"THE EFFECT OF PROCESSING ON AVAILABLE LYSINE AND PROTEIN QUALITY IN SUPPLEMENTED YEAST BREADS"

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We hereby recommend that the thesis prepared under our supervision by Eloisa Guerrero entitled The effect of processing on available lysine and protein quality in supplemented yeast breads be accepted as fulfilling this part of the requirements for the Degree of Master of Science.

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To my parents

and

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CHAPTER I

INTRODUCTION

In human nutrition, plant proteins have traditionally been considered less desirable than those from animals as sources of complete amino acid mixtures. Animal protein food is high priced food. Low protein quality vegetable diets have contributed to protein malnutrition. This is a major nutritional problem in the world today. Protein deficiency is continuously increasing, and affects mainly low income groups in both developed and underdeveloped societies (1).

There is a shortage of conventional protein food supplies throughout the world . Thus, there is considerable interest in developing new protein sources, and in improving the processing of such foods (2). However, time is needed to overcome the natural prejudice against drastic changes in food patterns, especially in those communities that enjoy little variety in their diets. Therefore, through gradual acceptance the new foods will come to play a significant role in the amelioration of the worldwide problem of hunger and malnutrition (3).

Another approach to solving the protein deficit is the genetic improvement of cereals, legumes and oilseeds.

Although this approach has already proved to be useful, the benefits of genetic changes must outweigh the potential reductions in crops or in their desirable agronomic characteristics (3,4,5,6,7). Digestibility is also an important factor in protein utilization. Among the factors that affect protein digestibility are types of pretreatment and heating.

Mild heating of oilseed flakes in processing is beneficial because it reduces antinutritional compounds, but severe heating is deleterious (8). This negative effect of overheating is due chiefly to a reduction in the level of lysine available to nonruminant animals (9,10). Due to the economic value of the oil, mill processing conditions are selected to maximize oil recovery at the expense of meal quality. Therefore, traditionally, after the extraction of vegetable oils, only a small portion of the oilseed meal is used as human food (9).

In the last decade the food use of oilseed meals, or protein derived from them, has expanded in the United States. This has occurred as a result of improved processing operations and development of new formula preparations for human consumption (9). These proteins have been evaluated for functional properties such as texture, acid solubility and foam stability when baking cookies,

doughnuts, cakes and breads, in which oilseed flour successfully replaced a portion of the wheat flour (11,12,13,2).

The purpose of this study was to test the protein quality of plant protein suplemented breads baked at Texas Woman's University by Eboh (14). She reported a high overall acceptability for the above breads, as a result of a sensory evaluation by a panel of Africans and non-Africans(14). Therefore, the present study tried to assess the nutritional value of those breads. The procedure consisted of the Protein Efficiency Ratio (PER) assay and laboratory analyses to determine the availability of lysine in the breads. The determination of the effect of food preparation on protein quality is important in order to modify formulations and processing technologies that could improve the nutritional value of baked products, and justify the extra cost of supplementary proteins.

CHAPTER II

LITERATURE REVIEW

Protein is an essential nutrient for humans, and its supply in the diet is necessary to maintain life (15). Unfortunately the cost of high quality protein sources is getting higher and higher everyday, and this is problematic for low income populations (15). Some solutions have been proposed to solve this problem. Among them can be mentioned the increment of traditional agricultural output along with a better efficiency of utilization of these products, e.g., nutritional upgrading of cereals (1). Also, a solution may be the utilization of protein from less conventional sources, although these good sources require increased sophistication of processing. These good sources provide increased possibilities of changes in food structures that must be controlled and evaluated in order to maintain the nutritional quality and safety of the product (16). There are many tables of chemical amino acid composition of foodstuffs, but they have a limited practical use in predicting protein quality, since the bioavailability of amino acids in food is affected by various processes (17, 18, 19, 20, 21).

Cereal protein quality has been improved by increasing

the concentration of the first and second limiting amino acids by addition of synthetic forms or proteins with higher concentration of the limiting amino acid. New genetic species high in lysine, one of the limiting amino acid in all cereals (6), are also available since 1964. However, none of these methods are free of potential nutritional or economic disadvantages, e.g., imbalance of amino acids due to excesses of other than the limiting amino acids, or undesirable agricultural characteristics of new genetic species, that can decrease viability of the crop (3, 7).

