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University of North Carolina at Greensboro, Ph.D., 1973
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IN VITRO AVAILABILITY OF ESSENTIAL AMINO ACIDS, AND THE PROTEIN EFFICIENCY RATIO OF COOKED *CICER ARIETINUM*

by

Meera Rao

A Dissertation Submitted to
the Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

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Approved by

Dissertation Adviser

APPROVAL PAGE

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RAO, MEERA. *In Vitro* Availability of Essential Amino Acids, and the Protein Efficiency Ratio of Cooked *Cicer arietinum*. (1972)
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The legume *Cicer arietinum* (chick-pea) is a commonly used source of dietary protein in India because of its high biological value and non-toxic nature. The objective of this investigation was to study the effects of cooking on the availability of four essential amino acids in chick-peas and on the utilization of its protein for tissue synthesis.

Acid hydrolysis for varying periods of time was used to determine the optimum level of each amino acid present in raw, roasted, boiled, and steam-cooked chick-peas. Enzymatic hydrolysis with pepsin and pancreatin for varying periods of time was used to study the quantitative release of the amino acids *in vitro*. The lysine, threonine, valine, and leucines present in the acid and enzyme hydrolysates were identified by 1-dimensional polychromatic paper chromatography using n-butanol, acetic acid, and water as the solvent for resolution. Quantitative estimations were based on the elution and colorimetric readings of the chromatograms. A growth experiment with weanling rats was employed to evaluate the overall quality of the protein. Using a randomized block design, five groups of 3 week old male rats of the Holtzman strain were fed a basal diet supplying 10% protein through casein, and experimental diets supplying 10% protein through raw, roasted, boiled, and steam-cooked chick-peas for a 4 week period.

Results showed that the amounts of lysine, threonine, valine, and leucines present in 100 g of raw chick-peas were 2.23 g, 2.10 g, 1.63 g, and 2.28 g, respectively. Optimum release of lysine was obtained after hydrolysis with acid for 6 hours, of threonine and valine after 48 hours, and of leucines after 24 hours. Statistical analysis of data showed a highly significant difference in the release of these amino acids after varying periods of hydrolysis.

Differences in the release of lysine and leucines after varying the duration of hydrolysis with enzymes was highly significant. Maximum release of lysine was obtained after 3 hour peptic digestion followed by 48 hour pancreatic digestion, and of leucines after 24 hour pancreatic digestion of the pepsin digest. Comparison of the percentage release of each amino acid after enzymatic hydrolysis showed that the differences between raw and cooked chick-peas were highly significant in the case of lysine and valine, the maximum amounts being found in the raw legume.

Results of the biological experiment showed a highly significant difference in the protein efficiency of raw and cooked legume. The protein efficiency ratio (PER) was lowest (1.87) on the roasted chick-pea diet, and maximum (2.66) on the diet in which the chick-pea had been cooked for 5 minutes at 15 p.s.i. The PER of raw chick-pea was lower than that of boiled and steam-cooked.

The investigation led to the following conclusions:

1. The release of lysine, threonine, valine, and leucines from raw and cooked chick-peas differs with the duration of acid hydrolysis.

2. Release of the four amino acids also differs with the duration of enzymatic digestion with pepsin and pancreatin.

3. Although raw chick-pea yields maximum release of the amino acids by *in vitro* enzyme digestion, it does not give the highest PER when fed to the intact animal.

4. Based on the *in vitro* analysis, cooking of chick-peas decreases the percentage release of the four amino acids. According to the PER determination, steam-cooking or boiling leads to an increase in the quality of protein above that of the raw chick-peas, while roasting has a detrimental effect.

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I. INTRODUCTION

Food proteins furnish the amino acids necessary for the anabolic processes of maintenance, repletion of tissues, and growth of new cellular proteins in the organism. The efficiency of utilization of dietary protein for tissue synthesis is influenced by the pattern of essential and non-essential amino acids provided by the diet, the nitrogen intake, the caloric intake, and the physiological state of the organism. Knowledge of the amino acid content of foods is important because it serves as a basis for planning diets for man and animals, in the development of novel protein foods used in feeding programs with vulnerable groups, and in the establishment of amino acid requirements of different species.

Various methods of analyses have been used extensively to determine the individual amino acids contained in raw foods. Since foods are generally subjected to heat treatment during preparation and processing, the amino acids may undergo changes by forming unusual chemical bonds, or by destruction. Hence, research concerning the effect of processing and household methods of cooking on the retention and availability of essential amino acids in commonly used sources of protein is of importance.

One of the main sources of protein among population groups dependent on vegetable protein foods due to cultural or economic reasons are leguminous seeds or pulses such as dried peas and beans. The legume Cicer arietinum, known as garbanzo, Egyptian pea, chick-pea or Bengal gram is cultivated in many tropical countries and has been shown to rate high in biological value compared to other vegetable protein foods. Legumes are cooked to increase palatability, to destroy toxic substances and enzyme inhibitors, and to render them more digestible.

Information available from earlier investigations is limited to the amino acid content of Cicer arietinum, and the effect of cooking procedures on the retention of these amino acids. Since various factors during processing might interfere with the quantity of an amino acid released by digestion, there is need for experimental evidence regarding the availability of essential amino acids from the cooked legume.

The objectives of this study were:

1. To determine the amounts of lysine, threonine, valine, and leucine present in chick-peas.

2. To determine the effect of cooking on the in vitro availability of these amino acids after enzymatic digestion.

3. To determine whether there is a difference in the protein efficiency ratio of raw and cooked chick-peas.

4. To compare the *in vitro* release of the amino acids with the quality of the protein as determined by the rat growth method.

The cooking treatments applied to the legume were based on the procedures commonly employed in India, i.e., dry roasting, boiling, and steam-cooking. The information obtained from the study will be of value in determining the method suitable for optimum conservation of the essential amino acids in chick-peas, and also serve as a basis for planning diets and supplementation of available foods with the limiting amino acids.

CHAPTER II

REVIEW OF LITERATURE

Legumes in Human Nutrition

Results of diet surveys in India have shown that a major part of the protein is derived from cereals and pulses with negligible amounts from animal proteins (1,2). Although legumes are an important source of amino acids in the vegetarian diets, supplementation with other protein foods is necessary to overcome the effects of certain limiting amino acids such as tryptophan and methionine. The most widely cultivated legumes used in the Indian diets are Bengal gram (Cicer arietinum), green gram (Phaseolus mungo), red gram (Cajanus cajan), dried peas (Pisum sativum), lentils (Lens esculenta), and Khesari dhal (Lathyrus sativus). Daily intakes range from 40-75 grams (3). Chick-peas are usually consumed in the form of soups in which the peas are soaked, boiled and mashed, toasted seeds either whole or milled, incorporated with cereal dishes, unleavened breads and fried foods (4, 5, 6).

Attempts to develop inexpensive, high quality protein foods at the Central Food Technological Research Institute in India have led to a product called the Indian Multi Purpose Food (IMPF), based on indigenous sources of protein such as Bengal gram grits, peanut, and sesame seed

cake fortified with minerals and vitamins (7). Organoleptic evaluation of the products showed mixtures based on peanut and chick-pea (75:25 parts) to be highly acceptable. A similar product made of peanut, chick-pea, and fish flours tested for shelf life at room temperature showed deterioration in nutritive value to be limited to losses of 25% vitamin A and 13% thiamine (8). A daily intake of 50 grams of a blend of peanut, chick-pea, soybean and sesame flour costing about 1.6 cents was estimated to supply the day's requirement for protein, calcium, and vitamins (9).

Daniels et al. (10) experimented with the fortification of blends of varying proportions of Bengal gram and wheat flour with lysine, methionine, and threonine. The protein efficiency ratio (PER) on Bengal gram and wheat flour mix (20:80) was 2.43, which increased to 2.96 when fortified with the amino acids. Results indicated that diets containing 80 parts of cereal and 20 parts of Bengal gram would provide adequate amounts of protein (14% on dry basis) for promoting optimal growth of preschool children. Investigators at the Instituto Mexicano de Investigaciones Technologicas used whole chick-pea and a spray dried oleoprotein to fortify dehydrated corn dough with the essential amino acids (11).

The nutritional value of the Indian Multi-Purpose Food has been demonstrated in several experimental feeding programs with vulnerable groups. Subrahmanyan (12)

reported that children receiving a cereal based diet supplemented with 2 oz. of IMPF showed significant increases in height, weight, and hemoglobin concentrations in contrast to children receiving an unsupplemented control diet. In an experiment designed to determine the effect of protein derived from processed Bengal gram, and skim milk on the control of edema in children from low income groups, Venkatachalam (13) observed no significant differences in the two groups of children fed the respective diets. However, the plasma albumin regeneration was higher in the children fed skim milk diets. Similar results were observed when a supplement of 4-5 oz. of IMPF was used to prevent edema and diarrhea in kwashiorkar patients (14). In addition to the disappearance of clinical signs in patients fed the IMPF supplements, there was also substantial gain in weight.

Nutritive Value of Chick-peas

The nutrient content of chick-peas has been investigated by several workers. Proximate analysis of the seed by Lal et al. (15) indicated that the seed coat was low in all nutrients except calcium. The embryo was found to be rich in protein, carbohydrates, iron, and phosphorus, but the amounts of these nutrients contributed were insignificant. In contrast, the cotyledons contained maximum food value. Thus, the common practice of removing the husk of

the legume by milling does not result in a significant nutrient loss. Soaking the seed in water appeared to have no effect on the migration of nutrients. Paper chromatographic estimation by Tawde and Cama (16) revealed that the soluble carbohydrate in chick-peas was approximately 6.7%, composed primarily of glucose and fructose.

Estimations of the amino acid content of the chick-pea appear to vary depending upon the type of analytical method used for determination. Tryptophan was found to be the limiting amino acid when the chick-pea was assayed by the microbiologic method (17). Chatterjee et al. (18) reported the presence of arginine, leucine, isoleucine, lysine, valine, phenylalanine, threonine, histidine, methionine, and tryptophan. Results from the 2-dimensional chromatographic estimation of Bagchi et al. (19) substantiated the presence of the amino acids identified by the microbiologic method (18). However, the essential amino acid levels in globulin fractions of Bengal gram assayed using electrophoretic methods by Tawde and Cama (20) were nearly twice the levels reported from microbiologic and chromatographic estimations.

Although chemical and microbiologic assays give an indication of the approximate amino acid content of foods, an animal growth experiment is often imperative to determine the actual biological value of the food protein. One of the most commonly used biological assays to evaluate

protein quality is the protein efficiency ratio in young rats. Jaffe (21) attempted to determine the limiting essential amino acids of various legume seeds. The seeds were ground and autoclaved at 10 p.s.i. for 20 minutes and supplied 10% protein in the diets. Kidney and hyacinth beans were soaked in water, autoclaved, dried, and ground to remove the toxic principle. On diets furnishing 10% chick-pea protein, 1.3 g of weight gain per day was noted, which increased to 2.9 g when the diets were supplemented with 0.3% methionine. Methionine supplements added to kidney and hyacinth bean diets raised the level of growth to be equal to or better than the performance on soybean diets, but had no effect on dried pea, lentil, and pigeon-pea diets, which suggested that other amino acids may be limited in these diets.

Results from a study by Russell (22) showed that addition of 0.1% methionine per 100 g of the diet containing chick-peas improved the PER to nearly that of soybeans. Adolph et al. (23) comparing the protein quality of legumes available in the Middle East, observed that diets composed of cooked parboiled wheat, chick-peas, and broad beans gave optimum PER. Mixtures of parboiled wheat and cooked chick-peas gave the next highest PER.

