maximum sensitivity. ii) Reagent A was protected from light to maintain the stability of the reagent. iii) Usually new Reagent A was prepared after one month if stored as in (ii) above.

4.3 Tryptophan Estimation from Cereal Proteins

Tryptophan contents from three enzymic cereal protein hydrolyzates of samples in Materials and Methods 3.1, Section II were estimated as in the Adopted Standard Procedure 4.2. A volume of 1.0 ml enzyme 'supernatant' was used. Quantitation was done using DL-tryptophan as

TABLE 19. Relative color intensity achieved with delayed incubation of tryptophan samples

$$\frac{A_{545}\ (\text{Delayed})}{A_{545}\ (\text{Immediately})} \times 100$$

Storage time (days)	Acetic Anhydride (% v/v)		
	8	16	32
0	96.0	95.8	87.7
4	95.4	91.1	84.0
8	89.6	88.2	82.8
16	81.3	80.1	77.3

the standard.

The results (Table 20) show that the tryptophan values obtained from both thermolysin and papain 'supernatants' were very similar. In a few samples, values obtained from papain 'supernatants' were slightly higher than those obtained from thermolysin 'supernatants'. The tryptophan values obtained from the Pronase 'supernatants' were in every case slightly lower than those from the other two enzymic 'supernatants' except for the sorghum X. The tryptophan value obtained from Pronase 'supernatant' of sorghum X was too high to account for except on the basis of possible interference from tannins. Sorghum X and Riosweet had a tannin content of 5.4 and 3.2%, respectively (Table 4).

The tryptophan values that were obtained from casein hydrolyzates of the three enzymes were found to be very close to some of the values reported in literature (Table 20). The three enzymes, however, gave slightly different values. Although values obtained from bovine albumin agreed very well for the three enzymes, these values were found to be slightly different from the literature values.

Of the cereal grains, 'supernatants' from the high tannin sorghum varieties, wheat and maize were found to be colored. It was not ascertained if these colored compounds in the 'supernatants' of the above mentioned cereals might in some way have interfered with the tryptophan estimation as was clearly apparent with the high tannin sorghum varieties.

The method that was used in this study for the tryptophan determination in the enzymic 'supernatants' has been reported by Opienska-Blauth *et al.* (1963) to equally detect free and/or bound tryptophan. The

TABLE 20. Tryptophan content of samples as determined from various enzyme 'supernatants'

Sample	Tryptophan Content (mg/100 mg Protein)			Literature values
	Thermolysin 'supernatant'	Papain 'supernatant'	Pronase 'supernatant'	
barley				
Herta	1.56	1.71	1.37	
Karl	1.55	1.57	1.36	
Risø 1508	1.84	1.87	1.64	
maize				
K_{26} x K_{52}	0.971	1.04	0.960	
A495 fl_2 x B8 fl_2	1.18	1.18	1.10	
$63o_2$ x $MS206o_2$	1.41	1.48	1.34	
rye				
Prolific	1.30	1.36	1.22	
Gazelle	1.29	1.36	1.23	
UC-90	1.29	1.25	1.15	
sorghum				
X	1.70	-	3.63	
Winner	1.49	1.52	1.29	
Riosweet	1.65	-	1.24	

TABLE 20 Continued.

Sample	Tryptophan Content (mg/100 mg Protein)			Literature values
	Thermolysin 'supernatant'	Papain 'supernatant'	Pronase 'supernatant'	
triticale				
Rosner	1.31	1.39	1.17	
Welsh	1.30	1.44	1.22	
Carman	1.36	1.48	1.25	
GA_{530} x UC-90	1.11	1.21	1.04	
wheat				
Neepawa	1.86	2.05	1.61	
Cocorit 71	1.54	1.68	1.40	
casein	1.36	1.50	1.46	1.36 ± 0.11[a], 1.34[b]
bovine albumin	0.70	0.70	0.68	0.57 - 0.62[c], 0.58[d]

[a]Amaya-F *et al.* (1977)

[b]Piombo and Lozano (1980)

[c]Spies (1967)

[d]Fischl (1960)

results that were obtained in this study (Table 20), however, suggested that both free and peptide bound tryptophan may not give the same color yield. Both papain and Pronase solubilized similar amounts of protein from cereal samples except for the high tannin sorghums (Table 5). However, the degree of hydrolysis of soluble protein obtained with Pronase was three times that obtained with papain (Table 7). That the tryptophan values determined from Pronase 'supernatants' were slightly lower than those determined from papain 'supernatants' suggested that the color yield of tryptophan in a more hydrolysed protein may be slightly lower.

4.4 Comparison of Results to Literature Findings

One factor that was not reported by Dalby and Tsai (1975) or Makinde *et al.* (1976) in relationship to the acetic anhydride requirements was the effect of light. In the work reported in this thesis it has been shown that the color forming ability of glacial acetic acid containing $FeCl_3$, or glacial acetic acid containing $FeCl_3$ and suboptimal levels of acetic anhydride, can be improved by exposure to light. It would therefore seem quite possible that at least some of the variability which has been attributed to the glacial acetic acid quality (Villegas and Mertz, 1971; Dalby and Tsai, 1975) was in fact due to variable degrees of exposure of Reagent A to light. Similarly, in those reagent mixtures containing acetic anhydride, the variable levels which have been reported to be required to obtain optimum color forming ability could depend on how much the reagent was exposed to light before it was used for tryptophan estimation.

The loss of color forming ability at higher levels of acetic anhydride than required for optimum color formation (Dalby and Tsai, 1975)

may have been related to delay before incubation at 65° C after reagent additions. In the work reported in this thesis, when standard tryptophan samples were incubated immediately after reagent additions, use of 32% v/v acetic anhydride gave slightly higher sensitivity than use of 16% v/v acetic anhydride (Fig. 13). However, when the samples were delayed for only 15 minutes before incubation but after reagent additions, color formation was more depressed at 32% v/v acetic anhydride than at 16% v/v (Table 19). Greater exposure of Reagent A to light also caused a more depressed color formation with delay in incubation.

Similar literature tryptophan values to the ones found here (Table 20) have been reported for barley (Concon, 1975), for rye (Concon, 1975; Shukla, 1975), for whole wheat flour (Amaya-F *et al.*, 1977) and for *Opaque*-2 maize (Shukla, 1975).

5. Summary

1) In the optimization of the tryptophan assay the standard procedure was modified as follows:
   a) Reagent C was made from Reagent A containing a minimum of 16% v/v acetic anhydride.
   b) Once made Reagent A was stored in light-proof containers. This way the reagent was found to be stable for at least 32 days.

2) Observation of the above two conditions in the standard procedure resulted in a minimum 20% improvement in relative sensitivity over the previous methods.

3) Delay in incubation after reagent additions resulted in the depression of color formation.

4) With enzymic hydrolyzates of cereal proteins, tryptophan values from both thermolysin and papain 'supernatants' agreed very closely. Pronase 'supernatants' gave slightly lower values.

5) Tryptophan could not be estimated from high tannin sorghum because of the supposed interferences from tannins.

6) The tryptophan values that were obtained using the method reported here were similar to some of those that have been reported in literature.

V. 'NON-DESTRUCTIVE' SINGLE KERNEL ANALYSES

1. Review of Literature

Several methods have been used for small scale, single seed or 'non-destructive' single seed analyses. Some of those methods that have been specifically used for the 'non-destructive' single seed analyses are reviewed below.

A micro-dye binding capacity (DBC) method which was useful for estimating both the protein and lysine contents in single grains such as rice, wheat and sorghum, without damaging the embryo was developed by Kaul *et al.* (1969). Protein values that were obtained from 10 mg of sample were similar to those obtained from 500 mg of sample. In the DBC methods, the dyes used react with the three basic amino acids histidine, lysine and arginine (Sharma and Kaul, 1971). The amount of the dye that reacts is correlated to both protein and lysine contents. In situations where the ratio of the basic amino acids to total protein is not constant, variable results would be obtained. Where there are higher levels of arginine and histidine but lower lysine content, the lysine would be overestimated and vice versa. Furthermore, before the method can be successfully used, the correlations between DBC and protein or lysine content have to be predetermined for each type of cereal tested (Udy, 1971).

Rhodes (1975) used a ninhydrin color test based on the higher

levels of free amino acids in high lysine grains and a turbidity test based on lower prolamine content to do a 'non-destructive' selection for high-lysine barley involving a cross with Risø 1508. The ninhydrin test was found less accurate than the turbidity test because even in the high lysine grains, the amount of free amino acids was found to further increase with the level of fertilization of the plant in the field (Rhodes, 1975). The turbidity test was found to be more sensitive than the ninhydrin test with grains of lower nitrogen content. Both tests were only sufficiently precise to distinguish lines differing in their genetic potential for lysine synthesis. In high lysine cereal varieties such as the *Hiproly* barley which does not significantly decrease in prolamine content as it increases in lysine content (Ma and Nelson, 1975) the turbidity test would not identify the high lysine seeds.

More recently, Ahokas (1978) presented a method that was applicable for the 'non-destructive' single kernel analyses of both tryptophan and protein contents in barley. Protein from the distal half of the seed was extracted with ethanolic hydroxide. However, since under the conditions of Ahokas (1978) only 64.3% of the total protein was extractable, estimation of tryptophan from the extracted protein may not have given a representative value. Furthermore, the method was not suited for routine analysis because of critical timing of some operations.

A 'non-destructive' single kernel estimation of tryptophan in maize is carried out at Cimmyt, Mexico (Villegas and Mertz, 1971). The amount of sample obtainable from maize kernels without destroying the embryo is however large enough to allow for this type of analysis. It was not reported (Villegas and Mertz, 1971) whether a 'non-destructive' single

kernel estimation of tryptophan in smaller kernels such as barley and wheat could also be achieved.

In a program for upgrading the nutritional quality of cereal grains, it would not only be necessary to determine the amount of essential amino acid(s) lysine and/or tryptophan but also the protein content (Mertz, 1968). The methods that have been discussed above, either cannot estimate these amino acids and protein from a part of a single kernel in most of the cereal grains or they are not accurate enough. For these reasons, more accurate 'non-destructive' assays suited for routine analysis would be desirable. Use of such assays would identify more precisely, the seeds that contain the right levels of these essential amino acids as well as the protein content.

2. Introduction

In the 'non-destructive' single kernel analyses, the methods and conditions established in the preceding four sections were to be applied. In the preceding sections, it was demonstrated that essentially all the protein could be solubilized from whole seed grists under selected conditions. It was further shown that total sample protein, tryptophan and available lysine could accurately be estimated from the solubilized protein. In the study that is to be undertaken in this section, only the distal half of the kernel would be used. The protein composition would therefore be slightly different from that of whole kernels. The amount of sample that would be available for analysis would be meagre. Associated with the smallness of sample are the other problems discussed in the general introductory section of this thesis.