The fortification of cereals with synthetic amino acids is not as efficient as expected (22, 6, 23). Therefore, mixing proteins in order to provide a better amino acid pattern seems to be an attractive alternative. Results from experimental animal growth studies show that mixtures of protein foods can minimize the deficiency of a particular essential amino acid (EAA) in the diet, although it is important to consider possible deleterious effects due to excesses of non-limiting amino acids (24). Nevertheless, some combinations of proteins have been of better quality than any of the proteins by itself. This indicates there is a synergistic effect that seems to be non-linear (24, 25).

Blends of proteins from legumes and cereals have the advantage of being conventional sources that can be used to

improve local diets without drastically affecting food patterns (26, 27). When legumes are mixed with wheat flour, there is an increase in amino acid availability; this phenomenon may be due to interactive effects between both proteins within the digestive tract. However, it has been shown that in wheat and wheat-legume blends the first limiting amino acid has an availability relatively inferior to other EAA , so that we can not expect to get straight line improvement by supplementation of wheat and legumes (28).

The evaluation of protein quality in mixtures of foods is difficult because protein quality is not dependent on a single factor (22). Thus, it is necessary to design sophisticated animal experiments that could be sensitive enough to detect little changes in protein sources. These protein changes could be as low as 5 percent of the total protein. The supplementary effect is achieved by adding small amounts of protein which are a rich source in the deficient amino acid(s); therefore, they supply what is lacking in the deficient protein (29). According to Bressani (29), the supplementary effect of proteins in mixtures can be measured by comparing animal growth after feeding different diets with a fixed level of imcomplete protein and increasing the amount of supplementary protein,

e.g., cottonseed flour (incomplete protein) and casein (supplementary protein) . There is a level of optimal supplementation, beyond which the increase in nitrogen content can also cause a decrease in the estimated protein quality. It is also possible to use a constant level of nitrogen to study the supplementary effect of proteins, by means of a different technique such as nitrogen balance.

Complementation among proteins is another method that can improve efficiency of protein utilization. Protein complementation is a method of combining protein sources so that they will mutually balance the other's deficiency or excesses. Complementation studies can be done using growth of animals fed a diet of fixed protein level in which the percentage of protein sources is variable. There are four different types of responses (29), but not all of these produce a true complementation effect. The true complementary combination is the one that shows a synergistic effect, i.e., there is an upgrading of nutritional value, and the protein quality is better than either of the individual components. Such is the case of the corn and soybean flour combination because these two proteins have a different first limiting amino acid, and at the same time are rich sources of the other's most deficient amino acid. In some cases it is possible to obtain a

partial complementation which can be expected whenever one component is able to complement the other, but can not fully do so because its partner is also slightly deficient in the same amino acid, e.g., casein and cereals. Casein is a rich source of lysine, and cereals are a better source of methionine.

The responses described can be correlated with the concentration of the limiting amino acid, but not entirely. Therefore, it is necessary to take into account other factors such as amino acid spectrum and protein intake as well as the relative needs of the animal. Thus, protein quality can not be considered constant, and must be tested for each different system in order to determine its potential use (22).

Cereal proteins are limited in lysine (6), and it is necessary to increase the lysine content to upgrade their quality. Wheat is one of the most common cereals all over the world, and its products are the staple food in many developed and underdeveloped countries (12). However, the capability of wheat protein to suit human protein needs is not sufficient for those populations of low protein intake; therefore, wheat products require the addition of supplementary protein to enhance their protein quality, especially in areas of limited protein sources (26, 14).

Oilseeds and legumes contain higher percentages of protein than cereals, and also have higher concentrations of lysine (30, 28). In spite of this they can not fully replace cereals. However, they can be useful in order to balance the deficiencies of cereal-based diets. Different combinations of cereals and legumes, or cereals and oilseeds, can enrich the product without greatly diminishing its acceptability (31, 32).

Oilseeds are easily available in most world markets, and also have a good nutritive value. Oilseeds such as glandless cottonseed low in gossypol are available in the U.S. and could become available in other markets. Consequently, they could become sourcess of supplementary protein (33, 31, 11, 13). Thus, it seems desirable to study the nutritive value of cottonseed and wheat blends to see how effectively wheat products can be improved.