Evaluation of the protein quality of different steam-cooked legume diets showed a higher biological value for Bengal gram than for unprocessed soybeans (24). This was

attributed to the antigrowth factor present in soybeans even after cooking for 15 minutes. The amounts of intake and digestibility on ad libitum feeding were also higher on Bengal gram diets.

Similar results were obtained when a mixture of chick-peas, sesame seed, and soybean flour developed for use in child feeding was tested with animals (25). If the chick-peas were autoclaved prior to incorporation into the diet, the resulting PER was higher than that obtained on a diet containing raw chick-peas. The sesame meal appeared to supply the sulfur containing amino acids that were low in chick-peas, while the limiting amino acid lysine was supplied by the legume.

Borchers and Ackerson (26) studied the effect of autoclaving on the trypsin inhibitor activity of legumes. The samples were prepared by autoclaving the legume powders for 30 minutes at 15 p.s.i. The peanut fat was removed by extraction. Results showed that the weight gain in rats was higher when peanut, jack-bean, horse-bean, lespedeza, lentil, velvet, and mung beans were autoclaved before feeding. The chick-pea, although found to contain a trypsin inhibitor (27), showed no difference in weight gain whether fed raw or autoclaved. The authors concluded that there was no correlation between improvement in nutritive value after autoclaving and the presence or absence of the trypsin inhibitor in the raw legume seed. Hence, the trypsin

inhibitor test would not indicate whether or not the nutritive quality of the legume would improve by autoclaving.

Blaizot (28) found that lentils, smooth peas, and chick-peas supported growth at subnormal levels when fed in mixed diets. Although chick-peas were found to be more adequate, diets consisting entirely of this legume revealed toxic effects in the animal after feeding for 20 days. Balance studies with autoclaved products revealed a lowering of nitrogen retention on all diets.

Effects of Heat on the Nutritive Value of Proteins

Although it is well known that the nutritive value of a protein can be altered by heat treatment, the nature of the alteration apparently varies with the conditions of the treatment. Some researchers have reported a decrease in nutritive value upon heating and attribute this to the deterioration of the native protein. Other investigators have reported an increase in nutritive value and suggest that the heat treatment either improves the digestibility or causes a greater release of the amino acids present in the protein.

The chemical changes which take place upon heating have been investigated in both isolated protein systems and in foods. Eldred and Rodney found that dry heat reduced the susceptibility of casein to both *in vitro* and *in vivo* enzymatic digestion (29). They found that casein

yielded 19% less amino nitrogen and 24-27% less lysine when it was heated at 150°C for 70 minutes. A similar effect has also been observed when edestin protein isolated from hemp seed was heated (3). In both instances, the detrimental effect of heat on the protein was attributed to the destruction of lysine. Greaves et al. (31) found that lysine was the most heat sensitive amino acid in casein and was destroyed when casein was heated to 130°C for 30 minutes, whereas losses of cystine, tyrosine, and tryptophan occurred at temperatures above 140°C. These researchers concluded that heat damage to lysine and other amino acids destroys the identity of the amino acid and decreases the absorbability of their nitrogen. The nature and extent of the damage apparently depends not only upon temperature and duration of heating, but also on the molecular structure of the original protein.

When a mixture of soybean protein and sucrose was autoclaved, a decrease in the amounts of all amino acids liberated by in vitro enzymatic digestion was observed by Evans and Butts (32). Although the exact reason for this decrease in amino acids was not apparent, the investigators suggested that either an inactivation of the diamino acids such as arginine and lysine occurred because of the free amino groups forming enzyme-resistant linkages with the carboxyl groups, or that actual destruction of the amino acids had taken place. There was some evidence that a similar binding of the amino groups of cystine,

methionine, and histidine rendered these non-available to the animal, while phenylalanine, threonine, leucine, isoleucine, and valine were unaffected.

Similar results were also obtained by Stevens et al. (33) who observed that autoclaving pure lysine for 4 hours at 120°C had no effect on the potency of this amino acid when it was added to a diet of overheated soybeans in which the lysine had been destroyed. Heating lysine in the presence of carbohydrates either destroyed the amino acid or rendered it unavailable for utilization. The browning occurring in the overheated mixtures was similar to the color changes noted in overheated soybean meal.

Schroeder et al. (34) concluded that browning was caused by the effect of pH on the carbohydrates while the Maillard reaction observed in some of the amino acid-carbohydrate mixtures resulted from amino-sugar linkages. The deteriorative changes seemed to be accelerated in an alkaline medium.

Baldwin et al. (35) reported that autoclaving a casein-dextrose complex resulted in the destruction of lysine and arginine. The results also showed that arginine, lysine, methionine, histidine, and threonine present in the mixture were rendered unavailable when autoclaved samples were fed to the growing rat. An increase in the moisture content of the casein-dextrose mixture during autoclaving was associated with a decrease in the extent to

which browning reaction occurred. Limiting the occurrence of browning led to an increase in the nutritive value of the complex. When the temperature of autoclaving was raised from 100°C to 121°C, an adverse effect on the nutritive value was observed. The authors concluded that the amino-sugar linkages formed when the mixture was heated for 80 minutes and that as heating was continued, a progressive type of binding occurred around the previously bound carbohydrates.

Patton (36) found that arginine, lysine, and tryptophan were inactivated to a considerable extent when refluxed with glucose and attributed this susceptibility to reaction with aldehydes to be caused by the presence of functional nitrogen groups unattached in polypeptide linkages. Thus, in the case of heat damage to proteins in the presence of reducing sugars, a combination of the reactive groups with aldoses resulted in degradation products or polymers.

Studies on the effects of heat on the proteins present in foods per se do not lead to any general conclusions due to the differences in the composition of individual foods and the variations in their chemical behavior. Several investigations of the amino-sugar reaction have been made with regard to meats, soybeans, and other legumes. That moderate heat processing of foods often leads to an improvement of its nutritive value has been borne out by these investigations, and the beneficial effect has been attributed

to the increased release of methionine where this is the limiting factor in the raw foods.

Osborne and Mendel (37) observed maximum growth rates in rats maintained on diets containing cooked soybeans as compared to raw and overheated samples. Mechanical breakdown of the beans into smaller particles enhanced its digestibility.

Melnick (38) reported that dry heating of soybean flour led to no improvement in biological value whereas autoclaving caused a pronounced increase due to enhanced availability of methionine. The differences in biological value was accounted for by the rates of release of individual amino acids during enzymatic digestion.

The nutritive value of cooked legumes other than soybeans has been investigated to a limited extent. Woods et al. (39) reported that field peas decreased in growth promoting properties after baking for 30 minutes at 14°C and autoclaving at 17 p.s.i. for 1 1/2 hr. They concluded that the deterioration was due to destruction of the sulfur containing amino acids which acted as a factor in utilization. Blaizot (28) also found a lower biological value in legumes after autoclaving.

Evans and St. John (40) observed progressive decreases in the availabilities of lysine and methionine after drastic autoclaving. Mild autoclaving led to no increase in the liberation of methionine, cystine, and lysine.

Waterman and Johns (41) found that cooking of Phaseolus protein for 5 minutes gave a noticeable increase in digestibility whereas cooking for 45 minutes led to optimum in vitro digestibility as well as weight gain in rats. The optimum cooking time for black beans as indicated by PER in rats was found to be 10-30 minutes (42). Animals on a diet of raw black beans were unable to survive despite dietary supplementation with methionine. Maximum nutritive value was obtained when beans were either cooked in a pressure cooker or in the household method of cooking in an open kettle.

Thus, the mechanisms involved in browning and in the Maillard reaction together with their effect on the nutritive value of proteins have been investigated extensively, yet much of the evidence is of a contradictory nature because of the variety of temperatures, media, and pH of the conditions used. The general conclusions reached by many of the investigators regarding the chemical changes that occur when proteins are heated in combination with carbohydrates are the destruction of certain amino acids, a change in the digestibility of the protein by proteolytic enzymes, a decrease in the availability of amino acids, and heat inactivation of enzyme inhibitors thereby enhancing the nutritive value.

Availability of Amino Acids

It is well recognized that the nutrient content of a food as determined by analyses does not necessarily

establish the amount of that nutrient available when consumed by man and animals. Although a protein may contain the amino acids in desirable proportions, interfering factors will influence the physiological availability of the amino acids, and thereby the ability of a protein to support growth and maintenance in the animal. Some of these factors are enzyme inhibitors, mechanical barriers such as cellulose, and the presence of excess non-amino nitrogen. Mouron (43) defined the availability of a given amino acid as the amount or percentage of that amino acid in the food which is utilized for protein synthesis in the organism when this amino acid is the only limiting factor in the diet.

Although the nutritional quality of a protein is best established with feeding trials, in vitro methods of protein evaluation are useful in assaying new protein foods and processing methods (43, 44). The two in vitro methods most commonly employed for estimating the biological availability of amino acids are based on either the measurement by Sanger's reaction of the percentage of free epsilon amino groups contained in the protein after hydrolysis or digestion, or a comparison of the rates at which amino nitrogen or free amino acids are released from proteins when incubated in vitro with proteolytic enzymes.

In vitro methods

The principle of the chemical method used by Carpenter (45, 46) for determining the availability of amino acids is

dependent on the reaction of fluoro-2:4-dinitrobenzene (FDNB) with the ε-amino groups of lysine in purified proteins. As a result of this reaction, a stable, colored, DNP-lysine compound is formed which is then measured in a colorimeter. Comparison of the results from the FDNB procedure with those obtained from animal experiments showed that a reduction in the number of free ε-amino groups was paralleled by a fall in the nutritional value.

Bujard et al. (47) found that availability values obtained by the enzymatic method correlated well with those obtained by the FDNB method when testing the availability of lysine in several roller dried milk powders. In all the heated samples, initial lysine content was much higher when compared to the amounts released by enzymatic hydrolysis. A later modification of the method by Carpenter (46) called the "corrected straight acid procedure" enables obtaining a blank value for non-lysine interfering color. The FDNB procedure has been widely used to estimate the effects of heat processing which lowers the availability of lysine by binding the ε-amino groups in all food proteins. This method, however, is restricted to the study of only lysine.

Proteolytic enzymes in the desiccated or crystalline forms have been employed by various investigators to study the availability of amino acids from proteins (48, 49, 50, 51). Eldred and Rodney (28) studied the proteolysis

in raw and heated casein by measuring the total nitrogen, amino nitrogen, and the amino acid lysine after enzymatic digestion with pepsin, trypsin, chymetrypsin, and crude pancreas. Although the digestibility of heated casein and the lysine content of acid hydrolysates were not significantly impaired, the amount of free lysine released by enzymatic hydrolysis was much lower than that released in raw casein. This was attributed to a change in the substrate available to the action of proteolytic enzymes after heating. The specificity of the enzyme lysine decarboxylase used for analysis prevented measurement of the ε-amino groups bound up in peptide linkages which remained unaffected by heat. Therefore, this measurement gave an indication of only the free lysine present in samples.

Pader et al. (48) reported a similar study in which the degree of protein hydrolysis in raw and heated casein was measured by formol titration, and the lysine content of hydrolysates was measured by microbiologic methods. Decrease in the nutritive value of the processed casein was explained to be due to the slower rate of release of lysine by enzyme digestion rather than to the extent of release.

Riesen et al. (49) determined the α-amino nitrogen in pancreatic hydrolysates of soybean protein given different heat treatments. A higher concentration of pancreatin

was needed with raw soybeans to overcome the effects of trypsin inhibitors. Results of this study showed that excessive heating of the samples prevented optimum pancreatic hydrolysis as well as the liberation of certain amino acids from the proteins. Although the values obtained by the α-amino nitrogen method were not strictly comparable to results from microbial assay, they showed a similar trend as far as deterioration by heat was concerned.