Preliminary tests were required to show that essentially all the

protein from the kernel distal halves could be solubilized by the enzymes. The method to be used for grinding the half kernels should reduce them fine enough to obtain maximum protein solubilization by the enzymes but without affecting the available lysine and tryptophan recoveries as compared to samples ground in Udy Cyclone mill.

After the above preliminary tests the methods would have to be scaled down to be able to estimate protein, available lysine and tryptophan from only 15 mg of sample flour. The size of sample chosen to work with is the minimum amount that would be obtained from most of the seed kernels used. All the methods that have been used in this study have the potential of being scaled down for 'non-destructive' single kernel analysis by alteration of some parameters.

Finally, it was to be shown that the embryo parts of the kernel left after slicing off the distal halves can germinate and grow into mature plants. What follows is therefore an account of what was done and the results that were obtained.

3. Materials and Methods

3.1 Materials

Only four cereals, Herta barley, Prolific rye, Rosner triticale and Neepawa wheat were used. A pulverizer similar to that described by Paulis and Walls (1979) for pulverization of single kernels was designed and made of hard steel in the University of Manitoba workshop. This pulverizer was used to reduce the kernel distal halves to a fine grist. Flasks with a 20 ml calibration marks that were used in the micro-Kjeldahl digestions were designed and made by the University of Manitoba

glass blower. Samples were solubilized with one enzyme, papain. Centrifugation of the assays were done in 12 x 40 mm test tubes. Incubations of both lysine and tryptophan assays were done in either 20 x 125 mm or 13 x 100 mm Kimax screw cap test tubes.

3.2 Sample Preparations for Chemical Analysis

Distal half seeds of each cereal were prepared by slicing kernels with a razor blade. Approximately 10 g of distal half seeds from each cereal type were pooled. These were mixed then divided into two lots. One lot was ground in Udy Cyclone mill as in Materials and Methods 3.2, Section II. The other lot was reduced to fine grist with the pulverizing mill. Samples were then defatted as in Materials and Methods 3.2, Section II before being subjected to enzymic hydrolysis.

3.3 Effect of Sample Pulverization on Soluble Protein, Available Lysine and Tryptophan Recoveries

To compare the pulverization method with the Udy Cyclone mill method duplicate samples (100 mg) were weighed from each of the preparation lots for each of the cereals. The comparison tests were to evaluate the effectiveness of the pulverization on subsequent sample operations, including protein solubilized, and recoveries of available lysine and tryptophan.

The amount of protein solubilized was determined as in Method 3.9, Section II. Available lysine values were estimated as in Standard Procedure 3.10, Section III. The amount of tryptophan was estimated as summarized in Section IV.

3.4 Scaled Down Procedures

3.4.1 Enzymic Solubilization of Protein. In the scaled down procedures for the 'non-destructive' single kernel analysis protein was solubilized from 15 mg of pulverized sample in 1.5 ml of the enzyme solution. This is a scaled down method of the one described in 3.8, Section II.

The sample protein to enzyme protein ratio was maintained the same as in Method 3.3, Section II. The conditions of incubations were the same as in Method 3.12, Section II. The 'supernatants' for analysis were recovered as in Method 3.2, Section III.

3.4.2 Estimation of the Amount of Solubilized Protein. The method that was used for the estimation of the solubilized protein is a scaled down version of the one described in Method 3.9, Section II. Protein was estimated from 0.25 ml of the sample 'supernatant'. The samples were digested in the 20 ml micro-Kjeldahl flasks using 0.6 ml concentrated sulphuric acid and 0.2 g of catalyst. The digests were diluted to 20 ml with distilled water.

Ammonia from the diluted digests was estimated by the colorimetric procedure summarized in Section I of this thesis, except that dilutions were done with 5.0 ml of water instead of 10.0 ml.

3.4.3 Estimation of Available Lysine. For available lysine estimation, 0.40 ml of sample 'supernatants' were used and reagents in the proportions indicated in the Standard Procedure 3.10, Section III added. After the amino group blocking stage with copper phosphate suspension the samples were centrifuged at 700 g for 5 minutes. From the clear supernatants 0.60 ml aliquots were pipetted into separate screw cap test

tubes and 0.05 ml of DNPyr in methanol (30 mg/ml) added. The incubation was done in a shaking water bath at 22-24° C in the dark for 2 hours followed by addition of 1.2 ml of 2 N HCl. The samples were washed three times with 2.0 ml portions of ethyl acetate as in Standard Procedure 3.10, Section III.

Absorbance readings of the samples were taken in 1.0 ml glass cuvettes with a 1 cm path length. Any minor details omitted in this procedure should be assumed to be the same as reported in the Standard Procedure 3.10, Section III.

3.4.4 Estimation of Tryptophan. Tryptophan content was estimated from 0.40 ml of the sample 'supernatants'. Reagent C_1 (1.2 ml) was added and the samples were mixed in a vortex mixer and immediately incubated at 65° C for 15 minutes. The samples were cooled in tap water and the absorbance readings were recorded at 545 nm in 1.0 ml cuvettes with a 1 cm path length. Reagent C_1 was prepared by mixing equal volumes of 36 N H_2SO_4 and Reagent A 1 to 2 hours before use.

3.4.5 Sample Preparation for Emergence and Survival Tests. Different proportions of individual kernels were removed by slicing from the distal end of randomly selected seeds of Herta barley, Prolific rye, Rosner triticale and Neepawa wheat. For each cereal 3 separate lots of 20 seeds were prepared such that the proximal end contained $\geq 30 < 40\%$, $\geq 40 < 50\%$ or $\geq 50\%$ by weight of the original seed. These together with 20 whole seeds (control) from the same seed lot were planted 1/4 inch deep in rows in a medium made of 1 part sand, 1 part peat and 2 parts loam soil in wooden boxes. These experiments were maintained in a green

house with watering as necessary. Two weeks after emergence the seedlings were topped with NPK (10:10:10) composite fertilizer. Before planting the seeds were not treated with fungicides or insecticides. The seedling emergence and survival rates were recorded.

In this context, the seedling emergence is defined as the appearance of the hypocotyl above the soil surface. Survival is defined as the successful flowering and heading of the plants.

4. Results and Discussion

4.1 The Effect of Sample Pulverization on the Amount of Protein Solubilized and Recoveries of Available Lysine and Tryptophan

The amounts of protein that were solubilized by papain enzyme from the distal half kernels ground by either the Udy Cyclone mill or pulverized are compared in Table 21. The percent protein solubilized was not affected by the method that was used to grind the samples.

The amount of available lysine and tryptophan from the supernatant obtained from papain hydrolyzate of samples ground by the two methods were also compared (Table 22). The two methods used in grinding the samples gave similar available lysine values. For Neepawa wheat, however, the amount of tryptophan obtained from pulverized samples was slightly higher than that obtained from samples ground in the Udy Cyclone mill (Table 22).

The reason for the difference in tryptophan value between the wheat sample ground in the Udy Cyclone mill and the pulverized one was not apparent. In this study no attempts were made to check the recovery rates of samples that were pulverized as compared to the ones that

TABLE 21. The effect of grinding methods on the amount of protein solubilized by papain

Sample	Protein Solubilized (%)	
	Grinding Method	
	Udy Cyclone mill	Pulverized
barley, Herta	93.0	94.9
rye, Prolific	96.1	95.3
triticale, Rosner	97.3	96.1
wheat, Neepawa	98.2	98.0

TABLE 22. The effect of grinding methods on the available lysine and tryptophan values obtained from papain 'supernatants'

Sample	mg Amino Acid/100 mg Protein			
	Available Lysine		Tryptophan	
	Udy Cyclone mill	Pulverized	Udy Cyclone mill	Pulverized
barley, Herta	3.50	3.48	1.56	1.48
rye, Prolific	3.32	3.28	1.25	1.22
triticale, Rosner	2.92	2.84	1.20	1.19
wheat, Neepawa	2.95	3.02	1.95	2.11

were ground in the Udy Cyclone mill. If the recoveries were however significantly different most probably even the available lysine obtained from wheat samples ground by the two methods would have been significantly different.

The two methods of grinding samples were found not to produce significantly different results. Therefore, the pulverizing mill can be successfully used to reduce the distal half of kernels to fine grist before estimation of soluble protein and the two essential amino acids.

4.2 Scaled Down Procedures

In all the scaled down analyses 15 mg of pulverized samples were weighed from the pooled distal half seeds as opposed to individual distal half seeds. This was found necessary to avoid any possible small variations from kernel to kernel. The aim of the work reported here was only to evaluate the suitability of the analytical methods that were to be applied to single kernels. When the methods are directly applied to single kernel analyses, it is hoped that the necessary precautions will have to be exercised.

In wheat, the kernels from the top third of the spike have been reported (McNeal and Davis, 1966) to have significantly lower protein contents than those from the bottom and middle portions. However, for both 2- and 6-rowed barley selections, Pomeranz *et al.* (1976) found that the position of the kernel on the spike had no significant effect on protein content or amino acid composition. Variation of up to 2.5% in protein content between tillers of the same plant in wheat have been reported (Bhatia *et al.*, 1970). Late tillers were found to be higher in protein content. In view of the findings reported by the above

workers, it is hoped that the seeds for the 'non-destructive' single kernel analyses will be selected in such a way as to avoid any variation from kernel to kernel.

4.2.1 Estimation of Solubilized Protein. Since only a small portion of the micro-Kjeldahl digest was used for the colorimetric estimation of protein, then provided the digestion was efficiently carried out at reduced volumes, this assay could be scaled down considerably. To maintain maximum sensitivity, however, it should be remembered that the aliquot of digest used will effect the pH of the colorimetric assay. The ratio of acid to final dilution volume should be kept as indicated in the summary of Section I of this thesis or the pH adjusted appropriately to maintain optimum pH in the colorimetric assay.

The duplicate values that were obtained from the estimation of solubilized protein as indicated in Method 3.4.2 are shown in Table 23. When these values were compared to the values obtained on a large sample basis (Table 21), it was found that the values were not significantly different. The values from the scaled down procedure, however, tended to be slightly lower. Under the conditions used here 15 mg of samples containing 14.0% protein gave an absorbance value of ~0.37 at 645 nm. Thus the absorbance values were still within an accurate part of the absorbance scale.

4.2.2 Estimation of Available Lysine. The available lysine values calculated from sample absorbance readings are shown in Table 24. The protein values given in the same table were from whole samples.