Non-enzymatic browning reactions can occur during the heating process of protein foods due to the presence of carbohydrates and/or oxidized fat. The reaction between amines and oxo- and oxycompounds (e.g., ketones and aldehydes) is called the Maillard reaction, which is very important in heat treatment of high carbohydrate systems, especially those containing sugars. The Maillard reaction is a series of complex sequential steps which are not fully

understood (34, 35). Among the factors that can affect the reactivity of these processes are the ratio of open chain sugar to ring form sugar concentration; the pH, which can decrease the decomposition of lysine and tryptophan by increasing hydrogen ion concentration; the type of amino acids in the protein, e.g., basic amino acids have a greater affinity for sugars; the moisture; presence of heavy metals; the temperature; and the concentration of carbohydrates. The interaction between sugars and amino acids decreases the nutritional value of proteins. Probably, this decrease is due to the biological unavailability of the products of such interactions, the formation of antinutritional factors and the formation of toxic compounds such as imidazoles and N-nitroso derivates. In return there is a production of color, flavor or off-flavor and antioxidant products (34)..

The baking process causes the formation of Maillard products (35) and destruction of lysine (36, 37, 38, 12, 39, 40) especially when one uses conventional versus microwave or steam baking. However, the organoleptic qualities of conventionally baked breads are generally rated much higher than microwave and slightly higher than steam-baked breads (12). Therefore, to remedy bread lysine deficiency it is not advisible to greatly modify the bread processing, but rather to fortify the bread. Nevertheless, it is mandatory

to test the availability of lysine in supplemented products before taking any action in this way (41). Tsen et al. (12) have found that lysine or soy fortification could effectively raise the PER of conventionally baked bread. Also Saab et al.(2) studied the losses of L-lysine hydrochloride during baking of fortified breads, and concluded that approximately one tenth of the added lysine was lost in spite of variation in formulation, processing or method to detect free lysine . Wolf et al.(21) considered the relative importance of food composition on free lysine and free methionine loss during elevated temperature processing, and reported that first order reaction kinetics can be applied to monitor free lysine and methionine losses. These losses were significantly influenced by protein, sugar, salt and water activity. Proteins and sugars worked as activators, while salt, and especially water activity had an inhibiting effect on the rate coefficient for these reactions.

The application of oilseed flour as supplementary protein is mainly meant to increase nutritional value through the increase in protein content; however, the interaction of supplementary protein with other components of the system can diminish the efficiency of supplementation. Consequently, it is necessary to assess

oilseed protein interactions with other reactive groups of the system under the conditions of processing.

Rhee and Rhee (39) evaluated the nutritional quality of oilseed protein heated with sugars by determining the extent of browning, in vitro protein digestibility, available lysine, total amino acids and computed PER (c-PER). It was concluded that there was no significant nutritional damage in the experimental complexes after heating them for 2 hours at 100°C. Therefore, oilseed supplemented food products could not be expected to show a substantial decrease in protein quality unless they contained or could produce significant amounts of reducing sugars during heat processing. Also, a high correlation was found between the available lysine content and c-PER. When the susceptibility of different oilseeds to damage by heat are compared the soy protein suffered greater damage than cottonseed or peanut protein with glucose complexes that were exposed to non-enzymatic browning by heat. Thus, Rhee and Rhee suggested that in order to better formulate protein enriched products, it is necessary to test the ultimate protein availability of the supplemented products, instead of merely increasing the protein concentration. Schuessler (42) assessed the nutritive value of cottonseed flour in baked products by biological value, digestibility, PER, protein

retention efficiency (PRE), liver protein utilization (LPU) and serum protein. Her data indicated that feeding products that were baked at 93° C for 18 minutes didn't have a detrimental effect on rat performance. She even suggested a trend toward slightly better nutritional index values than among rats consuming unbaked diets. However, after feeding diets heated at 163°C for 18 minutes she found a significant deterioration of the nutritional status of the rats. Martinkus (43) worked on the effect of heat treatments on cottonseed flour and L-lysine HCl supplemented cookies, and after analyzing protein, nitrogen, total amino acids, and available lysine, she found that lysine was destroyed due to the Maillard reaction, regardless of the type of heat treatment. The available lysine was proportionally decreased in the control and in the lysine supplemented cookies, and supplementation made a substantial improvement in both total and available lysine level. Marco (44) reported higher PER, PRE and LPU values for rats fed protein from cottonseed products which contained little or no sucrose, versus those fed relatively high sucrose products.