Hankes et al. (50), using procedures similar to those used by Riesen, compared the liberation of amino acids from raw and heated casein after acid and enzyme hydrolysis. Aliquots were removed after successive digestion of the samples with pepsin, pancreatin, and erepsin to study the progress of hydrolysis. The authors found no differences in the release of amino acids from raw and heated casein after pepsin digestion alone, but if pancreatin and erepsin were used, higher values were obtained for moderately heated samples of casein indicating that the amino acid bonds affected by heat were cleaved in the later stages of enzyme digestion.

Lowry et al. (51) investigated the nutritive impairment of casein and gluten heated with dextrose using pepsin, trypsin, chymotrypsin, pancreatin, and papain for in vitro digestion. Prior to digestion, the samples were boiled one hour to solubilize the protein. During incubation with enzymes, aliquots were removed at intervals and analyzed for

amino nitrogen. The casein-dextrose complex was found to be resistant to trypsin digestion when the samples were in a moist medium, but not in the dry state. An aqueous suspension of casein was easily digested by trypsin or by pepsin *in vitro*, whereas additional digestion with pepsin, chymotrypsin, and pancreatin was necessary to break down the dextrose complex. The authors attributed the differences in availability to the requirement of specific groups in the substrates for the enzymes to act upon.

Mouron *et al.* (52) removed the soluble carbohydrates from various processed milks by dialysis before enzymatic digestion of the protein with pepsin and pancreatin. Fractions were withdrawn to study the progress of hydrolysis, and in all of the roller-dried and evaporated milk samples tested, the amount of lysine liberated was lower than in fresh milk. The enzymatic liberation of amino nitrogen and methionine was lower in roller-dried milks, which were processed at a higher temperature. Amounts of tryptophan and tyrosine liberated did not differ among the samples. The loss of lysine in the processing of milks was explained to have occurred as a result of destruction by heat and inactivation due to formation of chemical bonds.

Using the FDNB methods and *in vitro* digestion procedures, Mouron *et al.* (43) estimated the effects of heat damage on the availability of amino acids in protein foods incorporated in child-feeding programs. Results showed that

the digestibility of samples of peanuts differed according to the varying levels of the antitryptic factor present. In the case of high protein biscuits, those cooked in the oven showed amino acid deterioration proportionate to the length of time, and the temperature used for heating. The loss in nutritive value was related to the destruction and inactivation of lysine, which was in agreement with the findings of other studies.

Sheffner et al. (53) attempted to develop an in vitro procedure based on the pattern of amino acids released by digesting the protein with enzymes to estimate the nutritive quality of proteins. The new method of computation termed the Pepsin Digest Residue (PDR) Index consisted of determining the amino acid content of a food after complete hydrolsis with acid and of the same food after pepsin digestion. The concentration of each amino acid estimated microbiologically in the pepsin digest was then subtracted from the concentration of the respective amino acid in the acid hydrolysate to give the "residue" fraction. Each amino acid was calculated as the percentage of the sum of the amino acids for the protein in the pepsin digest, and residue fractions. Applying this procedure to evaluate the proteins of whole egg, egg albumin, soy flour, casein, and white flour, the authors found good correlation between the PDR Index and the Net Protein Utilization (NPU) values.

Akeson and Stahman (44) modified the PDR Index procedure by employing the automatic amino acid analyzer for calculation of amino acids in the hydrolysates instead of the microbiological method, thus enabling evaluation of a large number of protein foods within a limited time.

Comparison of availability of amino acids from selected proteins using both microbiologic and chromatographic methods showed that microorganisms could utilize peptides as well as free amino acids (54). The values obtained for threonine and lysine were higher in enzymatic hydrolysates analyzed microbiologically. Thus, the use of microbiologic technique for amino acid assay from partial hydrolysates appears to be questionable. In vivo studies indicated that the threonine was not completely available from peanut meal to the protein-depleted rat, as determined by weight gain and nitrogen retention.

Evaluation of protein quality

Biological assays for evaluating protein quality have been widely used with the chick or the rat as the test animal. The main objectives of animal experiments are to explore the quality of a protein in relation to its amino acid content, to determine the effect of processing on the nutritive value of the protein, and to evaluate the protein of foods and diets as eaten (55). All measures of protein quality are a function of the limiting amino acid because

the usefulness of a protein is limited by the amino acid in shortest supply. The tests do not, therefore, yield any information about the other amino acids and the potential value of the protein if given in combination with other proteins.

Several excellent reviews have been written discussing the classical methods of Protein Efficiency Ratio (PER), Net Protein Utilization (NPU), Net Protein Retention (NPR), and Biological Value (BV), as well as various modifications of these methods (56, 57, 58).

Measurement of protein efficiency by gain in body weight is one of the simplest and most widely used procedures available. Osborne et al. (59) found a striking correlation between the weight gain in young rats and the quality of protein in the diet. Since food intake is regulated by caloric requirements of the animal, the same amount of food would be eaten given similar conditions, whether the diet contains a high or a low percentage of protein. A standard level of 10% protein is generally used in experiments on PER. Since the PER is influenced by age, weight, sex, and strain of the animal (60, 61), it is necessary to keep these variables under control. Keane et al. (62, 63) found that addition of 20% water to diets containing 6, 9, and 12% protein led to significant increases in PER compared to rats consuming the same amount of protein furnished by dry diets.

Bosshardt et al. (64) found that the PER in mice was influenced by changing the environment from the stock colony to individual cages, duration of the test period, level of protein in the diet, and the utilization of ingested protein for body protein gain. In an attempt to study the effect of caloric intake on the PER, Bosshardt and Barnes (65) compared the caloric intake and protein utilization in growing rats that were consuming isocaloric diets ad libitum. Protein utilization was measured as the percentage of absorbed protein nitrogen used for body nitrogen gain, and the caloric intake as the average daily consumption of calories per 100 cm^2 of body surface area. Proteins from four sources were fed at seven different levels to groups of rats. With each protein source there seemed to be a maximal caloric intake per unit body size at the level of maximum utilization of the given protein. Caloric intake was found to decrease at levels below or above the optimum level of protein utilization. Further investigation indicated that at a given level of protein intake increased caloric consumption could improve the utilization of that protein for growth.

Results from the study of Sure (66) showed that the PER of milled wheat flour was improved when supplemented with the limiting amino acids lysine, valine, and threonine.

Thus, a review of the studies dealing with the in vitro estimation of amino acids, and the biological experiments

to measure the effectiveness of a protein as fed to the intact animal, leads to the conclusion that while either method by itself would not yield sufficient information regarding the quality of a protein, a combination of the two methods would be of value in evaluating the nutritional adequacy of protein foods.

CHAPTER III

MATERIALS, METHODS, AND EXPERIMENTAL PROCEDURES

The essential amino acids lysine, threonine, valine and leucine in raw and cooked chick-peas were determined after acid hydrolysis, and after *in vitro* digestion with pepsin and pancreatin. The evaluation of protein quality of the raw and cooked legume was based on the protein efficiency ratio in weanling rats. Raw chick-peas were used as the control. The heat treatments applied to samples of chick-peas were roasting, boiling, and steam-cooking. The amino acids in the hydrolysates were identified by paper chromatography, and the quantities estimated by elution and measurement of light absorbance in a colorimeter.

Materials

The chick-peas (*Cicer arietinum*) were purchased from a grocery store in sufficient quantity to complete the study. The samples for raw and roasted variables were milled to a flour of 0.03" mesh size in a Fritz mill. The boiled and steam cooked samples were retained whole during cooking, then homogenized in a Waring blender, followed by drying in an oven to remove excess moisture. Preliminary experiments showed that cooking the chick-pea flour in the presence of moisture produced gelation of the starches

present, which hardened upon drying, making it difficult to be homogenized in the Waring blender. Therefore, the legume was cooked whole when a moist medium was used. On the other hand, roasting the chick-peas as whole seeds hardened them still further, thus preventing effective homogenization. It was necessary to employ the Fritz mill to powder the seeds for the raw and roasted samples.

Heat Treatments

The common methods of cooking chick-peas and other legumes employed in India have been reported in literature (4, 5, 6). Since leguminous seeds are dried to an extremely low moisture content in order to improve the keeping qualities, rehydration with water over a long period of time is necessary to soften the seed before cooking. In the case of roasting, dry heat is employed to dextrinize the starches and improve edibility. With increasing use of the pressure-cookers to shorten cooking time, the method of steam cooking under pressure is being widely applied.

The following cooking procedures were standardized after several trials under controlled conditions:

Roasting

The chick-pea flour (150 g) was roasted for 10 minutes, stirring constantly, in a 3-quart cast aluminum sauce-pan over the magic eye of a gas range. The sample appeared light brown with an enhanced aroma.

Boiling

Whole chick-peas were soaked in tap water (1:3 w/v) overnight for 16 hours in a 3-quart cast aluminum saucepan. The sample was heated gradually on top of a stove taking care to prevent loss of material by boiling over. The time was noted when boiling began, and the temperature was retained at that point (98°C) as determined by a thermometer calibrated from -10° to 260°C, for one hour. The cooked chick-peas were allowed to cool to room temperature, homogenized in a Waring blender for five minutes in order to obtain fine, moist grains, spread in 1/2" thick layers on enamel trays, and then dried overnight at 55°C.

Steam cooking

Whole chick-peas were soaked overnight in tap water (1:2 w/v) as described for the boiled sample. It was necessary to use a lesser amount of water than that used for boiling, since evaporation during cooking was negligible. The chick-peas were cooked for 5 minutes at 15 p.s.i. in a Vischer Flex Seal high pressure steamer, cooled to room temperature, homogenized, and dried following the same procedure used for the boiled sample.

The cooked chick-pea samples were prepared in adequate amounts to complete the *in vitro* analyses and the feeding experiment. The samples were stored in glass jars in the refrigerator.

The moisture levels in all samples were estimated using the A.O.A.C. procedure (67). The crude protein content was estimated by the Kjeldahl procedure of the A.O.A.C., after modification.

Acid Hydrolysis

Two methods are in common use for acid hydrolysis of proteins in preparation for amino acid analysis. In the method described by Block (68), the protein containing material is reflux-boiled with several hundred times its weight of 6 N HCl, in Erlenmeyer flasks. In order to eliminate losses that might occur when the sample is boiled in contact with air, and to reduce the amount of sample and HCl needed for hydrolysis, several investigators have used a modified method (69, 70). Moore and Stein (69) recommended using 5 mg of protein with 1 ml of 6 N HCl in 16 X 125 mm glass tubes. The mixture is frozen in the tube, the air evacuated, and the opening sealed. Hydrolysis is allowed to occur by placing the tubes in an oven at 110°C for the required number of hours. Smith et al. (71) found no difference when either constant boiling or 6 N HCl was used for the hydrolysis of proteins.

The method described by Moore and Stein (69) was followed for the acid hydrolysis of chick-pea proteins. The sample of chick pea weighing exactly 200 mg was transferred into a 10 ml ampoule of borosilicate glass, and 7 ml

of 6 N HCl was pipetted into the ampoule. The sample was well mixed by means of a test-tube shaker. The mixture was frozen solid by placing the ampoule in a beaker of acetone and dry ice. The air in the ampoule was evacuated by connecting it to a vacuum pump, and the neck sealed using an oxygen flame.