The absorbance values obtained from any one sample would depend

TABLE 23. Amount of protein solubilization obtained by the scaled down procedure

Sample	Sample weight	Protein solubilized (%)	Average protein solubilized (%)
barley, Herta	15.8	93.8	94.1
	15.4	94.3	
rye, Prolific	15.1	94.8	95.0
	15.9	95.1	
triticale, Rosner	15.4	95.6	95.6
	15.8	95.7	
wheat, Neepawa	15.7	97.8	97.7
	15.6	97.6	

TABLE 24. Available lysine values obtained by the scaled down procedure

Sample	Sample weight	Protein content (%)	mg Lysine per 100 mg protein	Average mg per 100 mg protein
barley, Herta	15.8	11.1	3.34	3.33
	15.4		3.31	
rye, Prolific	15.1	14.4	3.42	3.40
	15.9		3.39	
triticale, Rosner	15.4	14.6	2.98	2.99
	15.8		2.99	
wheat, Neepawa	15.7	10.6	3.01	2.94
	15.6		2.87	

on both the percent available lysine on a protein basis and on the percent protein on a sample basis. For Neepawa wheat with about 3.0 mg available lysine per 100 mg protein and a protein content of 10.6%, an absorbance reading of about 0.15 was recorded. Comparison of the scaled down procedure values (Table 24) to values obtained when 100 mg samples were used (Table 22), showed that the scaled down procedure gave very comparable available lysine values.

When the Standard Procedure (SP) 3.10, Section III is compared to the Scaled Down Procedure (SDP) 3.4.3 in this section, a few differences are apparent. To increase both the sample size and the measurable values, 3/4 of the sample was recovered in the SDP after α-amino group blocking stage as opposed to 1/2 in the SP. After dinitrophenylation stage the samples were diluted less in the SDP during acidification as compared to the SP. This was an attempt to raise the measurable absorbance values. The concentration of the acid used in the SDP was, however, adjusted to maintain the same concentration in the acidified samples as in the SP.

4.2.3 Estimation of Tryptophan. Unlike the nitrogen and available lysine assays, the tryptophan assay was not very versatile and therefore there were fewer factors that could be changed to raise the absorbance readings. In the standard tryptophan estimation no dilutions were made after the incubation of the samples with the reagents (Standard Procedure 3.2, Section IV).

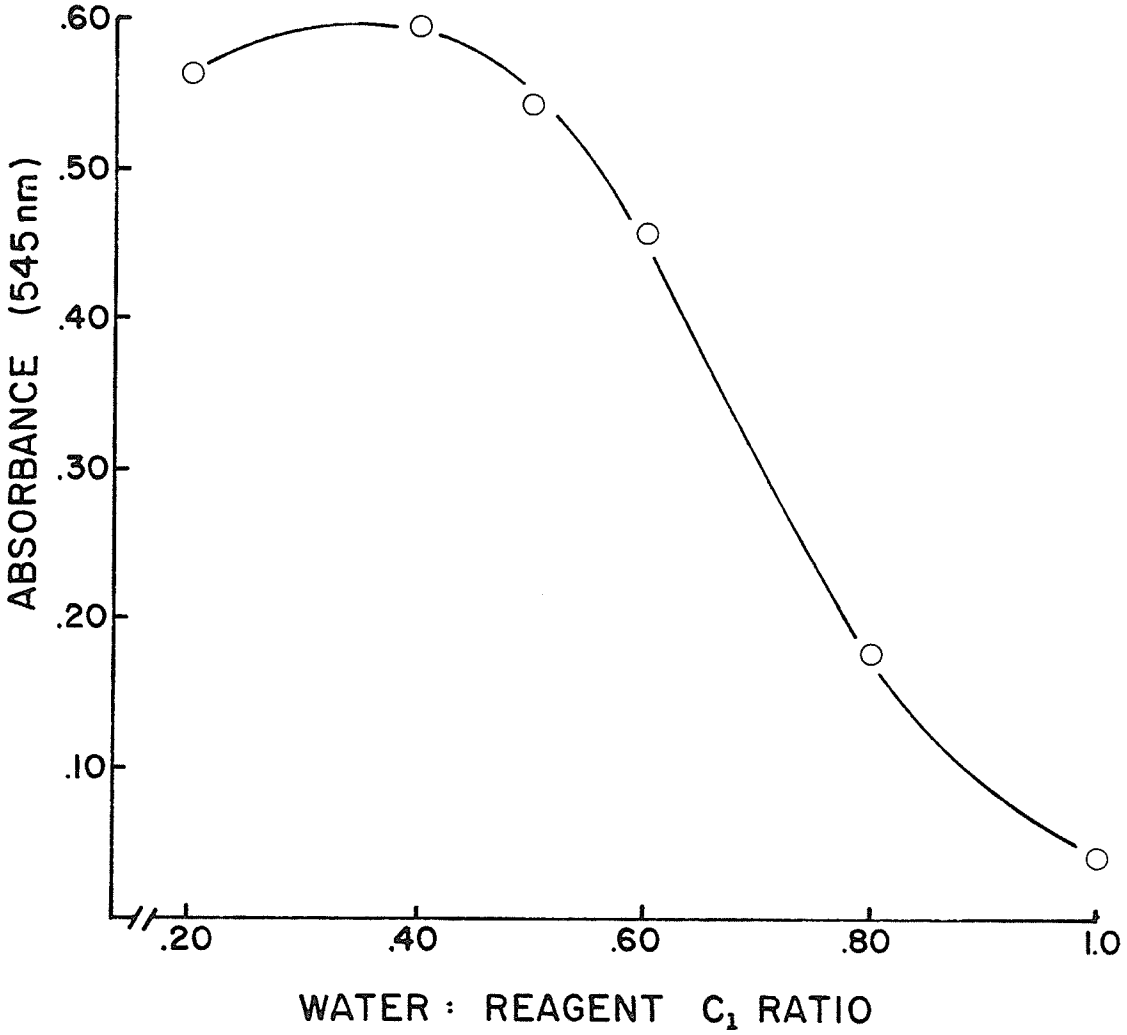
The ratio of water to Reagent C was shown to be critical to the values obtained (Opienska-Blauth *et al.*, 1963). This would mean that the volume of sample used cannot be altered without much effect on the

absorbance values. Decreasing the enzyme solution volume that is used to solubilize the protein from 15 mg samples, in an attempt to increase the tryptophan concentrations and hence the measurable values, has some limitations both in the volumes to be handled and the apparatus to be used.

Reagent C was made by mixing equal volumes of glacial acetic acid containing acetic anhydride (Reagent A) and 30 N sulphuric acid (Reagent B). It was thought that the dilution of Reagent B could be lowered at the expense of using more sample. Thus, assuming that making Reagent C from Reagent A and concentrated sulphuric acid (36 N) instead of 30 N H_2SO_4 (normal Reagent B) has no other side effects, this would allow addition of more sample to maintain the same water to acid ratio. This in turn would increase the measurable absorbance values.

Fig. 16 shows the absorbance values obtained from 50 µg tryptophan at different water to Reagent C_1 (made by mixing equal volumes of Reagent A and 36 N H_2SO_4) ratios. As the sample volumes (equivalent of water) increased, the absorbance readings increased slightly to a maximum value and then decreased with any further increase in sample volumes. Maximum absorbance values were obtained when sample to Reagent C_1 ratio was about 0.35. These absorbance values were similar in magnitude to those obtained at a sample to Reagent C (when Reagent C was made by mixing equal volumes of Reagent A and 30 N H_2SO_4) ratio of 0.25. Thus, under these new conditions of Reagent C_1 formulation, there was less dilution of sample which was a desired effect. Procedure 3.4.4 for estimation of tryptophan was therefore adopted. Under the conditions of this procedure the sample to Reagent C_1 ratio was 0.33.

Figure 16. The effect of water to Reagent C_1 ratio on the absorbance values. Each point represents the average absorbance of a duplicate sample containing 50 μg tryptophan.


.60
.50
.40
.30
.20
.10
ABSORBANCE (545 nm)
.20
.40
.60
.80
1.0
WATER : REAGENT C_1 RATIO

The tryptophan values that were obtained in the scaled down procedure (Table 25) were very similar to the values that were obtained when 100 mg of samples were used (Table 22). For Herta barley distal halves which had an average protein content of 11.1% on sample basis and tryptophan content of 1.43% on protein basis an absorbance of about 0.25 was obtained under the conditions of Procedure 3.4.4 above.

4.3 Seedling Emergence and Survival Rates

Table 26 shows the weight range of the seeds that were used, the proportions of the proximal half parts (as a percentage of the whole kernel weight) that were planted as well as the emergence and survival rates of these proximal halves. The weight of the control seeds were not recorded but since they were randomly selected their weight range would be expected to cover the range recorded for the other lots shown for each cereal.

For all the samples that were used except for the barley in which 30-40% of the proximal parts were left, once the seedlings emerged, they all reached maturity. For barley again when only 30-40% of the proximal parts of the kernels were left, emergence rate was drastically affected as compared to the controls.

The emergence rates for barley and rye as compared to the controls decreased with a decrease of the size of the proximal parts. Emergence rates for triticale and wheat were however not affected by the size of the proximal parts in the size range that was tested. The low emergence rates encountered for triticale must be accounted for by other factors not considered in this study and were not due to slicing off the distal ends of seeds since the control seeds had similar emergence rates.

TABLE 25. Tryptophan values obtained by the scaled down procedure

Sample	Sample weight	Protein content (%)	mg Try. per 100 mg protein	Average mg Try. per 100 mg
barley, Herta	14.8	11.1	1.41	1.43
	15.7		1.45	
rye, Prolific	15.2	11.4	1.25	1.24
	15.2		1.22	
triticale, Rosner	15.2	14.6	1.28	1.27
	15.6		1.26	
wheat, Neepawa	15.6	10.6	2.14	2.11
	15.9		2.09	

TABLE 26. The effect of removing different proportions of the distal end of the kernel on emergence and survival rates

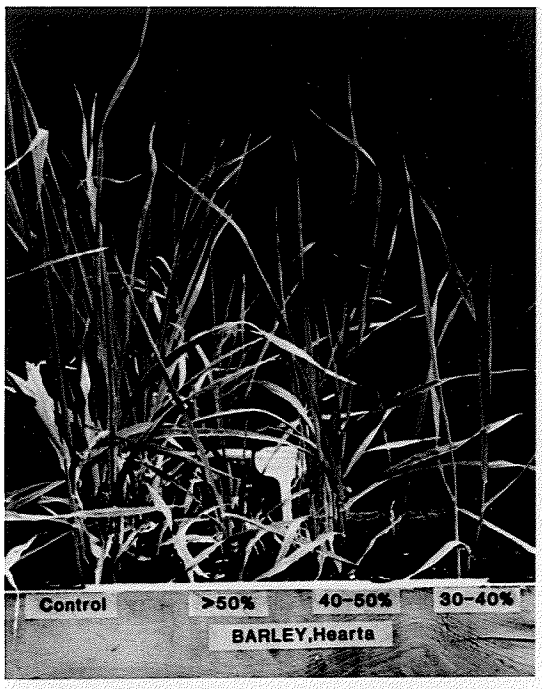
Sample	Seed weight range (mg)	Proportion of proximal part (%)	Emergence rate (%)	Survival rate (%)
barley, Herta	25.7 - 46.2	≥ 30 < 40	20	15
	28.6 - 40.6	≥ 40 < 50	65	65
	22.9 - 49.4	≥ 50	70	70
	not recorded	control	85	85
rye, Prolific	25.4 - 49.3	≥ 30 < 40	75	75
	21.1 - 42.0	≥ 40 < 50	80	80
	25.7 - 46.6	≥ 50	85	85
	not recorded	control	95	95
triticale, Rosner	39.9 - 52.2	≥ 30 < 40	30	30
	37.8 - 60.3	≥ 40 < 50	30	30
	37.6 - 59.6	≥ 50	40	40
	not recorded	control	30	30
wheat, Neepawa	25.8 - 45.7	≥ 30 < 40	95	95
	26.4 - 42.9	≥ 40 < 50	80	80
	21.8 - 40.0	≥ 50	90	90
	not recorded	control	90	90