In the chemical analysis of the protein, lysine is apparently present, but for some reason is unavailable. Lysine units that have reacted with sugars are no longer nutritionally useful, and consequently the ideal chemical

method of analysis should not measure such units. Only a comparison with the results of biological tests can be used to assess the validity of the chemical analysis (45, 46). The animal experiments to determine the nutritional value of protein can take place, basically, in two different ways: (1) by measuring the growth of developing animals or (2) by measuring the nitrogen balance of the full-grown animals. Nevertheless, the ability of foods as sources of available lysine must be studied in young growing animals because they have higher protein requirements than adult animals (47).

Lysine is one of the amino acids whose bioavailability can be determined chemically (16); however, no chemical has been discovered that will react only with the epsilon amino group of lysine in a protein (17). Using 1-fluoro-2,4-dinitrobenzene (FDNB), Sanger in 1945 identified and counted in proteins and peptides the N-terminal and free amino groups forming DNP (dinitrophenyl) amino acids, which resisted digestion with acid, and could be separated because of their color in column chromatography or photometrically by absorbance (17, 48). The principle of this test is the reaction of FDNB with free epsilon amino groups in proteins forming DNP-epsilon amino lysine which is stable to acid hydrolysis. The sample is acid hydrolyzed and available lysine, i.e., the DNP-bound lysine, is

determined. Carpenter and Ellinger (49) compared the measurement of lysine in whole food by using "FDNB-reactive" lysine versus feeding animal experiments, and the results showed an encouraging correlation. However, later studies suggest that there is no such correlation when protein is heated for a long time (50).

In many countries the ratio of dietary protein to kilocalories is constant and very similar to that found in most breads; besides that the EAA in wheat flour are almost sufficient to meet the suggested level for adults excluding lysine, which is deficient by about 8mg/g N (51). Therefore, without any other consideration, wheat flour of higher lysine concentration should be an adequate source of protein for adult populations.

The fortification of breads with free amino acids such as lysine and threonine have some advantages including the simplicity of the process and a minimal effect on acceptability of the product; however, some disadvantages including cost, damage during process, lessened absorption and toxicity limit the universal adoption of this method (52, 53).

Addition of supplementary proteins which are better sources of lysine than wheat flour can result in a blend of better protein quality and quantity; however, this is more

likely to affect acceptability of bread (54, 55, 56, 14). Therefore, the formulation of new recipes in order to overcome this adverse consequence is a major step in studies of protein supplementation (2).

Eboh (14) baked wheat flour yeast bread supplemented with 15 percent of six different flours. The breads were considered by an African tasting panel, which reported that the overall acceptability of supplemented breads was almost as good as high gluten wheat bread. All breads were rated in the range of "like very well", on the Hedonic scale. However, the non-African panel ratings were significantly different from the African group scores. The overall acceptability of supplemented breads was only "like slightly", except for soybean bread; which was ranked as "like very well", as was the high gluten wheat bread that served as control.

Most of the reports on the nutritional value of supplemented breads have used male weaning rats. The techniques have been various and the results were not always in total agreement; however, it can be concluded that addition of supplementary protein such as cottonseed or soybean flour can improve weight gain, although weight gain sometimes is not related to the lysine concentration (57, 51, 30). Existing human studies are few and also subjected

to many different factors that make them difficult to judge. Nevertheless, quite often it has been claimed that rat studies can be extrapolated to humans without major modifications (29). Therefore, new protein blends can be evaluated very well by experimental animals, although it's important to keep in mind the limitations of each method.