The ampoule was then placed in a hot-air oven which had been preheated to 110°C. The sample was allowed to hydrolyze for 6, 24, or 48 hours.

After hydrolysis for the specified length of time, the ampoule was cooled and broken open by filing at the neck. The hydrolysate was decolorized with a small amount of charcoal and filtered through Whatman no. 12 filter paper into an evaporating dish.

The clear solution was evaporated to dryness over warm sand, and the residue was dissolved in 4 ml of 10% isoprepyl alcohol. The hydrolysate thus prepared was analyzed for the essential amino acids using 1-dimensional paper chromatography. Four replicates of each sample were hydrolyzed for different lengths of time.

Enzymatic Hydrolysis

Hydrolysis using proteolytic enzymes was done to investigate whether there was a difference in the extent and the rate of release of the essential amino acids after different periods of incubation. The procedure consisting

of the following steps was adapted from that described by Akeson and Stahman (44):

The enzyme digest was prepared by weighing out 500 mg of the sample plus 7.5 mg of dry pepsin[1] (1:10,000) into a 250 ml Erlenmeyer flask. The sample was well mixed after the addition of 75 ml of 0.1 N HCl and a few drops of toluene to prevent bacterial growth. The flask was incubated in a water bath maintained at 37°C for 3 hours.

After 3 hours of pepsin digestion, the pH of the solution was adjusted to 8.3 using about 1.5 ml of 5 N NaOH. Twenty mg of pancreatin[2] was added to the alkaline enzyme digest, and the incubation continued for 6, 24, or 48 hours in a shaker water bath maintained at 37°C.

The digestion flask was removed from the water bath after the specified time, and 50 ml of 1% picric acid was added to deproteinize the solution. The digest was filtered through Whatman no. 12 filter paper into beakers of 400 ml capacity. The clear yellow solution was desalted by passing through a column of anion exchange resin (Amberlite IRA 401 S) into an evaporating dish.

After rinsing the column with 5 ml portions of 0.02 N HCl 3 times to ensure complete recovery of the

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[1]Sigma Chemical Company.

[2]Porcine Pancreas, Sigma Chemical Company.

desalted solution, the sample was evaporated over warm sand and taken up in 10 ml of 10% isopropyl alcohol.

The hydrolysate obtained from the final step was analyzed for the essential amino acids using 1-dimensional paper chromatography.

Four replicates of the raw, roasted, boiled, and steam-cooked chick-peas were hydrolyzed for each of the three periods of digestion employed, i.e., 6, 24, and 48 hours. Enzyme blanks were run with each batch of digests to correct for the amino acids contributed by the enzymes.

Chromatography for Identification

Whatman no. 1 chromatography paper measuring 9" X 18" was marked with a line 2 1/2" from the 9" edge. The four replicate hydrolysates of each sample were applied using a 5 lambda pipette. Aliquots of 1% solutions of the pure amino acids were also applied on the same sheet of paper to facilitate identification of the unknown amino acids. After air drying the spots, the papers were suspended in the solvent composed of n-butanol, acetic acid, and water (30:5:25 v/v) for descending chromatography in a jar saturated with vapors of the same solvent, at room temperature.

After resolution for about 16 hours, the papers were air dried in a hood for 30 minutes, heated for 1 minute at 100°C in a pre-heated oven, cooled to room temperature, and sprayed with a mixture of ethyl alcohol containing 0.2%

ninhydrin, 1% acetic acid, and 0.2% 2, 4, 6-collidine, and a 1% solution of cupric nitrate in absolute ethyl alcohol. The two solutions were combined (25:1.5 v/v) just before using (72). The characteristic color of each amino acid was revealed by heating the chromatogram in the oven at 100°C for two minutes.

Quantitative Estimation

Standard curves of the amino acids lysine, threonine, valine, and leucine were prepared by applying 5 µl through 25 µl aliquots prepared by dissolving 10 mg of the crystalline amino acid in 10 ml of 1% HCl. The papers were developed using the same procedure as described for identification. The reagent used for the development of color in all quantitative chromatography was 0.25% ninhydrin in n-butanol. The colored areas were cut from the chromatogram, placed in separate test tubes, and the amino acid eluted with 10 ml of 75% ethyl alcohol containing 5 mg $CuSO_4.5H_2O$ per 100 ml by shaking with a test tube shaker until all the color was extracted. Concentrations were determined by standard colorimetric methods using a wave length of 570 mm (19).

The procedure was repeated using 5 µl aliquots of the chick-pea hydrolysates. The areas on the chromatogram identified as lysine, threonine, valine, and leucine were eluted, the optical density read in the colorimeter, and

the concentration of each amino acid calculated by referring to the standard curve of the known amino acid.

Evaluation of Protein Quality

The effect of cooking procedures on the quality of chick-pea protein as measured by the rat growth method was examined according to the procedure described by Campbell (55).

Weanling male rats,[1] 50-60 grams in weight were used for the experiment. The rats were housed in individual wire bottom cages and given free access to food and water. At the beginning of the experiment the animals were randomized into replications according to initial body weights. The test diets within each replication were randomly assigned to individual cages. The duration of the feeding experiment was four weeks. The animals were weighed weekly, and their food consumption was recorded. Records on spillage were also kept on each animal during the experiment.

The composition of the basal casein diet used for comparison, and the diets containing chick-peas given the different cooking treatments, is given in Table 1. The basal diet I contained a 10% level of protein derived from 10% of casein. Since the moisture and protein content of the raw and cooked chick-peas did not differ significantly, quantities of the raw, roasted, boiled, and

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[1]Holtzman strain rats purchased from the Holtzman Company, Madison, Wisconsin.

TABLE 1

COMPOSITION OF THE BASAL AND EXPERIMENTAL DIETS

Constituents	Per Cent Basal Diet	Per Cent Experimental Diets
Cornstarch[a]	70	30
Vegetable oil[b]	10	10
Mineral mix[c]	4	4
Vitamin mix[d]	1	1
Cellulose[e]	5	5
Casein[f]	10	0
Chick-pea	0	50
Oeum percomorphum[g]		

[a]Powdered Cornstarch 105-A, Clinton Corn Processing Company, Clinton, Iowa.

[b]Crisco, Proctor and Gamble Company, Cincinnati, Ohio.

[c]Salt Mixture W, Nutritional Biochemicals Corporation, Cleveland, Ohio. The composition of this salt mixture is listed as: (in per cent) $CaCO_3$ 21.000; $CuSO_4.5H_2O$, 0.039; $FePO_4.2H_2O$, 1.470; $MnSO_4$, 0.020; $MgSO_4$, 9.000; $KAl(SO_4)_2.12H_2O$, 0.009; KCl, 12.000; KH_2PO_4, 31.000; KI, 0.005; NaCl, 10.5000; NaF, 0.057; and $Ca_3(PO_4)_2$, 14.900.

[d]Each 100 gm of vitamin mix contained: (in milligrams) 0.1% vitamin B_{12} (with mannitol),0.1; biotin, 1; folic acid, 5; Thiamine. HCl, 25; pyridozine.HCl, 25; 2-methyl-naphthaquinone, 50; riboflavin, 50; nicotinic acid, 50; Ca pantothenate, 150; p-aminobenzoic acid, 500; (in grams) inositol, 5; choline chloride, 7.5; DL-methionine, 30; and cornstarch, 56.6. All vitamins and methionine were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

[e]Alphacel, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[f]Vitamin Test Casein, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[g]Each kilogram of diet contained 24 drops of oleum percomorphum, Mead Johnson and Company, Evansville, Indiana.

steam-cooked chick-peas necessary to supply 10% protein were used instead of the casein, for diets II, III, IV, and V, respectively. Because of the marked difference in the protein content of casein and chick-peas part of the cornstarch in diets II through V was replaced by chick-peas.

The PER of individual animals in each group were calculated at weekly intervals.

CHAPTER IV

RESULTS AND DISCUSSION

The purpose of this investigation was to determine the effect of cooking on the availability of the essential amino acid present in chick-peas, which would influence the protein efficiency ratio of the legume. Raw chick-peas were used as the control with which the amounts of amino acids released after the cooking treatments were compared. It was necessary to establish the initial levels of crude protein and the essential amino acids lysine, threonine, valine, and leucine in the chick-peas used in this investigation. The crude protein content was estimated by the Kjeldahl procedure, and the amino acids by acid hydrolysis for varying periods of time followed by paper chromatographic separation.

Results from the polychromatic paper chromatography of chick-pea hydrolysates revealed the characteristic color of the amino acids. The presence of lysine (reddish brown which develops a pink ring upon standing), threonine (greenish brown which turns to purplish brown), valine (purple), and the leucines (light purple with yellow ring, or light blue) were identified by comparison of color and ratio of frontal movement (R_f) values with the chromatograms

of the pure amino acids. Bagchi et al. (19), using 2-dimensional paper chromatography of amino acids, found inadequate separation of leucine and isoleucine and classified them as leucines. Data obtained in this investigation also revealed that leucine merged with isoleucine on the chromatogram to give a spot of indefinite color and R_f value.

One-dimensional chromatography was selected after preliminary experiments with thin layer chromatography on pre-coated plates and resolution with several combinations of solvents, and 2-dimensional paper chromatography with butanol, acetic acid and water for the first phase, and phenol and water for the second, had proved to be unsatisfactory. Pilot studies with 1-dimensional paper chromatography gave values in conformity with findings of investigators using other methods. Since certain amino acids having the same R_f value might make identification difficult in 1-dimensional chromatography, the polychromatic method of Moffat and Lytle (72) was employed.

Acid Hydrolysis

Results of quantitative estimation from 1-dimensional chromatograms of acid hydrolysates showed that the amounts of lysine, threonine, valine and the leucines contained in 100 g of raw chick-peas were 2.23 g, 2.10 g, 1.63 g, and 2.28 g respectively. These values were higher than the

values reported by previous investigations especially the microbiologic method of Chatterjee et al. (18). A comparison of amino acid values reported by several workers is presented in Table 2. It was evident from a review of studies that the number and quantities of the essential amino acids found in raw chick-peas vary markedly depending on the conditions used for the hydrolysis of proteins, equipment, and procedures for the identification and quantitative estimation of the amino acids. Differences in the moisture, protein, and other chemical constituents of the sample may also contribute to the variation in results. Information pertaining to the determination of the essential amino acid content of chick-peas by 1-dimensional paper chromatography is particularly limited and practically nonexistent.

The amino acid levels obtained in this study seemed to be closest to the data obtained by electrophoretic separation (20). The high values obtained for threonine could be due to the presence of another amino acid of the same R_f value in a lower concentration, the color of which was masked by the presence of a high concentration of threonine. Color development using the polychromatic reagent yielded a heavy purple spot identified as threonine. Since the leucine and isoleucine values could not be calculated individually in this study, it was comparable to the

TABLE 2

QUANTITIES OF LYSINE, THREONINE, VALINE, AND LEUCINES IN CHICK-PEAS DETERMINED BY 1-DIMENSIONAL PAPER CHRCMATOGRAPHY COMPARED WITH LITERATURE VALUES USING OTHER METHODS

% Protein Content	Amino Acids (g/100 g of sample)					Method of estimation
	Lysine	Threonine	Valine	Leucine	Iso-leucine	
20.34	2.23	2.10	1.63	2.28		1-dimensional paper chromatography
17	1.97	0.67	0.68	1.62		2-dimensional paper chromatography (19)
..	2.88	2.50	2.78	3.79	2.88	Electro-phoresis (20)
21.80	1.39	1.04	1.17	1.75	1.31	Microbio-logic (17)
19	0.26	0.60	0.85	1.28	1.07	Microbio-logic (18)
20.10	1.38	0.76	1.03	1.54	1.19	Column chroma-tography (73)

total values for leucine and isoleucine reported by other workers.