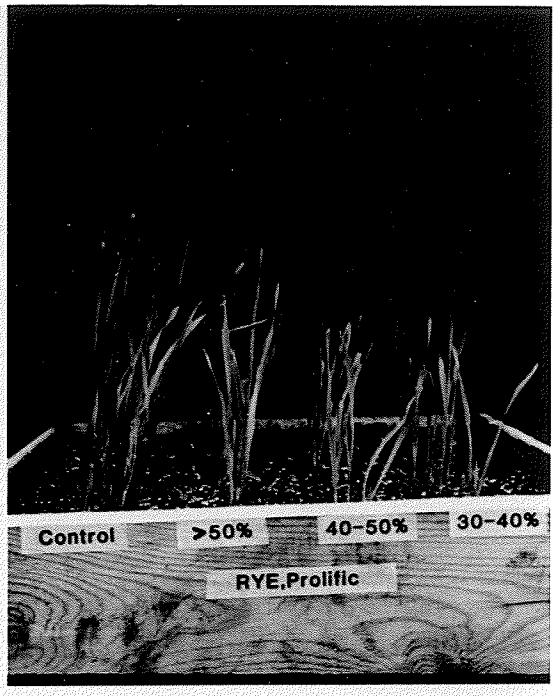
Slicing off the distal halves of the kernels caused a reduction in the seedling vigour in the early stages of growth for all the cereal types that were tested. The vigour was most reduced for those seedlings which had most of the distal halves of the kernel sliced off (Fig. 17). However, at the advanced stages of growth the plants attained the same height almost at the same time. At maturity (Fig. 18) all the seedlings had attained approximately the same height and had approximately the same spike size.

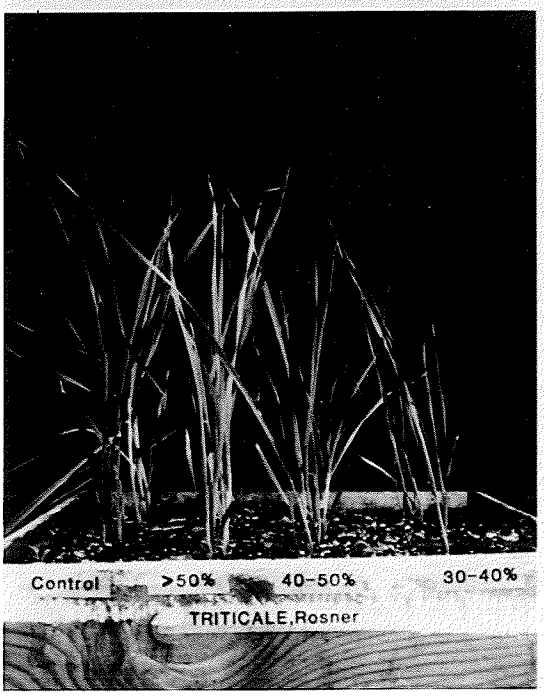
5. General Discussion

The studies that were done in Sections I-IV of this thesis culminated in the 'non-destructive' single kernel estimations of total protein and two essential amino acids all from a part of a single kernel. In this Section (V), it has been clearly demonstrated that the values that were obtained from an equivalent of 15 mg sample were very similar to values obtained on a large sample (100 mg) basis. This accuracy, coupled with the versatility of performing three types of assays from a part of a single kernel were found to supersede the achievements of the earlier 'non-destructive' single kernel assays (Literature Review). Identification of kernels with the right characteristics would be more easily accomplished. Furthermore, it is the author's view that the simultaneous estimation of these amino acids and the protein content from a single kernel is a better approach to upgrading cereal grain nutritional quality than previous methods. Only kernels with the right amounts of both the essential amino acids and protein content would therefore be considered as candidates for further breeding work.

Figure 17. Seedlings obtained from seeds in which different proportions of proximal kernels were left after slicing off distal parts. Barley is shown at 25 days, rye at 7 days and triticale and wheat at 20 days post emergence, respectively.


Control
>50%
40-50%
30-40%
BARLEY,Hearta


Control
>50%
40-50%
30-40%
RYE,Prolific


Control
>50%
40-50%
30-40%
TRITICALE,Rosner

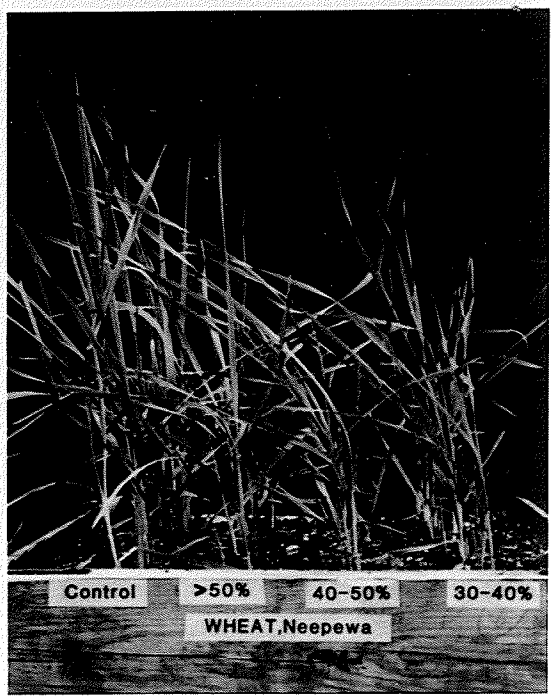
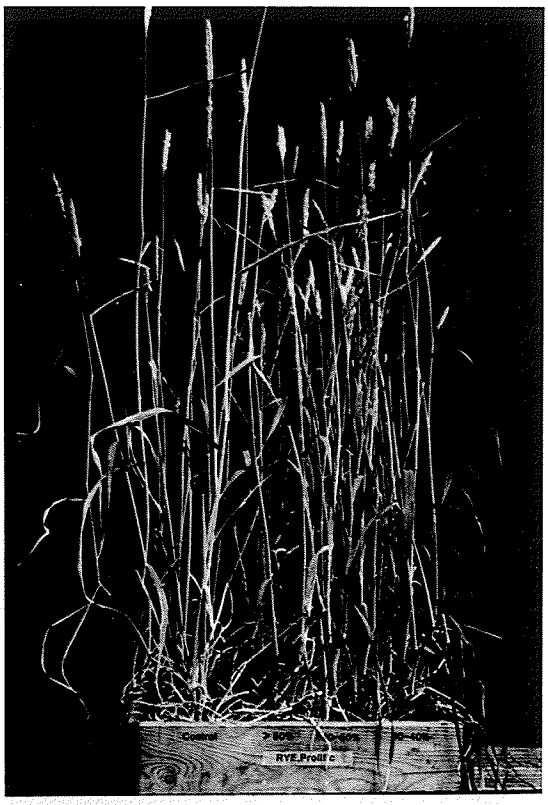
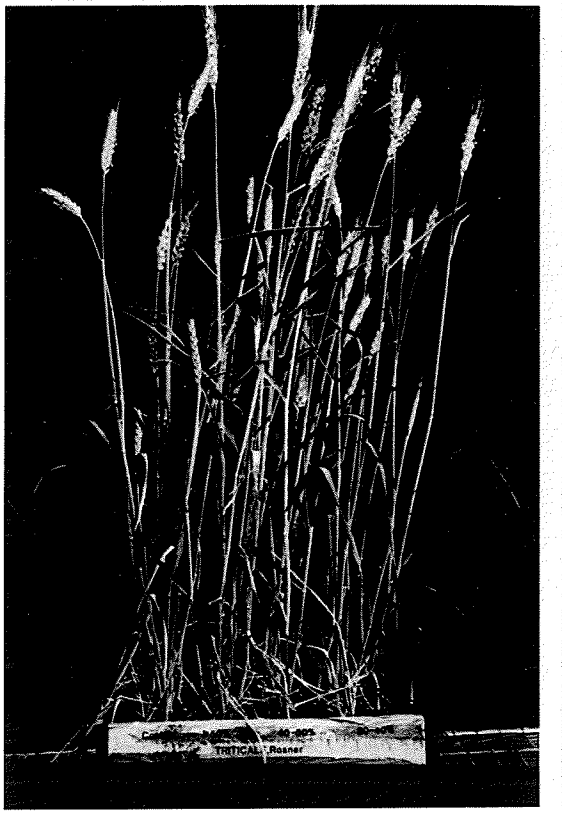

Control
>50%
40-50%
30-40%
WHEAT,Neepewa

Figure 18. Mature plants obtained from seeds in which different proportions of proximal kernels were left after slicing off distal parts.


Control
>50%
40-50%
BARLEY,Hearts




TRITICAL,Rosner


Control
> 50%
40-50%
30-40%
WHEAT,Neepewa

To select the kernels for analysis in a segregating ear or spike, the precautions as pointed out in 4.2 above would have to be taken. Once the kernels have been selected the distal half of a kernel would have to be obtained and pulverized as in 3.2 above. The pulverization mill that was used in this study could reduce the samples to fine grist in less than 3 minutes. Solubilization of protein from samples took 24 hours but samples could be solubilized every day. The assays that were used were all colorimetric and are therefore endowed with the speed of colorimetric procedures. Thus not only are these assays suitable for routine analysis but many samples could be analyzed each time. It was estimated that one person could analyze for either protein and tryptophan content or available lysine and tryptophan content in at least 30 samples in a normal working day.

Single seed analysis would offer a possibility of back-crossing type of breeding operation. The seeds would be back-crossed and then selfed. Single kernel analysis would then be done on the seeds from the selfed plants. Since the number of samples generated in this type of breeding operation are not as many as with most of the other breeding systems, these samples could easily be handled by the assays presented in this thesis.

The only other problems that would be considered in the above type of analyses would be the possible variable moisture content of samples and defatting of samples before they were analyzed. It would not be necessary to determine the moisture content of samples before analysis, provided the moisture content of samples was equilibriated to a constant value.

Tsai *et al.* (1972) reported that defatting of maize samples was not essential in the estimation of available lysine values. In the study reported in this thesis, preliminary investigations (unrecorded data) showed that when the distal half samples were used, defatting of samples did not give significantly different available lysine values as compared to undefatted samples. Thus the defatting of samples could be omitted. However, when whole samples were used, it was found that available lysine values could not be accurately estimated from oat samples (Section III).