Classically, the nutritive value of protein has been tested in animals by determination of the growth rate, e.g., the PER introduced by Osborne et al. in 1919 (58). Young rats are fed a protein and their gain in body weight is divided by the weight of protein consumed. This ratio is customarily reported by most researchers, is easy to perform and internationally accepted, but it has been criticized due to insufficient accuracy. Critics most often fault this evaluation for not considering the part of the intake lost during tissue replenishment due to decomposition of body protein. Bender and Doell (59) corrected this deficiency by introducing the Net Protein Ratio (NPR), which included a group of animals consuming a protein free diet. The decrease in weight during the first two weeks of the experiment is considered as body maintenance expense and is added to the increase in weight of the experimental groups.

There are several factors that can affect the results of the PER, decreasing its precision. Among them is the

variation in food intake, which is particularly important for poor quality proteins (58) . The method of the Association of Official Analytical Chemists (AOAC)(60) has limits; however, it is sufficiently flexible to allow the assay of materials as diverse as processed ready-to-eat cereals (with or without milk) and grain legume products (61). The uniformity of the diets consumed by the different experimental groups is of critical importance; the content of nitrogen, fat, moisture, crude fiber and ash in the diets must be identical. Thus, every food product represents a different problem in adjusting the basal percentage of the nutrients in the diet.

The PER line represents a dose-dependent reaction until it plateaus as the growth rate reaches the maximum, and then falls slowly at higher intakes. There are continuous challenges to the reliability of the PER as a predictive test for humans. Bodwell (50) reported a poor agreement between nutritional value as estimated in adult men and as estimated by various rat assays or by chemical amino acid scores. No single proposed procedure for protein assessment is uniform enough to evaluate the acceptability of new protein products. However, determination of the PER can still yield useful information as an estimate of the protein value of a given food, especially when compared to those of

other protein sources.

The nutritional value of protein has been evaluated by postprandial amino acid concentration in blood, and the response curve has been used to detect changes in availability (62). It is believed that amino acids are removed from blood proportionally to their requirement (63), and since blood serves as the medium for transport of nutrients between tissues it might also be reflecting amino acid abnormalities due to protein malnutrition.

McLaughlan (64) found the concentration of a plasma amino acid to be correlated to the concentration of that amino acid in the protein fed; however, he suggested that comparison of protein quality can not always be achieved by this method due to the difference in composition of foods, e.g., differences in percentages of fat, carbohydrates or moisture. Nevertheless, he recommended the measurement of plasma amino acid for evaluation of the processing effect on amino acid availability of a single food.

Imbalances of amino acids and dietary deficiency of proteins can be similarly reflected in blood amino acid concentration, and also may be exaggerated, due to the decrease in food consumption that is observed in these conditions. These effects could be directly determined by the blood amino acid pattern (65).

Ostrowski's report (66) summarized analyses for availability of amino acid supplements in foods and feeds, and studied the biochemical and nutritional implications of these findings. Among biochemical techniques to assess the availability of an amino acid, the blood level is one of the most appealing tools. Ostrowski showed that it is possible to find a high correlation coefficient in blood, liver and muscle amino acid concentrations. He suggested that blood lysine can be a good indicator of lysine "sufficiency" in diets, and might also be possible to draw a formula to predict amino acid availability from supplements knowing some of the main factors affecting amino acid concentration, e.g., sulfur amino acid and other EAA concentrations in the diet. In this article he considered that the most likely factors involved in supplementary amino acid utilization are the administration system, methionine concentration and food intake. At the same time he reviewed common problems and inconsistencies in blood lysine quantitation. For example, in the postprandial measurement the maximum concentration was found to be between the first and third hour after intake. However, in old animals or animals that have been treated for a long time, the lysine peak appeared before this time. The use of younger animals for lysine absorption studies could result in a more consistent response to

dietary lysine.

Despite the correlation shown by some authors (67) the use of blood lysine as an indicator of available lysine in the diet, is still very controversial. A main objection to its reliability is the incomplete knowledge of factors affecting blood lysine concentration. Part of the variability found in these studies is due to climatic and physical stress, that can be responsible for a short-term decrease in blood lysine concentration. Particular techniques for killing experimental animals after a meal can drastically decrease the lysine level in blood. There are questions without answers: Why do animals on a free protein diet show a big lysine peak after a meal? Why is it not always possible to correlate blood lysine concentration and available lysine in the diet? Ostrowski concluded that it is important to consider the limits of short term biochemical test before any conclusions are drawn (66).