Acid hydrolysis for different lengths of time was done in order to establish the optimum level of lysine, threonine, valine, and the leucines found in chick-peas. Results from the 6, 24, and 48 hour hydrolyses supported the hypothesis that the amount of amino acid released was influenced by the duration of hydrolysis. Optimum amounts of lysine were obtained after 6 hour acid hydrolysis, with the levels decreasing as heating was continued to 24 and 48 hours (Table 3). Threonine and valine were released to the maximum when chick-peas were hydrolyzed for 48 hours, whereas the leucines were susceptible to destruction if hydrolysis was continued beyond the 24 hour period. Differences in the amounts released after each period of hydrolysis were found to be highly significant ($p \leq 0.01$) in the case of all four amino acids (Appendix B, Table 1). The rates of release of the amino acids did not vary from the raw to cooked samples.

The marked differences observed in the amounts of amino acids in the 6, 24, and 48 hour hydrolysates could be due to several factors. Since the protein was not pure, the presence of soluble carbohydrates, starches, and other materials present in the legume could have affected the release of amino acids. The characteristics of the protein which would thereby influence the configuration

TABLE 3

AMINO ACIDS[a] RELEASED FROM RAW AND COOKED CHICK-PEAS AFTER ACID HYDROLYSIS FOR 6, 24, AND 48 HOURS

Treatment of Sample	Duration of Hydrolysis		
	6 hours	24 hours	48 hours
	Lysine (g/100 g chick-pea)		
Raw	2.410	2.228	1.905
Roasted	2.300	2.181	1.805
Boiled	2.300	2.181	1.810
Steam-cooked	2.315	2.191	1.810
	Threonine (g/100 g chick-pea)		
Raw	1.565	1.630	2.100
Roasted	1.565	1.530	2.200
Boiled	1.515	1.500	2.200
Steam-cooked	1.515	1.530	2.210
	Valine (g/100 g chick-pea)		
Raw	0.908	1.270	1.628
Roasted	0.844	1.170	1.600
Boiled	0.812	1.182	1.527
Steam-cooked	0.880	1.270	1.600
	Leucines (g/100 g chick-pea)		
Raw	1.870	2.400	2.280
Roasted	1.840	2.340	2.220
Boiled	1.870	2.340	2.280
Steam-cooked	1.840	2.340	2.280

[a]Each value is the mean of 4 determinations.

of the peptide bonds contained may determine the ease with which the amino acids are broken off. The weaker peptide linkages that are easily split by heat and acid may release the individual amino acids within a shorter period than stronger peptide bonds which take a longer period to break down. In contrast, the amino acids sensitive to heat may undergo destruction at high temperatures and long periods of heating and be converted to compounds that do not react with ninhydrin.

Studies of other investigators led them to conclude that lysine did not decrease with increasing hydrolysis time, that leucines and valine had a slow rate of release, and that threonine was decomposed at a rate which is linear to the duration of hydrolysis (74, 75, 76). These observations do not agree with the findings of the present investigation because of the variation in periods of hydrolysis used. Most of the researchers continued hydrolysis for 70 hours, whereas in this study the maximum period was 48 hours. Variation in results also point to the fact that the structure of the protein and amino acid sequence differ from one food to another. Some linkages may be more protected from contact with acid than are others because of peculiarities in the folding of the protein molecule.

Enzymatic Hydrolysis

Enzymatic digestion with pepsin for 3 hours was followed by digestion with pancreatin for 6, 24, or 48 hours using raw and cooked chick-peas as the substrates.

The amino acid levels in the enzymatic hydrolysates which are presented in Table 4 were markedly lower than the content in corresponding acid hydrolysates. Although rates of release of the amino acids upon enzymatic digestion for 6, 24, and 48 hours differed from those obtained by acid hydrolysis, in general optimum releases of all four of the amino acids were obtained upon hydrolysis with pepsin for 3 hours followed by pancreatin for 24 to 48 hours. Differences due to the duration of digestion were highly significant ($p \leq 0.01$) in the case of lysine and the leucines (Appendix B, Table 2). Maximum release of lysine from raw as well as cooked chick-peas was obtained after 48 hours pancreatic digestion. Maximum release of threonine from raw and steam-cooked chick-peas was obtained after 24 hours, whereas in the roasted and boiled samples after 48 hours. Optimum release of valine was found after 24 hours in all samples except those steam-cooked. The 24 hour enzymatic digestion was adequate to release the optimum amount of the leucines contained in all the samples.

The data in Table 5 show the amount of each amino acid released after 6, 24, and 48 hours of enzymatic

TABLE 4

AMINO ACIDS[a] RELEASED FROM RAW AND COOKED CHICK-PEAS AFTER ENZYME[b] HYDROLYSIS FOR 6, 24, AND 48 HOURS

Treatment of Sample	Duration of Hydrolysis		
	6 hours	24 hours	48 hours
	Lysine (g/100 g chick-pea)		
Raw	0.710	0.750	1.375
Roasted	0.500	0.500	1.114
Boiled	0.500	0.600	1.133
Steam-cooked	0.575	0.650	1.175
	Threonine (g/100 g chick-pea)		
Raw	0.413	0.535	0.513
Roasted	0.341	0.403	0.413
Boiled	0.392	0.461	0.426
Steam-cooked	0.392	0.461	0.426
	Valine (g/100 g chick-pea)		
Raw	0.476	0.505	0.426
Roasted	0.402	0.413	0.406
Boiled	0.439	0.402	0.413
Steam-cooked	0.439	0.402	0.413
	Leucines (g/100 g chick-pea)		
Raw	1.003	1.204	0.835
Roasted	0.904	1.125	0.759
Boiled	0.954	1.110	0.775
Steam-cooked	0.954	1.183	0.779

[a]Each value is the mean of 4 determinations.

[b]Enzyme hydrolysis of all samples included digestion with pepsin for 3 hours prior to pancreatic digestion for varying periods of time.

digestion expressed as a percentage of the optimum amount of that amino acid obtained from acid hydrolysis which was 6 hours for lysine, 48 hours for threonine and valine, and 24 hours for the leucines. Analysis of variance showed a highly significant difference between raw and cooked chick-peas ($p \leq 0.01$) in the case of lysine and valine. It is evident that the release of certain amino acids were lowered after 48 hours enzymatic digestion in raw, roasted, boiled and steam-cooked chick-peas (Table 5). This indicates that the amino acids are affected differently by the method of cooking employed.

The factors responsible for the lower percent release obtained in the case of 48 hour enzyme hydrolysis were, either conversion of the amino acids to compounds that were removed when the solutions were deproteinized with picric acid, or due to the interference of proteolysis caused by accumulation of the end products which depressed the substrate-enzyme interaction. The release of individual amino acids varied substantially with different periods of hydrolysis (Appendix B, Table 3). Those peptide linkages which are most susceptible to the action of pepsin and pancreatic enzymes were broken, thus releasing certain amino acids more rapidly than others.

The values obtained from enzyme digestion were much lower when compared to the levels present in acid hydrolysates.

This could be due to the fact that the substrate contains materials other than proteins which interfere with the proteolytic action of the enzymes. Also, certain peptide bonds which are broken by the high temperature and concentration of the HCl used in acid hydrolysis are not cleaved to the optimum extent by the enzymes in pepsin and pancreatin. Low yields suggest that incomplete hydrolysis by enzymes and/or non-specific degradation may have occurred.

The action of pepsin is non-specific in cleaving peptide bonds. The bonds most susceptible to pepsin involve the aromatic amino acids tyrosine, phenylalanine, tryptophane, and leucine. The linkages involved with both the carboxyl and amino groups of these amino acids can be hydrolyzed by pepsin (77). However, in the present study pepsin digestion alone could not be used because of inadequate separation of the amino acids in paper chromatography.

Pancreatin normally consists of the enzymes trypsin, chymotrypsin, elastase, and carboxypeptidases A and B. These enzymes cleave specific peptide linkages more readily than others. Trypsin hydrolyzes peptide bonds at the carboxyl end of lysine. Chymotrypsin attacks the carboxyl groups of aromatic amino acids and leucine. It also cleaves bonds of several other amino acids including lysine and threonine. Carboxypeptidase A hydrolyzes the carboxy-terminal lysine. These enzymes would thus be

TABLE 5

PERCENTAGE RELEASE OF THE AMINO ACIDS[a] FROM RAW AND COOKED CHICK-PEAS AFTER DIFFERENT PERIODS OF ENZYME HYDROLYSIS

Treatment of Sample	Duration of Hydrolysis		
	6 hours	24 hours	48 hours
	Lysine (%)		
Raw	29.46	31.12	57.05
Roasted	20.74	21.74	46.26
Boiled	20.74	24.89	47.05
Steam-cooked	23.86	26.97	48.76
	Threonine (%)		
Raw	18.69	24.21	23.21
Roasted	15.43	18.24	18.69
Boiled	17.78	19.91	18.69
Steam-cooked	17.78	20.86	19.28
	Valine (%)		
Raw	29.26	31.04	26.24
Roasted	24.71	25.38	24.95
Boiled	26.98	25.26	24.95
Steam-cooked	26.98	24.71	25.45
	Leucines (%)		
Raw	41.79	50.17	34.79
Roasted	37.67	46.92	31.63
Boiled	39.75	46.25	32.29
Steam-cooked	39.75	49.29	32.46

[a]The amino acids released after varying periods of enzyme hydrolysis are expressed as a percentage of the amino acid levels established after acid hydrolysis.

able to attack the residues of peptides formed by the action of trypsin and chymotrypsin, and liberate free amino acids. Since pancreatin does not contain amino-peptidases, dipeptidases, or enzymes cleaving specific linkages such as prolidase present in the intestinal mucosa, a 100% yield of free amino acids would not be obtained upon digestion of a protein with pepsin and pancreatin.

Since several structural features of the native protein determine the nature of the hydrolytic product, it is not possible to predict the mechanism of proteolysis occurring in a particular system. The folding of the polypeptide chains can allow a potentially susceptible bond to resist cleavage by restricting the availability of the groups that can react with the enzyme. The configuration that makes certain bonds readily available at the surface of the protein enables hydrolysis by enzymes of the appropriate specificity.

Geiger et al. (78) attempted the isolation of peptides containing proline and threonine from enzymatic hydrolysates of zein. The end products of in vitro pepsin and trypsin digestion were separated by 2-dimensional paper chromatography. The chromatograms revealed the absence of proline and threonine, but there were three unidentified spots. Subsequent acid hydrolysis of the enzyme digest showed the presence of proline and threonine instead of the unidentified spots on the chromatogram. The investigators

concluded that threonine and proline were combined in a peptide linkage not readily hydrolyzed by pancreatic enzymes. Thus, there are differences in the extent to which peptides are broken down to yield the amino acids, by acid and enzyme hydrolyses.

Reports of several workers (44, 53) indicate that the nutritive value of proteins calculated on the basis of the release of amino acids after enzymatic hydrolysis of the protein is comparable to the nutritive value based on biological assays. One of the purposes of this study was to determine if the in vitro release of amino acids from chick-peas could be related to the growth promoting properties of the protein. Hankes et al. (50) reported that 77% of the total amino acids in raw casein was released after digestion with pepsin for 2 hours, pancreatin for 2 hours, and erepsin for 5 days. Denton and Elvehjem (79) found that the rate and extent of amino acids released by enzymatic hydrolysis of casein, beef, and zein did not differ greatly when digestion with pepsin, pancreatin, and duodenal powders were used. However, they did not employ the enzymes for complete proteolysis. The lower protein quality of zein as compared to beef and casein was not reflected in the patterns of amino acids released, thereby suggesting that enzymatic hydrolysis could not be used to predict protein efficiency. In the present investigation the maximum period of hydrolysis employed was for 48 hours.