Eventually, the available lysine and tryptophan values would have to be expressed as a percent on a protein basis. Since almost complete solubilization of protein in samples was obtained (Table 23) the amount in hydrolyzate supernatant would be approximately equal to total sample protein. This value could be used to express the two essential amino acids on a protein basis. The procedures presented here, however, were found not sensitive enough to do a 'non-destructive' single kernel analysis of sorghum. Neither could the 'non-destructive' single kernel estimation of available lysine be done on oats because of the problems discussed in Sections II and III of this thesis.

6. Summary

1) It has been demonstrated that total protein, available lysine and tryptophan content could be estimated from the distal parts of barley, rye, triticale and wheat. The assays would be applicable to other seeds of the same size or larger.

2) All the assays were found suited for routine analysis, because they are fast and do not require any critical timing of the operations except for tryptophan assay where the incubations have to immediately follow reagent additions.

3) The proximal parts of the seed kernels that were left after analyzing the distal parts were shown to be able to germinate and grow to mature plants without any special treatments.

4) The 'non-destructive' single seed analysis performed here would offer good possibilities of back-cross type of a breeding operation.

BIBLIOGRAPHY

AHOKAS, H. 1978. A simple and rapid screening method for the determination of protein and tryptophan in kernel halves and small samples of meal. J. Sci. Food Agric. 29: 47-52.

AMAYA-F, J., YOUNG, C.T. and CHICHESTER, C.O. 1977. Automated determination of tryptophan in legumes and cereals. J. Agric. Food Chem. 25(1): 139-143.

AMBLER, R.P. and MEADWAY, R.J. 1968. The use of thermolysin in amino acid sequence determination. Biochem. J. 108: 893-895.

ARNON, R. 1970. Papain. In G.E. Perlmann and L. Lorand (eds.). Methods in Enzymology Vol. XIX. Proteolytic Enzymes, pp. 226-244. Academic Press, New York and London.

BAILEY, J.L. 1967$_a$. Selective cleavage of peptide chains. In Techniques in Protein Chemistry, 2nd revised and expanded edition, pp. 134-162. Elsevier Publishing Company, Amsterdam-London-New York.

————. 1967$_b$. Miscellaneous analytical methods. In Techniques in Protein Chemistry, 2nd revised and expanded edition, pp. 342-352. Elsevier Publishing Company, Amsterdam-London-New York.

BALARAVI, S.P., BANSAL, H.C., EGGUM, B.O. and BHASKARAN, S. 1976. Characterisation of induced high protein and high lysine mutants in barley. J. Sci. Food Agric. 27: 545-552.

BASHA, S.M.M. and ROBERTS, R.M. 1977. A simple colorimetric method for the determination of tryptophan. Anal. Biochem. 77: 378-386.

BENCZE, W.L. and SCHMID, K. 1957. Determination of tyrosine and tryptophan in proteins. Anal. Chem. 29(8): 1193-1196.

BENDELOW, V.M. and MEREDITH, W.O.S. 1955. Reliability of prediction tests for malting quality of barley. Can. J. Agric. Sci. 35: 252-258.

BENNETT, H.P.J., ELLIOT, D.F., EVANS, B.E., LOWRY, P.J. and McMARTIN, C. 1972. Complete amino acid analysis of peptides and proteins after hydrolysis by a mixture of sepharose-bound peptidases. Biochem. J. 129: 695-701.

BENOITON, L. and DENEAULT, J. 1966. The hydrolysis of two ε-N-Methyl-L-lysine derivatives by trypsin. Biochim. Biophys. Acta. 113: 611-613.

BHATIA, C.R., JAGANNATH, D.R. and GOPAL-AYENGAR, A.R. 1970. Induced micro-mutations for major protein fractions in wheat. In Improving Plant Proteins by Nuclear Techniques, pp. 96-106. IAEA, Vienna.

BHUMIRATANA, S., HILL, Jr. C.G. and AMUNDSON, C.H. 1977. Enzymatic solubilization of fish protein concentrate in membrane reactors. J. Food Sci. 42(4): 1016-1021.

BIER, M. and NORD, F.F. 1951. On the mechanism of enzyme action XLVI. The effect of certain ions on crystalline trypsin and reinvestigation of its isoelectric point. Arch. Biochem. Biophys. 33: 320-332.

BIETZ, J.A. 1974. Micro-Kjeldahl analysis by an improved automated ammonia determination following manual digestion. Anal. Chem. 46: 1617-1618.

BLOM, L., HENDRICKS, P. and CARIS, J. 1967. Determination of available lysine in foods. Anal. Biochem. 21: 382-400.

BLOW, D.M. 1971. The structure of chymotrypsin. In P.D. Boyer (ed.). The Enzymes Vol. III. Hydrolysis: Peptide Bonds, 3rd edition, pp. 185-212. Academic Press, New York and London.

BOCCU, E., VERONESE, F.M., FONTANA, A. and BENASSI, C.A. 1970. Sulfenyl halides as modifying reagents for polypeptides and proteins. Quantitative evaluation of tryptophan and cysteine residues in proteins. Eur. J. Biochem. 13: 188-192.

BONDI, A. and BIRK, Y. 1954. Studies on the action of papain on protein feeds. Bull. Res. Counc. Isr. iv: 57-62.

BOOTH, V.H. 1971. Problems in the determination of FDNB-available lysine. J. Sci. Food Agric. 22: 658-664.

BRUNO, D. and CARPENTER, K.J. 1957. A modified procedure for the estimation of 'available lysine' in food proteins. Biochem. J. 67: 13 p.

BUCHANAN, R.A. and BYERS, M. 1969. Interference by cyanide with the measurements of papain hydrolysis. J. Sci. Food Agric. 20: 364-367.

BÜCHMANN, N.B. 1979. *In vitro* digestibility of protein from barley and other cereals. J. Sci. Food Agric. 30: 583-589.

BURNS, E.A. 1969. Inorganic nitrogen-hydrogen compounds. In C.A. Streuli and P.R. Averell (eds.). The Analytical Chemistry of Nitrogen and its Compounds. Part I. Chemical Analysis vol. 28, pp. 51-83. Wiley-Interscience.

BYERS, M. 1967. The *in vitro* hydrolysis of leaf proteins. I. The action of papain on protein extracted from the leaves of *Zea mays*. J. Sci. Food Agric. 18: 28-33.

CARPENTER, K.J. and ELLINGER, G.M. 1955. The estimation of 'available lysine' in protein concentrates. Biochem. J. 61: xi-xii.

CARPENTER, K.J. 1960. The estimation of the available lysine in animal-protein foods. Biochem. J. 77: 604-610.

CHILDS, E.A. 1975. An enzymatic-chemical method for extraction of cottonseed protein. J. Food Sci. 40: 78-80.

———— and FORTE, J.F. 1976. Enzymatic and ultrasonic techniques for solubilization of protein from heat-treated cottonseed products. J. Food Sci. 41: 652-655.

CLARK, Jr. J.M. 1963 (ed.). Proteins and amino acids. In Experimental Biochemistry, pp. 69-87. W.H. Freeman and Company, San Fransisco.

COCKING, E.C. and YEMM, E.W. 1954. Estimation of amino acids by ninhydrin. Biochem. J. 58: xii.

CONCON, J.M. 1975. Rapid and simple method for the determination of tryptophan in cereal grains. Anal. Biochem. 67: 206-219.

CONKERTON, E.J. and FRAMPTON, V.L. 1959. Reaction of gossypol with free ε-amino groups of lysine in proteins. Arch. Biochem. Biophys. 81: 130-134.

DALBY, A. and TSAI, C.Y. 1975. Acetic acid requirement in the colorimetric determination of tryptophan. Anal. Biochem. 63: 283-285.

DATTA, S.C. 1976. Dinitrophenylation of the compounded chick starter rations for the estimation of available lysine. J. Agric. Food Chem. 24(3): 542-544.

DAVIES, M.G. and THOMAS, A.J. 1973. An investigation of hydrolytic techniques for the amino acid analysis of foodstuffs. J. Sci. Food Agric. 24: 1525-1540.

DELAAGE, M. and LAZDUNSKI, M. 1968. Trypsinogen, trypsin, trypsin-substrate and trypsin-inhibitor complexes in urea solutions. Eur. J. Biochem. 4: 378-384.

DESNUELLE, P. 1960$_a$. Chymotrypsin. In P.D. Boyer, H. Lardy and K. Myrbäck (eds.). The Enzymes Vol. 4. Hydrolytic Cleavage (Part A), 2nd edition, pp. 93-118. Academic Press, New York and London.

————. 1960$_b$. Trypsin. In P.D. Boyer, H. Lardy and K. Myrbäck (eds.). The Enzymes Vol. 4. Hydrolytic Cleavage (Part A), 2nd edition, pp. 119-132. Academic Press, New York and London.

DOHAN, D.A., STANDING, K.J. and BUSHUK, W. 1976. A new method of analysis of protein content in grain by protein activation. Cereal Chem. 53(1): 91-100.

DOLL, H. and KØIE, B. 1977. Influence of the high lysine gene from barley mutant 1508 on grains, carbohydrates and protein yield. In Seed Protein Improving by Nuclear Techniques, pp. 107-120. IAEA, Vienna.

DRENTH, J., JANSONIUS, J.N., KOEKOEK, R., SWEN, H.M. and WOLTHERS, B.G. 1968. Structure of papain. Nature (Lond.). 218: 929-932.

DRUCKER, H. and BORCHERS, S.L. 1971. The role of calcium on thermolysin: Effect on kinetic properties and autodigestion. Arch. Biochem. Biophys. 147: 242-248.

DUGGAN, D.E. and UDENFRIEND, S. 1956. The spectrophotofluorometric determination of tryptophan in plasma and of tryptophan and tyrosine in protein hydrolyzates. J. Biol. Chem. 223: 313-319.

EDELHOCH, H. 1967. Spectroscopic determination of tryptophan and tyrosine in proteins. Biochemistry. 6(7): 1948-1954.

ENDO, S. 1962. Studies on protease by thermophilic bacteria. J. Ferment. Technol. 40: 346-353.

FAFUNSO, M.A., BASSIR, O. and OKE, O.L. 1976. *In vitro* digestibility of some leaf protein preparations using papain. Nutr. Rep. Int. 14(3): 353-357.

FAWCETT, J.K. and SCOTT, J.E. 1960. A rapid and precise method for the determination of urea. J. Clin. Pathol. 13: 156-159.

FEATHERSTON, W.R. and ROGLER, J.C. 1975. Influence of tannins on the utilisation of sorghum grain by rats and chicks. Nutr. Rep. Int. 11(6): 491-497.

FEDER, J. and SCHUK, J.N. 1970. Studies on the *Bacillus subtilis* neutral-protease- and *Bacillus thermoproteolyticus* thermolysin-catalyzed hydrolysis of dipeptide substrates. Biochemistry. 9 (14): 2784-2791.

————, GARRET, L.R. and WILDI, B.S. 1971. Studies on the role of calcium in thermolysin. Biochemistry. 10(24): 4552-4556.

FELKER, P. 1977. Micro determination of nitrogen in seed protein extracts with the salicylate-dichloisocyanurate color reaction. Anal. Chem. 49: 1080.

FINKLE, B.J. and SMITH, E.L. 1958. Crystalline papain: number and reactivity of thiol groups; chromatographic behaviour. J. Biol. Chem. 230: 669-690.

FINLEY, J.W., FERREL, R.E. and FELLERS, D.A. 1972. Determination of added lysine in fortified wheat and bulgar. Cereal Chem. 49: 514-521.

FISCHL, J. 1960. Quantitative colorimetric determination of tryptophan. J. Biol. Chem. 235(4): 999-1001.

FONTANA, A., BOCCU, E. and VERONESE, F.M. 1975. Effect of EDTA on the conformational stability of thermolysin. Exper. Suppl. (Basel). 26: 55-59.

FRIEDMAN, M. and FINLEY, J.W. 1971. Methods of tryptophan analysis. J. Agric. Food Chem. 19(4): 626-631.

FRITZ, A., BAUMER, M., ULONSKA, E. and LENZ, W. 1973. A contribution to the analytical determination of the lysine content in barley. In Nuclear Techniques for Seed Protein Improvements, pp. 371-378. IAEA, Vienna.

GAITONDE, M.K. and DOVEY, T. 1970. A rapid and direct method for the quantitative determination of tryptophan in the intact protein. Biochem. J. 117: 907-911.

GOODWIN, T.W. and MORTON, R.A. 1946. The spectrophotometric determination of tyrosine and tryptophan in proteins. Biochem. J. 40: 628-632.

GREEN, N.M. and NEURATH, H. 1953. The effects of divalent cations on trypsin. J. Biol. Chem. 204: 379-390.

HAGBERG, A., KARLSSON, K.-E. and MUNCK, L. 1970. Use of *Hiproly* in barley breeding. In Improving Plant Protein by Nuclear Techniques, pp. 121-132. IAEA, Vienna.

HALL, R.J., TRINDER, N. and GIVENS, D.I. 1973. Observations on the use of 2,4,6-trinitrobenzenesulphonic acid for the determination of available lysine in animal protein concentrates. Analyst (Lond.). 98: 673-686.

————, ———— and WOOD, M.R. 1975. The determination of available lysine in carbohydrate-rich materials. Analyst (Lond.). 100: 68-70.

———— and HENDERSON, K. 1979. An improvement in the determination of available lysine in carbohydrate-rich samples. Analyst (Lond.). 104: 1097-1100.

HESS, G.P. 1971. Chymotrypsin-chemical properties and catalysis. In P.D. Boyer (ed.). The Enzymes Vol. III. Hydrolysis: Peptide Bonds, 3rd edition, pp. 213-248. Academic Press, New York and London.

HILL, R.L. and SCHMIDT, W.R. 1962. The complete enzymic hydrolysis of proteins. J. Biol. Chem. 237(2): 389-396.

————. 1965. Hydrolysis of proteins. Adv. Protein. Chem. 20: 37-107.

HINKEL, Jr. E.T. and ALFORD, C.E. 1951. Studies of methods for measuring the proteolytic activity of papain. Ann. N.Y. Acad. Sci. 54: 208-227.

HOLM, H. 1971. Micro-method for determination of non-N-terminal lysine in milk and similar products. J. Food Sci. Agric. 22: 378-381.

HOLMQUIST, B. and VALLEE, B.L. 1974. Metal substitutions and inhibition of thermolysin: Spectra of the cobalt enzyme. J. Biol. Chem. 249(14): 4601-4607.

HOWE, E.E., JANSEN, G.R. and GILFILLAN, E.W. 1965. Amino acid supplementation of cereal grains as related to the world food supply. Am. J. Clin. Nutr. 16: 315-320.

HSU, H.W., VAVAK, D.L., SATTERLEE, L.D. and MILLER, G.A. 1977. A multienzyme technique for estimating protein digestibility. J. Food Sci. 42(5): 1269-1273.

HUGLI, T.E. and MOORE, S. 1972. Determination of tryptophan content of proteins by ion exchange chromatography of alkaline hydrolyzates. J. Biol. Chem. 247(9): 2828-2834.

HWANG, K. and IVY, A.C. 1951. A review of the literature on the potential therapeutic significance of papain. Ann. N.Y. Acad. Sci. 54: 161-207.

JOHNSON, V.A., MATTERN, P.J. and SCHMIDT, J.W. 1970. The breeding of wheat and maize with improved nutritional value. Proc. Nutr. Soc. 29: 20-31.

———— and LAY, C.L. 1974. Genetic improvement of plant protein. J. Agric. Food Chem. 22(4): 558-566.

KAKADE, M.L. and LIENER, I.E. 1969. Determination of available lysine in proteins. Anal. Biochem. 27: 273-280.

———————. 1974. Biochemical basis for the differences in plant protein utilization. J. Agric. Food Chem. 22(4): 550-555.

KALCKAR, H.M. 1947. Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies of the enzymes of purine metabolism. J. Biol. Chem. 167: 461-475.

KAMRA, O.P. 1971. Genetic modification of seed protein quality in cereals and legumes. Z. Pflanzenzuecht. 65: 293-306.

KASARDA, D.D., NIMMO, C.C. and KOHLER, G.O. 1971. Proteins and the amino acid composition of wheat fractions. In Y. Pomeranz (ed.). Wheat Chemistry and Technology, 2nd edition, pp. 227-299. Am. Assoc. of Cereal Chemists, St. Paul, Minnesota.

KAUL, A.K., DHAR, R.D., SWAMINATHAN, M.S. and AHNSTRÖM, G. 1969. A rapid dye-binding method of screening single grains for protein characteristics. Curr. Sci. 38(14): 330-331.

KAY, C.M., SMILLIE, L.B. and HILDERMAN, F.A. 1961. The molecular weight of trypsinogen. J. Biol. Chem. 236: 118-121.

KEIL, B. 1971. Trypsin. In P.D. Boyer (ed.). The Enzymes Vol. III. Hydrolysis: Peptide Bonds, 3rd edition, pp. 249-275. Academic Press, New York and London.

KIMMEL, J.R. and SMITH, E.L. 1954. Crystalline papain: I. Preparation, specificity and activation. J. Biol. Chem. 207: 515-531.

KOTAKI, A. and SATAKE, K. 1964. Acid and alkaline degradation of the TNP-amino acids and -peptides. J. Biochem. (Tokyo). 56(4): 299-307.

KOOPS, J., KLOMP, H. and ELGERSMA, R.H.C. 1975. Rapid determination of nitrogen in milk and dairy products by colorimetric estimation of ammonia following an accelerated digestion procedure. Neth. Milk Dairy J. 29: 169-180.

KRAUT, J. 1971. Chymotrypsinogen: X-ray structure. In P.D. Boyer (ed.). The Enzymes Vol. III. Hydrolysis: Peptide Bonds, 3rd edition, pp. 165-183. Academic Press, New York and London.

KREIL, G. and TUPPY, H. 1961. Peptides, terminal and internal, released by digestion with trypsin. Nature (Lond.). 192: 1123-1125.

KREIS, M. 1977. Starch and free sugars during kernel development of Bomi barley and its high-lysine Mutant 1508. In Seed Protein Improvement by Nuclear Techniques, pp. 115-120. IAEA, Vienna.

LABERGE, D.E., MACGREGOR, A.W. and MEREDITH, W.O.S. 1973. Changes in the free sugar content of barley kernels during maturation. J. Inst. Brew. 79: 471-477.

LEIN, K.-A., BRUNKHORST, K. and SCHÖN, W.J. 1973. Assessment of different techniques for estimation of total nitrogen and lysine in cereals. In Nuclear Techniques for Seed Protein Improvement, pp. 363-369. IAEA, Vienna.

LIGHT, A., FRATER, R., KIMMEL, J.R. and SMITH, E.L. 1964. Current status of the structure of papain: The linear sequence, active sulfhydryl group, and the disulphide bridges. Proc. Nat. Acad. Sci. U.S.A. 52: 1276-1283.

LIU, T.Y. and CHANG, Y.H. 1971. Hydolysis of protein with p-toluenesulfonic acid. Determination of tryptophan. J. Biol. Chem. 246 (9): 2842-2848.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

MA, Y. and NELSON, O.E. 1975. Amino acid composition and storage proteins in two new high-lysine mutants in maize. Cereal Chem. 52: 412-419.

MAKINDE, M.A., HARE, L.B. and LACHANCE, P.A. 1976. The critical role of acetic anhydride in the rapid determination of tryptophan. Nutr. Rep. Int. 14(5): 547-552.

MASUDA, T. 1959. Studies on active group of papain. I. Effect of activators on inhibition by aldehyde reagents. J. Biochem. (Tokyo). 46(11): 1489-1496.

MATHESON, N.A. 1968. II. Determination of available lysine in feeding stuffs by dinitrophenylation. J. Sci. Food Agric. 19: 496-502.

MATSUBARA, H., SINGER, A., SASAKI, R. and JUKES, F.H. 1965. Observation on the specificity of a thermostable bacterial protease "Thermolysin". Biochem. Biophys. Res. Commun. 21(3): 242-247.

————, SASAKI, R., SINGER, O. and JUKES, F.H. 1966. Specific nature of hydrolysis of insulin and tobacco mosaic virus protein by thermolysin. Arch. Biochem. Biophys. 115: 324-331.

————. 1967. Some properties of thermolysin. In C.L. Prosner (ed.). Molecular Mechanism of Temperature Adaptation, pp. 283-294. Publication No. 84. Am. Assoc. for the Advancement of Science, Washington, D.C.

———— and SASAKI, R.M. 1969. High recovery of tryptophan from acid hydrolyzates of proteins. Biochem. Biophys. Res. Commun. 35(2): 175-181.

————, SINGER, A. and SASAKI, R.M. 1969. Effect of proline residue on the hydrolysis of substrates by thermolysin. Biochem. Biophys. Res. Commun. 34(5): 719-724.

————. 1970. Purification and assay of thermolysin. In G.E. Perlmann and L. Lorand (eds.). Methods in Enzymology Vol. XIX. Proteolytic Enzymes, pp. 642-650. Academic Press, New York and London.

McMILLAN, W.W., WISEMAN, B.R., BURNS, R.E., HARRIS, H.B. and GREENE, G.R. 1972. Bird resistance in diverse germplasm of sorghum. Agron. J. 64: 821-822.

McNEAL, F.H. and DAVIS, D.J. 1966. Protein content of wheat kernels from different parts of the spike. Agron. J. 58: 635-636.

MERTZ, E.T., BATES, L.S. and NELSON, O.E. 1964. Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science (Washington, D.C.). 145: 279-280.

————. 1968. High lysine corn. Agric. Rev., Coop. State Res. Serv. U.S. Dep. Agric. 6(3): 1-6.