The amount of lysine released was linear to the duration of hydrolysis. Further digestion with other proteolytic enzymes may have yielded higher amounts. Hence, the use of partial enzymatic hydrolysis to determine nutritive value of proteins, and the amino acids ultimately available to the intact animal, has certain limitations.

Protein Efficiency Ratio

The grams of weight gained per gram of protein consumed on diets supplying 10% protein from casein, and from raw, roasted, boiled, and steam cooked chick-peas were estimated in weanling rats for a 4 week experimental period.

Results of the animal experiment showing the PER on different diets and the mean weight gain of each group of animals for the 4 week period are presented in Table 6. The maximum PER obtained was on the casein diet, followed by the performance on steam-cooked, boiled, raw, and roasted chick-peas in the order mentioned. The differences in PER due to the method used to cook the legume was highly significant ($p \leq 0.01$). Findings of other investigators regarding the effect of cooking on the PER of chick-peas are conflicting. Hirve and Magar (80) reported that autoclaving the legume for 15 minutes at 15 p.s.i. improved the growth promoting property significantly as compared to the raw legume. This was attributed to the destruction of a trypsin inhibitor which has been identified

TABLE 6

MEAN WEIGHT GAINS AND PROTEIN EFFICIENCY RATIO OF CASEIN AND CHICK-PEA DIETS FED TO WEANLING RATS

Diet	Type of Protein	Mean Weight Gain[a] at 4 Weeks (grams)	PER
I	Casein	92	2.75
II	Raw chick-pea	75	2.21
III	Roasted chick-pea	60	1.87
IV	Boiled chick-pea	81[b]	2.42
V	Steam-cooked chick-pea	87[c]	2.66

[a]Each value is the mean of six animals unless otherwise indicated.

[b]Mean value of 5 animals.

[c]Mean value of 4 animals.

by Borchers *et al*. (27). The data from the present investigation show that cooking in the presence of moisture improves the PER of chick-peas. This may be due to the inactivation of the trypsin inhibitor present which would prevent its interference with enzymatic digestion *in vivo*. Although other workers (80) found that autoclaving without moisture had a beneficial effect, results of this study revealed that cooking by dry heat as in roasting lowered the PER significantly, suggesting that the beneficial effect of destroying the trypsin inhibitor was overcome by the destructive effect on the essential amino acids present. In contrast, Jaffe (21) found that cooking did not improve the biological value of chick-peas above that of the raw legume and concluded that the trypsin inhibitor present did not interfere with the enzymatic digestion considerably.

Studies on other legumes have shown that cooking does not improve the protein efficiency of cow-peas, and English peas (81). Weight losses were evident in rats on diets of raw lima beans and pinto beans. Feeding the same legumes after cooking led to significant increases in weight. Another investigator reported loss of weight and death within 4 weeks in animals fed uncooked kidney beans, navy beans, or pinto beans at a 10% protein level (82). Increasing the protein level to 15% caused earlier death.

The biological value of all the legumes except English peas improved upon heating at 15 p.s.i. for 45 minutes.

Thus, it is apparent that a wide range in growth promoting properties exists among the vegetable protein foods, and the effect of cooking differs according to the chemical constituents of individual legumes. In chick-peas dry heat as in roasting appeared to have a deleterious effect on the PER when compared to the legume in the raw state or when cooked in the presence of moisture.

General Discussion

Review of the results from the *in vitro* hydrolysis of chick-pea protein and the animal experiment to illustrate the protein efficiency ratio of the legume shows that the cooking treatment which caused maximum impairment in the release of lysine was reflected in the significantly lower weight gains in rats when fed a diet of roasted chick-peas. The PER data in Table 6 show that steam-cooking under pressure leads to maximum conservation of the nutritive value. The PER on chick-peas boiled for 45 minutes was lower than that of the shorter cooking period. The quantities of lysine, threonine, and valine released *in vitro* were significantly lowered after all three methods of cooking compared to the raw sample; the PER was higher in steam-cooked and boiled samples than in the raw. This suggests the presence of an interfering factor in raw chick-peas that influences the performance of intact

animals when the protein is consumed. The significantly lower PER found on the roasted chick-pea diet corresponds to the lower release of lysine *in vitro*. The higher *in vitro* release of amino acids on raw chick-peas was not accompanied by higher PER when fed to rats. Hence, this can be attributed partly to changes in the essential amino acid content.

That there is a destruction of amino acids when protein is heated with carbohydrates has been shown conclusively by many investigators (34, 35, 83). The changes in proteins were found to be accompanied by a color change and was termed non-enzymatic browning, or the Maillard reaction. Experiments with proteins that underwent this reaction showed a destruction of the amino acids or the development of enzyme resistant linkages. Schroeder *et al*. (34) explained that browning was independent of the Maillard reaction. Browning was only a color change when proteins and carbohydrates were heated together, whereas Maillard reaction was accompanied by a loss of amino nitrogen as revealed by low color intensity of spots in paper chromatography. Results of animal experiments have shown that lysine is particularly susceptible to destruction. The data from this study support these observations.

Various attempts have been made to study the exact chemical steps involved in the Maillard reaction. Baldwin (35) found that the change in free amino groups

was reflected in the ability of the protein to bind the orange G dye. The protein lost free amino groups rapidly during the first few minutes of heating, then reached a plateau. The dye binding curve corresponded well with the biological value curve from animal experiments and the microbiologic estimation curve of lysine. According to Lea (83), in the beginning of the reaction free amino groups combined with the carbohydrates. Prolonged heating tended to bind the carbohydrates by condensation around already bound molecules. The rate of combination of sugars with the side chains of lysine was found to be maximum at the beginning of heating, gradually involving arginine, histidine, tyrosine, and methionine. It was possible to regenerate the tyrosine,methionine, and part of the lysine, but the remaining lysine, arginine, and histidine were completely destroyed.

Browning reaction was found to be high in an alkaline medium as a result of the formation of hydrolxymethyl-furfural (36). Long periods of heating even at low temperatures were more destructive when estimated microbiologically although small amounts of hydroxymethylfurfural and browning were present. This pointed to the conclusion that browning and Maillard reaction often occurred simultaneously. Frankel-Conrat (84) found that formaldehyde reacted first with the free amino acids of protein to form amino-hydroxymethylfurfural groups. These reacted with other reactive groups in peptide chains

and protein molecules forming cross linkages or methylene bridges, thereby increasing the molecular weight and decreasing solubility.

The reaction that occurred in cooked chick-peas may have been similar to those reported to have occurred in soybeans and cake mixes (37, 85). There is the possibility that the reducing sugars present in the carbohydrate part of the chick-pea formed enzyme-resistant linkages with the free amino groups, thus preventing enzymatic digestion, and absorption in vivo. The decreased absorption of the essential amino acids would therefore be reflected in a lower tissue synthesis in the intact animal.

The destructive effects of heating appeared to be drastic in the case of lysine since significant differences were found in the enzymatic release of this amino acid from cooked chick-peas. Threonine, valine, and the leucines were affected to varying degrees. Since precautions were taken to prevent loss of nutrients by mechanical means such as discarding the cooking water or any part of the sample, the change in the amino acid content can be attributed to be solely due to the temperature, duration of treatment, and the medium used in each of the cooking methods.

CHAPTER V

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS FOR FURTHER RESEARCH

Summary

A combination of *in vitro* techniques of acid and enzyme hydrolyses and a biological experiment with weanling rats was used to study the nutritive value of proteins present in the legume *Cicer arietinum*. Since proteins undergo physical and chemical changes when subjected to heat and moisture, the effects of commonly used methods of cooking were investigated. In the case of legumes such as the chick-pea, the changes in proteins are further complicated because of the possible interference of enzymatic digestion by the presence of naturally occurring, heat-sensitive enzyme inhibitors.

The lysine, threonine, valine and leucines contained in chick-peas were identified by 1-dimensional, descending, paper chromatography using n-butanol, acetic acid, and water as the solvent. The amino acids were identified after resolution, by development of color using a freshly-mixed reagent of cupric nitrate and ninhydrin. Color development in the chromatograms for quantitative estimation was by ninhydrin in butanol. The quantities of lysine, threonine, valine, and the leucines present in the

acid and enzyme hydrolysates of chick-peas were determined by the colorimetric method.

Results showed that optimum release of lysine was obtained after 6 hour acid hydrolysis from raw as well as cooked chick-peas. Hydrolysis for 48 hours was necessary for maximum release of threonine and valine, and 24 hours for the release of the leucines.

Enzymatic hydrolysis for 3 hours with pepsin followed by varying periods of pancreatic digestion showed optimum release of lysine after 48 hours, and of the leucines after 24 hours in all samples of the legume. Maximum amounts of threonine from raw and boiled chick-peas were released after 24 hour digestion, and from roasted samples after 48 hour digestion. The valine in roasted and raw chick-peas was released after 24 hours, whereas in the boiled and steam-cooked samples only 6 hour digestion was sufficient for optimum release.

When diets furnishing the raw, and cooked chick-peas were fed to groups of weanling rats at a 10% protein level, the protein efficiency ratio of steam-cooked chick-peas proved to be the maximum, and of the roasted samples the minimum, while that of the raw and boiled samples fell between these.

Other studies on the effects of heat on the nutritive value of proteins point to the fact that the amino-sugar reaction is more pronounced in the presence of carbohydrates,

leading to changes in the availability of amino acids (34, 35, 36). Chick-peas have been shown to contain soluble carbohydrates as well as starches (15, 16). Therefore, it is reasonable to assume that browning and Maillard reactions can occur in roasted chick-peas, preventing complete availability of the amino acids when fed to the intact animal. The formation of enzyme resistant linkages prevents *in vitro* as well as *in vivo* release of the amino acids upon enzymatic digestion. This is not evident in acid hydrolysates since the temperature and concentration of the acid used are sufficient to break down such linkages.

Conclusions

Examination of the data from *in vitro* enzymatic release of the amino acids and from the weight gains of weanling rats leads to the following conclusions:

1. Hydrolysis with acid for varying periods of time is necessary to establish the optimum amounts of individual amino acids present in chick-peas.

2. The quantities of amino acids released from chick-peas by digestion with pepsin and pancreatin varies with the period of hydrolysis.

3. The amino acids released by *in vitro* enzyme digestion were maximum in raw chick-peas, whereas the PER was maximum on steam-cooked chick-peas. The *in vitro* percentage release of lysine and valine was significantly

lowered by all methods of cooking, whereas the lowest PER was obtained on roasted chick-peas. Therefore, use of the *in vitro* enzymatic method to predict the nutritive quality of chick-peas does not appear to be effective.

4. The enzymes in pepsin and pancreatin do not yield complete hydrolysis of chick-pea protein. Certain peptides need specific enzymes for their cleavage and the subsequent release of the amino acids.

5. The short period of heating under pressure employed in the case of steam-cooking led to optimum efficiency of the protein in *Cicer arietinum* when fed to growing rats, implying that the household pressure cooker method may be the most beneficial for conservation of nutritive value of the legume.

6. Dry heat leads to a decrease in the nutritive value of chick-peas indicating that the destruction or inactivation of heat sensitive amino acids such as lysine may be responsible for the change.

Recommendations for Further Research

The results obtained from this investigation indicated that further research should be done to completely characterize the amino acid availability in chick-peas.