————, MISRA, P.S. and JUMBANATHAN, R. 1974. Rapid ninhydrin color test for screening high lysine mutants of maize, sorghum, barley and other cereal grains. Cereal Chem. 51: 304-307.

MILLER, G.E. and RICE, Jr. J.D. 1963. Determination of the concentration of ammonia nitrogen in plasma by means of a simple ion exchange method. Am. J. Clin. Pathol. 39(1): 97-103.

MORIHARA, K. and TSUZUKI, H. 1966. Proteolytic substrate specificity and some elastolytic properties of a thermostable bacterial protease. Biochim. Biophys. Acta. 118: 215-218.

———— and EBATA, M. 1967. Comparison of substrate specificities of a thermostable bacterial proteinase and a bacterial elastase. J. Biochem. (Tokyo). 61(1): 149-151.

———— and OKA, T. 1968. The complex active sites of bacterial neutral proteases in relation to their specificities. Biochem. Biophys. Res. Commun. 30(6): 625-630.

————, TSUZUKI, H. and OKA, T. 1968. Comparison of the specificities of various neutral proteinases from microorganisms. Arch. Biochem. Biophys. 123: 572-588.

MOSSE, J. 1966. Alcohol-soluble proteins of cereal grains. Fed. Proc. 25: 1663-1669.

MURRAY, D.R.P. 1933. Activation of papain by cyanide and other substances. Biochem. J. 27: 543-556.

NAIR, B.M., ÖSTE, R., ASP, N.G. and DAHLQVIST, A. 1976. Enzymatic hydrolysis of food protein for amino acid analysis. I. Solubilization of food protein. J. Agric. Food Chem. 24(2): 386-389.

NARAHASHI, Y. and YANAGITA, M. 1967. Studies on proteolytic enzymes (Pronase) of *Streptomyces griseus* K-1. I. Nature and properties of the proteolytic enzyme system. J. Biochem. (Tokyo). 62(6): 633-641.

————, SHIBUYA, K. and YANAGITA, M. 1968. Studies on proteolytic enzyme (Pronase) of *Streptomyces griseus* K-1. II. Separation of exo- and endo-peptidases of Pronase. J. Biochem. (Tokyo). 64(4): 427-437.

NATIONAL RESEARCH COUNCIL. 1977. Protein quality and quantity. In Supporting Papers: World Food and Nutrition Study Vol. I, pp. 46-53. National Academy of Sciences, Washington, D.C.

NELSON, O.E. 1969. Genetic modification of protein quality in plants. Adv. Agron. 21: 171-194.

NELSON, Jr. O.E. 1970. Improvement of plant protein quality. In Improving Plant Protein by Nuclear Techniques, pp. 43-51. IAEA, Vienna.

NOMOTO, M. and NARAHASHI, Y. 1959. A proteolytic enzyme of *Streptomyces griseus*. IV. General properties of *Streptomyces griseus* protease. J. Biochem. (Tokyo). 46(12): 1645-1651.

————, NARAHASHI, Y. and MURAKAMI, M. 1960_a. A proteolytic enzyme of *Streptomyces griseus*. VI. Hydrolysis of protein by *Streptomyces griseus* protease. J. Biochem. (Tokyo). 48(4): 593-602.

————, NARAHASHI, Y. and MURAKAMI, M. 1960_b. A proteolytic enzyme of *Streptomyces griseus*. VII. Substrate specificity of *Streptomyces griseus* protease. J. Biochem. (Tokyo). 48(6): 906-918.

NORTHROP, J.H., KUNITZ, M. and HERRIOTT, R.M. 1955. Chymo-trypsinogen and chymo-trypsin. In Crystalline Enzymes, 2nd edition, revised and enlarged, pp. 96-124. Columbia University Press, New York.

OELSHLEGEL, Jr. F.J., SCHROEDER, J.R., and STAHMANN, M.A. 1970. A simple procedure for basic hydrolysis of proteins and rapid determination of tryptophan using a starch column. Anal. Chem. 34: 331-337.

OKE, O.L. and UMOH, I.B. 1974. Nutritive value of leaf protein: A note on the comparison of *in vitro* and *in vivo* methods. Nutr. Rep. Int. 10(6): 397-403.

OKUYAMA, T. and SATAKE, K. 1960. On the preparation and properties of 2,4,6-trinitrophenyl-amino acids and -peptides. J. Biochem. (Tokyo). 47(4): 454-466.

OPIEÑSKA-BLAUTH, J., CHAREZIÑSKI, M. and BERBEC̄, H. 1963. A new, rapid method of determining tryptophan. Anal. Biochem. 6: 69-76.

OSTROWSKI, H., JONES, A.S. and CADENHEAD, A. 1970. Availability of lysine in protein concentrates and diets using Capenter's method and a modified Silcock method. J. Sci. Food Agric. 21: 103-107.

PATTON, C.J. and CROUCH, S.R. 1977. Spectrophotometric and kinetics investigation of the Berthelot reaction for the determination of ammonia. Anal. Chem. 49(3): 464-469.

PAULIS, J.W. and WALL, J.S. 1979. Note on mill for pulverizing single kernels of cereals for isoelectric focusing. Cereal Chem. 56(5): 497-498.

PAYNE, R.E. and HILL, Jr. C.G. 1978. Enzymatic solubilization of leaf protein concentrate in membrane reactors. J. Food Sci. 43: 385-389.

PENKE, B., FERENCZI, R. and KOVACS, K. 1974. A new acid hydrolysis method for determining tryptophan in peptides and proteins. Anal. Biochem. 60: 45-50.

PIOMBO, G. and LOZANO, Y.F. 1980. Automated procedure for routine analysis of tryptophan in cereal and legume food samples. J. Agric. Food Chem. 28(3): 489-496.

PIRIE, N.W. 1971. A survey of other experiments on protein production. In N.W. Pirie (ed.). Leaf Protein: Its Agronomy, Preparation, Quality and Use. IBP Handbook No. 20, pp. 29-43. Blackwell Scientific Publications, Oxford and Edinburgh.

POMERANZ, Y., WESENBERG, D.M., SMITH, R.T., ROBBINS, G.S. and GILBERTSON, J.T. 1976. Amino acid composition of barley kernels from different parts of the spike. Cereal Chem. 53(6): 839-845.

PRICE, M.L., SCOYOC, S.V. and BUTLER, L.G. 1978. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. J. Agric. Food Chem. 26(5): 1214-1218.

PYM, R.V.E. and MILHAM, P.J. 1976. Selectivity of reaction among chlorine, ammonia, and salicylate for determination of ammonia. Anal. Chem. 48(9): 1413-1415.

RAMACHANDRA, G., VIRUPAKSHA, T.K. and SHADAKSHARASWAMY, M. 1977. Relationship between tannin levels and *in vitro* protein digestibility in finger millet (*Eleusine coracana* Gaertn). J. Agric. Food Chem. 25(5): 1101-1104.

RAO, S.R., CARTER, F.L. and FRAMPTON, V.L. 1963. Determination of available lysine in oilseed meal proteins. Anal. Chem. 35(12): 1927-1930.

REARDON, J., FOREMAN, J.A. and SEARCY, R.L. 1966. New reactants for the colorimetric determination of ammonia. Clin. Chim. Acta. 14: 403-405.

REED, G. and THORN, J.A. 1971. Enzymes. In Y. Pomeranz (ed.). Wheat Chemistry and Technology, 2nd edition, pp. 453-491. Am. Assoc. of Cereal Chemists, St. Paul, Minnesota.

RHODES, A.P. 1975. A comparison of two rapid screening methods for the selection of high-lysine barleys. J. Sci. Food Agric. 26: 1703-1710.

ROACH, A.G., SANDERSON, P. and WILLIAMS, D.R. 1967. Comparison of methods for the determination of available lysine value in animal and vegetable protein sources. J. Sci. Food Agric. 18: 274-278.

ROBEL, E.J. 1967. Ion-exchange chromatography for the determination of tryptophan. Anal. Biochem. 18: 406-413.

ROBYT, J.F. and WHELAN, W.J. 1968. The α-amylases. In J.A. Radlely (author). Starch and its Derivatives, 4th edition, pp. 430-476. Chapman and Hill Ltd.

ROCHE, R.S. and VOORDOUW, G. 1978. The structural and functional roles of metal ions in thermolysin. CRC Crit. Rev. Biochem. 1: 1-23.

ROSTAGO, H.S., FEATHERSTON, W.R. and ROGLER, J.C. 1973. Studies in the nutritional value of sorghum grains with varying tannin contents for chicks. 1. Growth studies. Poult. Sci. 52: 765-772.

SANGER, F. 1945. The free amino groups of insulin. Biochem. J. 39: 507-515.

SANNER, T. and PHIL, A. 1963. Studies on the active-SH group of papain on the mechanism of activation by thiols. J. Biol. Chem. 238(1): 165-171.

SASAKI, T., ABRAMS, B. and HORECKER, B.L. 1975. A fluorometric method for the determination of the tryptophan content of proteins. Anal. Biochem. 65: 396-404.

SAUNDERS, R.M., CONNOR, M.A., BOOTH, A.N., BICKOFF, E.M. and KOHLER, G.O. 1972. Measurement of digestibility of alfalfa protein concentrates by *in vivo* and *in vitro* methods. J. Nutr. 103: 530-535.

———— and KOHLER, G.O. 1972. *In vitro* determination of protein digestibility in wheat millfeeds for monogastric animals. Cereal Chem. 49: 98-103.

SCHECHTER, I. and BERGER, A. 1968. On the active site of proteases. III. Mapping the active site of papain; specific peptide inhibitors of papain. Biochem. Biophys. Res. Commun. 32(5): 898-902.

SCHWERT, G.W. and EISENBERG, M.A. 1949. The kinetics of the amidase and esterase activities of trypsin. J. Biol. Chem. 179: 665-672.

SEARCY, R.L., SIMMS, N.M., FOREMAN, J.A. and BERGQUIST, L.M. 1965. Specificity of the Berthelot color reaction. Clin. Chim. Acta. 12(2): 170-175.

SELIM, A.S.M. 1965. Microdetermination of lysine in protein hydrolyzates. J. Agric. Food Chem. 13(5): 435-438.

SHAPIRA, E. and ARNON, R. 1969. Cleavage of one specific disulfide bond in papain. J. Biol. Chem. 244(4): 1026-1032.

SHARMA, T.R. and KAUL, A.K. 1971. Correlation between some quality characters and protein determinants in bread wheat. Curr. Sci. 40(7): 150-152.

SHELTON, K.R. and ROGERS, K.S. 1971. Tryptophanyl fluorescence of sodium dodecyl sulfate treated and 2-mercaptoethanol reduced proteins: A simple assay for tryptophan. Anal. Biochem. 44: 134-142.

SHUKLA, T.P. 1975. Cereal proteins: Chemistry and food applications. CRC Crit. Rev. Food Technol. 6: 1-75.

SINGH, R. and AXTELL, J.D. 1973. High lysine mutant gene (*hl*) that improves protein quality and biological value of grain sorghum. Crop Sci. 13: 535-539.

SIPOS, T. and MERKEL, J.R. 1970. An effect of calcium ions on the activity, heat stability, and structure of trypsin. Biochemistry. 9(14): 2766-2775.

SLUYTERMAN, L.A.AE. 1967. The activation reaction of papain. Biochim. Biophys. Acta. 139: 430-438.

SMITH, E.L. and KIMMEL, J.R. 1960. Papain. In P.D. Boyer, H. Lardy and K. Myrbäck (eds.). The Enzymes Vol. 4. Hydrolytic Cleavage (Part A), 2nd edition, pp. 133-173. Academic Press, New York and London.

SPIES, J.R. and CHAMBERS, D.C. 1949. Chemical determination of tryptophan in proteins. Anal. Chem. 21(10): 1249-1266.

————. 1967. Determination of tryptophan in proteins. Anal. Chem. 39: 1412-1416.

STOCKELL, A. and SMITH, E.L. 1957. Kinetics of papain action. I. Hydrolysis of benzoyl-L-argininamide. J. Biol. Chem. 227: 1-26.

TSAI, C.Y., HANSEL, L.W. and NELSON, O.E. 1972. A colorimetric method of screening maize seeds for lysine content. Cereal Chem. 49: 572-579.

UDY, D.C. 1971. Improved dye method for estimating protein. J. Am. Oil Chemists' Soc. 48: 29A-33A.

VILLEGAS, E. and MERTZ, E.T. 1971. Chemical screening methods for maize protein quality at CIMMYT. Res. Bull. No. 20. CIMMYT.

VOORDOUW, G. and ROCHE, R.S. 1974. The cooperative binding of two calcium ions to the double site of apothermolysin. Biochemistry. 13(24): 5017-5021.

WADDELL, W.J. 1956. A simple ultraviolet spectrophotometric method for the determination of protein. J. Lab. Clin. Med. 48: 311-314.

WALL, Sr. L.L. and GEHRKE, C.W. 1975. An automated total protein nitrogen method. J. Assoc. Off. Anal. Chem. 58(6): 1221-1226.

WANG, S.S. and CARPENTER, F.H. 1967. Kinetics of the tryptic hydrolysis of the oxidized B chain of bolvine insulin. Biochemistry. 6(1): 215-224.

WEATHERBURN, N.W. 1967. Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. 39(8): 971-974.

YAMAGUCHI, R., SUZUKI, H. and HIRASHIMA, H. 1970. Colorimetric determination of ammonia. IV. A new method with o-chlorophenol and 2,6-dichloroisocyanurate. Chem. Pharm. Bull. (Tokyo). 18: 1886-1871.

YAMAMOTO, T. and IZUMIYA, N. 1967. Action of trypsin on synthetic substrates. III. Hydrolysis of homologous peptides ranging from diglycyl-L-lysyl-glycine to diglycyl-L-lysyl-tetraglycine. Archiv. Biochem. Biophys. 120: 497-502.

YAMASHITA, K. 1960. Action of chymotrypsin on synthetic substrates. IV. Action of α-chymotrypsin on acyl-L-tyrosylglycines and acyl-L-tyrosylglycylglycines. J. Biochem. (Tokyo). 48(5): 651-661.

YEMM, E.W. and COCKING, E.C. 1955. The determination of amino acids with ninhydrin. Analyst (Lond.). 80: 209-213.

APPENDIX 1

Papain Enzyme Activators

Because the enzyme hydrolyzates were to be analyzed for total nitrogen after micro-Kjeldahl digestions, the activators of papain that contained nitrogen would be undesirable. Cysteine·HCl was found to be the most effective of several activators that were tried. Thioglycolic acid and 2,3-dimercaptopropanol were slightly less effective (Buchanan and Byers, 1969). The reagent 2,3-dimercaptopropanol has been reported to cause maximum activation of papain (Stockwell and Smith, 1954); however, applications for routine operations are restricted by its strong smell.

Cyanide is an effective activator of papain enzyme (Byers, 1967). It was supposed to escape as hydrogen cyanide gas during the micro-Kjeldahl digestion of samples (Byers, 1967). Later Buchanan and Byers (1969) showed that in the presence of reducing sugars, the cyanide which formed cyanohydrins would be detected in the micro-Kjeldahl digests. The error due to cyanide diminished as the ratio of cyanide N to substrate N in the digest decreased. When the ratio of cyanide N to substrate N was 1:15 the apparent increase in protein content was found to be 3-5% (Buchanan and Byers, 1969).

To avoid errors due to cyanide Buchanan and Byers (1969) recommended the use of thioglycolic acid as an activator for papain. In this

study when thioglycolic acid was used, the recoveries of lysine were affected (see below). Therefore use of cyanide was considered as the best alternative although it caused some increase in apparent protein content.

To mimimize the error due to cyanide it was desirable to add only enough to activate the enzyme. The concentration of cyanide required to give papain maximum activity using maize (K_{26} x K_{52}) protein as a substrate was determined. Fig. A1 shows the amount of protein solubilized in 24 hours using various cyanide concentrations. Any further increase in cyanide concentration above 1 mM did not cause any significant increase in the amount of protein solubilized. A cyanide concentration of 1.5 mM was used to activate papain in all the studies that were done. At this concentration the cyanide N to substrate N ratio under the conditions used was 1:22. At this ratio, all other factors remaining constant, the increase in nitrogen content of samples would be expected to be lower than under Buchanan and Byers (1969) conditions. When the total N from the solubilized protein plus residue protein obtained from the papain hydrolyzate was compared to the N from whole samples there was no significant difference between the two values.

Effect of Thioglycolic Acid on the Available Lysine Recoveries

When thioglycolic acid at a concentration of 0.01 M (less than half the amount recommended by Buchanan and Byers, 1969) was used to activate papain, lower recoveries of available lysine were obtained. The recoveries from standard lysine solutions were more seriously affected than from samples (Table A1). Recoveries from different samples were also not equally affected (Table A1).

Figure A1. Effect of the amount of KCN used for papain activation on the amount of protein solubilized from maize (K_{26} x K_{52}) in 24 hours at 65° C.

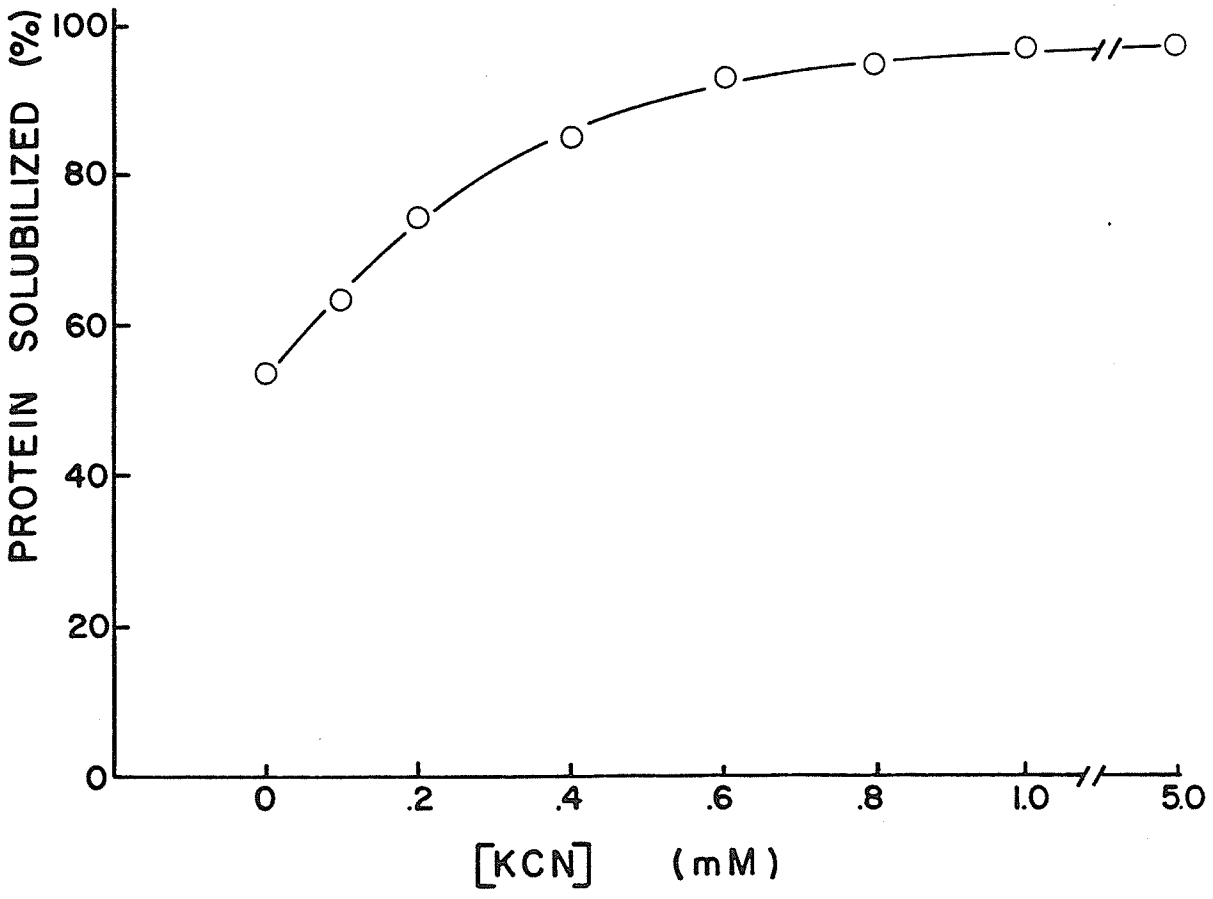

PROTEIN SOLUBILIZED (%)
100
80
60
40
20
0
0
.2
.4
.6
.8
1.0
5.0
[KCN] (mM)

TABLE A1. Effect of thioglycolic acid on the available lysine recoveries

Sample	Lysine Recoveries (%)	
	Papain Activated with	
	KCN (1.5 mM)	Thioglycolic acid (0.01 M)
Herta barley	100	93.5
Risø 1508 barley	100	95.9
Maize $63o_2$ x $MS206o_2$	100	83.4
Prolific rye	100	90.8
Lys·HCl (100 µg)	101.2	69.1
Lys·HCl (200 µg)	100.4	72.5

For comparison purposes only, the available lysine values obtained from samples in CN-activated enzyme were assumed to be 100% while the recoveries of standard lysine were based on the absolute amount added before incubations. Because of the low lysine recoveries thioglycolic acid was found unsuitable for papain activation if available lysine values were to be determined from the hydrolyzate so obtained.

APPENDIX 2

TABLE A2. Solubilized protein as determined from supernatant or pooled supernatant and washings

Sample	Protein Solubilized (%)	
	Supernatant	Supernatant and washings
Maize, $K_{26} \times K_{52}$	98.1	98.7
Wheat, Neepawa	97.8	98.3
Triticale, Rosner	97.1	97.3