The *in vitro* release of the other essential as well as non-essential amino acids in chick-peas could be studied using a method that would yield separation of these amino

acids. Since several techniques are available for the identification and quantitative estimation of amino acids, additional information concerning the amino acid composition of chick-peas might be obtained using microbiologic assay, column chromatography, or the automatic amino acid analyzer. Results obtained by other workers have indicated differences in the composition of chick-peas with regard to number as well as quantities of the amino acids. This was attributed partly to be due to differences in the chemical constituents of the sample itself. Hence, the amino acid content of chick-peas should be analyzed using different methods on the same sample in order to compare the results.

Animal experiments to investigate the in vivo availability of individual amino acids should be done. Since evaluation of food proteins by the PER yields information only on the overall quality of the protein, further research should attempt to investigate whether the biological availability of individual amino acids for tissue synthesis are affected differently with the methods of cooking used.

Since legumes vary in their biological value, the supplementary value of chick-peas with other sources of proteins should be evaluated. Proteins in the Indian diet are mainly contributed from a wide variety of legumes, cereals, nuts and oilseeds, and dairy products. The biological value of combinations of these foods and chick-peas in proportions representative of the Indian diet should be

investigated. Biological experiments should also be done with diets containing different levels of chick-pea protein.

Fortification of the chick-pea with its limiting amino acids in the synthetic form, and the effects of subsequent storage and cooking methods on the *in vitro* availability of essential amino acids as well as the protein quality should be evaluated. Since storage conditions and household cooking procedures vary considerably among population groups, there is a possibility that these would have an effect on the conservation of nutrients. Surveys need to be conducted to gain background knowledge and information regarding the actual cooking procedures used in different geographical regions of India.

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APPENDIX A

DATA FROM THE AMINO ACID DETERMINATIONS AFTER ACID AND ENZYME HYDROLYSIS, AND THE PROTEIN EFFICIENCY RATIO

TABLE 1

AMOUNTS[a] OF LYSINE PRESENT IN RAW AND COOKED CHICK-PEAS DETERMINED[b] AFTER 6, 24, AND 48 HOURS OF ACID HYDROLYSIS[c]

Treatment of Sample	Replications				Mean
	1	2	3	4	
	Hydrolysis for 6 hours				
Raw	2.600	2.600	2.220	2.220	2.410
Roasted	2.600	2.000	2.200	2.600	2.300
Boiled	2.000	2.000	2.600	2.600	2.300
Steam-cooked	2.220	2.220	2.220	2.600	2.315
	Hydrolysis for 24 hours				
Raw	2.228	2.228	2.228	2.228	2.228
Roasted	2.228	2.228	2.228	2.040	2.181
Boiled	2.040	2.228	2.228	2.228	2.181
Steam-cooked	2.228	1.680	2.228	2.628	2.191
	Hydrolysis for 48 hours				
Raw	2.000	1.620	2.000	2.000	1.905
Roasted	2.000	2.000	1.220	2.000	1.805
Boiled	1.620	1.620	2.000	2.000	1.810
Steam-cooked	2.000	2.000	1.620	1.620	1.810

[a]Lysine values reported in g/100 g chick-pea.

[b]Based on colorimetric readings after 1-dimensional paper chromatography.

[c]Hydrolyzed with 6 N HCl.

TABLE 2

AMOUNTS[a] OF THREONINE PRESENT IN RAW AND COOKED CHICK-PEAS DETERMINED[b] AFTER 6, 24, AND 48 HOURS OF ACID HYDROLYSIS[c]

Treatment of Sample	Replications				Mean
	1	2	3	4	
	Hydrolysis for 6 hours				
Raw	1.760	1.500	1.500	1.500	1.565
Roasted	1.760	1.500	1.500	1.500	1.565
Boiled	1.500	1.500	1.760	1.300	1.515
Steam-cooked	1.300	1.760	1.500	1.500	1.515
	Hydrolysis for 24 hours				
Raw	1.760	1.500	1.500	1.760	1.630
Roasted	1.760	1.760	1.300	1.300	1.530
Boiled	1.500	1.500	1.500	1.500	1.500
Steam-cooked	1.300	1.760	1.760	1.300	1.530
	Hydrolysis for 48 hours				
Raw	2.600	2.600	1.820	1.820	1.630
Roasted	2.200	2.200	2.200	2.200	2.200
Boiled	2.200	2.200	2.200	2.200	2.200
Steam-cooked	1.820	1.820	2.600	2.600	2.210

[a]Threonine values reported in g/100 g chick-pea.

[b]Based on colorimetric readings after 1-dimensional paper chromatography.

[c]Hydrolyzed with 6 N HCl.

TABLE 3

AMOUNTS[a] OF VALINE PRESENT IN RAW AND COOKED CHICK-PEAS DETERMINED[b] AFTER 6, 24, AND 48 HOURS OF ACID HYDROLYSIS[c]

Treatment of Sample	Replications				Mean
	1	2	3	4	
	Hydrolysis for 6 hours				
Raw	0.608	1.008	1.008	1.008	0.908
Roasted	0.880	0.880	1.008	0.608	0.884
Boiled	0.880	0.880	0.880	0.608	0.812
Steam-cooked	0.880	0.880	0.880	0.880	0.880
	Hydrolysis for 24 hours				
Raw	1.240	1.240	1.300	1.300	1.270
Roasted	1.300	1.300	0.840	1.240	1.170
Boiled	0.840	1.240	1.240	1.408	1.182
Steam-cooked	1.300	1.300	1.240	1.240	1.270
	Hydrolysis for 48 hours				
Raw	1.700	1.700	1.700	1.408	1.627
Roasted	1.700	1.700	1.300	1.700	1.600
Boiled	1.808	1.300	1.700	1.300	1.527
Steam-cooked	1.700	1.700	1.300	1.700	1.600

[a]Valine values reported in g/100 g chick-pea.

[b]Based on colorimetric readings after 1-dimensional paper chromatography.

[c]Hydrolyzed with 6 N HCl.

TABLE 4

AMOUNTS OF LEUCINES[a] PRESENT IN RAW AND COOKED CHICK-PEAS DETERMINED[b] AFTER 6, 24, AND 48 HOURS OF ACID HYDROLYSIS[c]

Treatment of Sample	Replications				Mean
	1	2	3	4	
	Hydrolysis for 6 hours				
Raw	1.900	1.900	1.780	1.900	1.870
Roasted	1.900	1.900	1.780	1.780	1.840
Boiled	1.900	1.900	1.780	1.900	1.870
Steam-cooked	1.780	1.780	1.900	1.900	1.840
	Hydrolysis for 24 hours				
Raw	2.400	2.400	2.400	2.400	2.400
Roasted	2.400	2.400	2.400	2.160	2.340
Boiled	2.160	2.400	2.400	2.400	2.340
Steam-cooked	2.400	2.400	2.400	2.160	2.340
	Hydrolysis for 48 hours				
Raw	2.400	2.400	2.160	2.160	2.280
Roasted	2.400	2.160	2.160	2.160	2.220
Boiled	2.160	2.160	2.400	2.400	2.220
Steam-cooked	2.160	2.160	2.400	2.400	2.280

[a]Values reported are a combination of leucine and isoleucine in g/100 g chick-pea.

[b]Based on colorimetric readings after 1-dimensional paper chromatography.

[c]Hydrolyzed with 6 N HCl.

TABLE 5

AMOUNTS[a] OF LYSINE PRESENT IN RAW AND COOKED CHICK-PEAS DETERMINED[b] AFTER VARYING PERIODS OF ENZYMATIC HYDROLYSIS[c]

Treatment of Sample	Replications				Mean
	1	2	3	4	
	Hydrolysis for 6 hours				
Raw	0.600	0.600	0.820	0.820	0.710
Roasted	0.500	0.500	0.500	0.500	0.500
Boiled	0.350	0.350	0.650	0.650	0.500
Steam-cooked	0.650	0.650	0.650	0.350	0.575
	Hydrolysis for 24 hours				
Raw	1.050	0.650	0.650	0.650	0.750
Roasted	0.350	0.350	0.650	0.650	0.500
Boiled	0.700	0.500	0.500	0.700	0.600
Steam-cooked	0.700	0.700	0.700	0.500	0.650
	Hydrolysis for 48 hours				
Raw	1.400	1.200	1.400	1.500	1.375
Roasted	0.850	1.504	1.104	1.000	1.114
Boiled	2.050	0.850	1.020	0.614	1.133
Steam-cooked	1.700	1.000	1.000	1.000	1.175

[a]Lysine values reported in g/100 g chick-pea.

[b]Based on colorimetric readings after 1-dimensional paper chromatography.

[c]Enzyme hydrolysis of all samples included digestion with pepsin for 3 hours prior to pancreatic digestion for varying periods of time.

TABLE 6

AMOUNTS[a] OF THREONINE PRESENT IN RAW AND COOKED CHICK-PEAS DETERMINED[b] AFTER VARYING PERIODS OF ENZYMATIC HYDROLYSIS[c]

Treatment of Sample	Replications				Mean
	1	2	3	4	
	Hydrolysis for 6 hours				
Raw	0.302	0.450	0.450	0.450	0.413
Roasted	0.304	0.450	0.304	0.304	0.340
Boiled	0.450	0.450	0.220	0.450	0.392
Steam-cooked	0.450	0.450	0.450	0.220	0.392
	Hydrolysis for 24 hours				
Raw	0.650	0.420	0.420	0.650	0.535
Roasted	0.502	0.502	0.304	0.304	0.403
Boiled	0.650	0.304	0.304	0.502	0.440
Steam-cooked	0.420	0.420	0.502	0.502	0.461
	Hydrolysis for 48 hours				
Raw	0.650	0.500	0.450	0.450	0.512
Roasted	0.450	0.450	0.450	0.304	0.413
Boiled	0.302	0.450	0.450	0.450	0.413
Steam-cooked	0.302	0.450	0.450	0.502	0.426

[a]Threonine values reported in g/100 g chick-pea.

[b]Based on colorimetric readings after 1-dimensional paper chromatography.

[c]Enzyme hydrolysis of all samples included digestion with pepsin for 3 hours prior to pancreatic digestion for varying periods of time.

TABLE 7

AMOUNTS[a] OF VALINE PRESENT IN RAW AND COOKED CHICK-PEAS DETERMINED[b] AFTER VARYING PERIODS OF ENZYMATIC HYDROLYSIS[c]

Treatment of Sample	Replications				Mean
	1	2	3	4	
	Hydrolysis for 6 hours				
Raw	0.450	0.450	0.502	0.502	0.476
Roasted	0.502	0.302	0.302	0.502	0.402
Boiled	0.502	0.450	0.302	0.502	0.539
Steam-cooked	0.502	0.502	0.302	0.450	0.439
	Hydrolysis for 24 hours				
Raw	0.450	0.450	0.620	0.502	0.505
Roasted	0.304	0.450	0.450	0.450	0.413
Boiled	0.302	0.420	0.502	0.420	0.411
Steam-cooked	0.502	0.302	0.302	0.502	0.402
	Hydrolysis for 48 hours				
Raw	0.304	0.450	0.450	0.502	0.426
Roasted	0.420	0.304	0.450	0.450	0.406
Boiled	0.304	0.420	0.450	0.450	0.406
Steam-cooked	0.304	0.450	0.450	0.450	0.413

[a]Valine values reported in g/100 g chick-pea.

[b]Based on colorimetric readings after 1-dimensional paper chromatography.

[c]Enzyme hydrolysis of all samples included digestion with pepsin for 3 hours prior to pancreatic digestion for varying periods of time.

TABLE 8

AMOUNTS[a] OF LEUCINE PRESENT IN RAW AND COOKED CHICK-PEAS DETERMINED[b] AFTER VARYING PERIODS OF ENZYMATIC HYDROLYSIS[c]

Treatment of Sample	Replications				Mean
	1	2	3	4	
	Hydrolysis for 6 hours				
Raw	1.102	0.904	1.102	0.904	1.003
Roasted	0.904	0.904	0.904	0.904	0.904
Boiled	0.904	1.102	0.904	0.904	0.953
Steam-cooked	0.904	0.904	1.102	0.904	0.953
	Hydrolysis for 24 hours				
Raw	1.202	1.202	1.310	1.100	1.203
Roasted	1.100	1.100	1.202	1.100	1.125
Boiled	0.910	0.910	1.310	1.310	1.110
Steam-cooked	0.910	1.202	1.310	1.310	1.183
	Electrolysis for 48 hours				
Raw	0.810	0.810	0.910	0.810	0.835
Roasted	0.910	0.702	0.910	0.514	0.759
Boiled	0.500	0.500	1.050	1.050	0.775
Steam-cooked	0.508	0.508	1.050	1.050	0.779

[a]Values reported are a combination of leucine and isoleucine in g/100 g chick-pea.

[b]Based on colorimetric readings after 1-dimensional paper chromatography.

[c]Enzyme hydrolysis of all samples included digestion with pepsin for 3 hours prior to pancreatic digestion for varying periods of time.

TABLE 9

PERCENTAGE OF LYSINE RELEASED[a] FROM RAW AND COOKED CHICK-PEAS AFTER VARYING PERIODS OF ENZYMATIC HYDROLYSIS

Treatment of Sample	Replications				Mean
	1	2	3	4	
	Hydrolysis for 6 hours				
Raw	24.90	24.90	34.02	34.02	29.46
Roasted	20.75	20.75	20.75	20.75	20.75
Boiled	14.52	14.52	26.97	26.97	20.74
Steam-cooked	26.97	26.97	26.97	14.52	23.86
	Hydrolysis for 24 hours				
Raw	43.57	26.97	26.97	26.97	31.12
Roasted	14.52	14.52	26.97	26.97	20.74
Boiled	29.05	20.75	20.75	29.05	24.90
Steam-cooked	29.05	29.05	29.05	20.75	26.97
	Hydrolysis for 48 hours				
Raw	58.09	49.79	58.09	62.24	57.05
Roasted	35.27	62.41	45.81	41.49	46.24
Boiled	85.06	35.27	42.32	25.49	47.04
Steam-cooked	70.54	41.49	41.49	41.49	48.75

[a]Quantities of lysine released calculated as a percentage of the optimum amount contained in the acid hydrolysates.

TABLE 10

PERCENTAGE OF THREONINE RELEASED[a] FROM RAW AND COOKED CHICK-PEAS AFTER VARYING PERIODS OF ENZYMATIC HYDROLYSIS

Treatment of Sample	Replications				Mean
	1	2	3	4	
	Hydrolysis for 6 hours				
Raw	13.67	20.36	20.36	20.36	18.70
Roasted	13.76	20.36	13.76	13.76	15.41
Boiled	20.36	9.96	20.36	20.36	17.76
Steam-cooked	20.36	20.36	20.36	9.96	17.76
	Hydrolysis for 24 hours				
Raw	29.41	19.01	19.01	29.41	24.21
Roasted	22.72	22.72	13.76	13.76	18.24
Boiled	29.41	13.76	13.76	22.72	19.91
Steam-cooked	19.01	19.01	22.72	22.72	20.86
	Hydrolysis for 48 hours				
Raw	29.41	22.63	20.36	20.36	23.19
Roasted	20.36	20.36	20.36	13.76	18.71
Boiled	13.67	20.36	20.36	20.36	18.69
Steam-cooked	13.67	20.36	20.36	22.72	19.28

[a]Quantities of threonine released calculated as a percentage of the optimum amount contained in the acid hydrolysates.

TABLE 11

PERCENTAGE OF LEUCINES RELEASED[a] FROM RAW AND COOKED CHICK-PEAS AFTER VARYING PERIODS OF ENZYME HYDROLYSIS

Percentage of Sample	Replications				Mean
	1	2	3	4	
	Hydrolysis for 6 hours				
Raw	45.92	37.67	45.92	37.67	41.79
Roasted	37.67	37.67	37.67	37.67	37.67
Boiled	37.67	45.92	37.67	37.67	39.73
Steam-cooked	37.67	37.67	45.92	37.67	39.73
	Hydrolysis for 24 hours				
Raw	50.08	50.08	54.58	45.83	50.14
Roasted	45.83	45.83	50.08	45.83	46.89
Boiled	37.92	37.92	54.58	54.58	46.25
Steam-cooked	37.92	50.08	54.58	54.58	49.29
	Hydrolysis for 48 hours				
Raw	33.75	33.75	37.92	33.75	34.79
Roasted	37.92	29.25	37.92	21.42	31.63
Boiled	20.83	20.83	43.75	43.75	32.28
Steam-cooked	21.17	21.17	43.75	43.75	32.46

[a]Quantities of leucine in combination with isoleucine released calculated as a percentage of the optimum amounts contained in the acid hydrolysates.

TABLE 12

PERCENTAGE OF VALINE RELEASED[a] FROM RAW AND COOKED CHICK-PEAS AFTER VARYING PERIODS OF ENZYMATIC HYDROLYSIS

Treatment of Sample	Replicates				Mean
	1	2	3	4	
	Hydrolysis for 6 hours				
Raw	27.66	27.66	30.86	30.86	29.26
Roasted	30.86	18.57	18.57	30.86	24.72
Boiled	30.86	27.66	18.57	30.86	26.99
Steam-cooked	30.86	30.86	18.57	27.66	26.99
	Hydrolysis for 24 hours				
Raw	27.66	27.66	38.11	30.86	31.07
Roasted	18.69	27.66	27.66	27.66	25.42
Boiled	18.57	25.82	30.86	25.82	25.27
Steam-cooked	30.86	18.57	18.57	30.86	24.71
	Hydrolysis for 48 hours				
Raw	18.69	27.66	27.66	30.86	26.22
Roasted	25.82	18.69	27.66	27.66	24.96
Boiled	18.69	25.82	27.66	27.66	24.96
Steam-cooked	18.69	27.66	27.66	27.66	25.42

[a]Quantities of valine released calculated as a percentage of the optimum amounts contained in the acid hydrolysates.

TABLE 13

GRAM WEIGHT GAINS IN RATS ON BASAL AND EXPERIMENTAL DIETS AT FOUR WEEKS OF FEEDING

Diets	Replications						Mean
	1	2	3	4	5	6	
I	88	106	103	75	99	80	92.00
II	65	89	79	77	84	58	75.33
III	69	66	35	63	65	59	59.50
IV	80	(98)[a]	75	83	77	92	81.00
V	90	(107)	62	106	(96)	89	86.75

[a]( ) indicates calculated missing plot value.

TABLE 14

FOOD CONSUMPTION IN GRAMS

Diet	Replications						Mean
	1	2	3	4	5	6	
I	328	354	347	325	351	297	333.66
II	333	353	340	350	359	306	340.16
III	320	322	288	345	334	296	315.83
IV	334	(350)[a]	331	319	334	362	338.33
V	338	(370)	284	364	(365)	306	337.83

[a]( ) indicates calculated missing plot value.

APPENDIX B

ANALYSIS OF VARIANCE OF DATA OBTAINED FROM *IN VITRO* EXPERIMENTS AND THE PROTEIN EFFICIENCY RATIO

TABLE 1

ANALYSIS OF VARIANCE OF DATA FROM ACID HYDROLYSATES OF CHICK-PEAS

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square
Lysine			
Total	47	4.528	
Replications	3	.242	
Treatments	3	.061	.020
Raw vs cooked	1	.060	.060
Duration of hydrolysis	2	2.127	1.064**
Error	39	2.095	.054
Threonine			
Total	47	9.552	
Replications	3	0.062	
Treatments	3	0.026	.008
Raw vs cooked	1	.022	.022
Duration of hydrolysis	2	4.664	2.332**
Error	39	4.800	0.123
Valine			
Total	47	5.417	
Replications	3	0.032	
Treatments	3	0.064	0.021
Raw vs cooked	1	.031	.031
Duration of hydrolysis	2	4.232	2.116**
Error	39	1.089	0.028
Leucines			
Total	47	2.694	
Replications	3	0.004	
Treatments	3	0.016	0.005
Raw vs cooked	1	0.010	0.010
Duration of hydrolysis	2	2.273	1.136**
Error	39	0.401	0.010

** Highly significant difference ($p \leq 0.01$).

TABLE 2

ANALYSIS OF VARIANCE OF DATA FROM ENZYME HYDROLYSATES OF CHICK-PEAS

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	
		Lysine		
Total	47	6.639		
Replications	3	0.224		
Treatments	3	0.398	0.133	
Raw vs cooked	1		0.343	0.343
Duration of hydrolysis	2	3.884	1.942**	
Error	39	2.133	0.055	
		Threonine		
Total	47	0.502		
Replications	3	0.009		
Treatments	3	0.065	0.022	
Raw vs cooked	1		0.019	0.019
Duration of hydrolysis	2	0.049	0.024	
Error	39	0.379	0.010	
		Valine		
Total	47	0.305		
Replications	3	0.004		
Treatments	3	0.027	0.009	
Raw vs cooked	1		0.014	0.014
Duration of hydrolysis	2	0.005	0.002	
Error	39	0.269	0.007	
		Leucines		
Total	47	2.254		
Replications	3	0.318		
Treatments	3	0.049	0.016	
Raw vs cooked	1		0.038	0.038
Duration of hydrolysis	2	1.090	0.545**	
Error	39	0.797	0.020	

**Highly significant difference ($p \leq 0.01$).

TABLE 3

ANALYSIS OF VARIANCE OF PERCENTAGE RELEASE OF AMINO ACIDS BY ENZYME HYDROLYSIS

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	
		Lysine		
Total	47	11430.07		
Replications	3	386.10		
Treatments	3	684.76	228.25	
Raw vs cooked	1	589.640		589.640*
Duration of hydrolysis	2	6681.04	3340.52**	
Error	39	3677.37	94.29	
		Threonine		
Total	47	1015.80		
Replications	3	20.10		
Treatments	3	133.06	44.35	
Raw vs cooked	1	11.232		11.232
Duration of hydrolysis	2	100.52	50.26	
Error	39	762.12	19.54	
		Valine		
Total	47	1151.79		
Replications	3	131.54		
Treatments	3	569.46	189.82**	
Raw vs cooked	1	101.47		101.47**
Duration of hydrolysis	2	22.94	11.47	
Error	39	427.85	10.97	
		Leucines		
Total	47	79450.93		
Replications	3	572.73		
Treatments	3	105.58	35.19	
Raw vs cooked	1	65.313		65.313
Duration of hydrolysis	2	8153.55	4076.77	
Error	39	70619.07	1810.74	

*Significant difference ($p \leq 0.05$).
**Highly significant difference ($p \leq 0.01$).

TABLE 4

ANALYSIS OF VARIANCE OF DATA FROM THE EXPERIMENT ON PROTEIN EFFICIENCY RATIO OF CASEIN AND CHICK-PEA DIETS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square
Total	29	4.514	
Between methods	4	2.690	0.672**
Error	25	1.824	0.073

**Highly significant difference ($p \leq 0.01$).