CHAPTER III

PROCEDURE

The PER method described by AOAC (60) was used for the evaluation of the protein quality of various combinations of whole kernel cottonseed flour and high gluten wheat flour (studies 1 and 2), and protein supplemented yeast breads (study 3). The diet is described in table 1. The proximate analysis of whole kernel cottonseed, high gluten wheat flour, and the experimental breads is attached (Appendix). The experimental animals were male, albino Holtzman rats in the age range of 21-28 days. On arrival they were weighed and individually housed in standard screen bottom galvanized cages measuring 25 x 18 x 18 cm. For the first two to three days, they were fed a basal diet (Table 2). After the acclimation period, they were divided into different groups, each group having the same average weight, and assigned to experimental diets (68). They were supplied with water and food ad libitum. Food balance was completed at the end of each seven day period and an accumulative balance was made at 28 days. The temperature of the room was controlled at $72^{\circ}F \pm 2$. The animals had a 12 hour light/12 hour dark cycle (60). The PER was evaluated for 28 days using ANRC casein as the reference protein. One group was fed a diet

Table 1.- Diet composition for PER assay[a]

Ingredients	Percent composition
Protein source	$S = \frac{1.60 \times 100}{\%\ \text{nitrogen of sample}}$
Corn oil	$8.00 - \frac{S \times \%\ \text{ether extract}}{100}$
Salt mixture	$5.00 - \frac{S \times \%\ \text{ash}}{100}$
Vitamin mixture	1.00
Cellulose	$1.00 - \frac{S \times \%\ \text{crude fiber}}{100}$
Water	$5.00 - \frac{S \times \%\ \text{moisture}}{100}$
Carbohydrate to make	100.00

[a] Calculated according to the procedure given by the Association of Official Analytical Chemists (60).

Table 2.- Basal diet

Ingredients	Percent in diet
Casein (a)	20.0
Corn oil (b)	8.0
Cellulose	1.0
Mineral mix (c)	5.0
Water	5.0
Choline (d)	0.2
Corn starch (e)	59.8
Vitamin mix (f)	1.0
	100.0

a Animal Nutrition Research Council (ANRC) Casein:Sheffield Chemical, Lyndhurst, N.J. 07071.

b Mazola corn oil .

c American Institute of Nutrition (AIN) mineral mixture (70).

d Choline bitartrate : ICN Nutritional Biochemicals.

e U.S.Biochemical Corp.

f American Institute of Nutrition vitamin mixture (70).

containing casein and 9.06% fat as a control treatment to be compared with diets of high fat content (69).

Studies 1 and 2 were equally designed to find the PER response to feeding various combinations of cottonseed and high gluten wheat flours at a constant level (10%) of dietary protein (Table 3). Study 1 included 13 groups and study 2 included 10 of the groups in study 1, and one new group that was added in order to better define the shape of the distribution curve of PER values. The number of animals per group was 5 (Table 3). The calculation of PER was done by the following equation:

PER= Weight gained(g)/Protein consumed(g)

This was done for each animal, and a mean and standard deviation were then calculated for each treatment. The treatment PER values were adjusted on the basis of a standard PER value of 2.50 for the casein control groups (8% and 9.06% fat).

There were 70 experimental animals used for the PER evaluation of bread protein whose initial weights varied from 63-75 g. After the acclimation period, they were divided and assigned to experimental diets in seven groups of ten, each group having the same average weight (68). Three of the groups were fed diets in which the protein

Table 3. Treatment administered to each group of experimental rats in the PER assay of various combinations (%) of cottonseed flour (FFCS) and high gluten wheat flour (HGWF)[a].

Group	FFCS	HGWF	No of animals Study 1	Study 2
1	100	0	5	5
12	95	5		10
2	90	10	5	5
3	80	20	5	5
4	70	30	5	5
5	60	40	5	5
6	50	50	5	
7	40	60	5	5
8	30	70	5	5
9	20	80	5	5
10	10	90	5	
11	0	100	5	5

[a] Study 1 was conducted by Mei-Feng Kuo as part of a research assistantship assigment. Study 2 was conducted by Jeong-Sook Yoo as an individual research project.

content was supplied by yeast breads baked at Texas Woman's University (14). The three flours used in the breads were : high gluten wheat flour (HGWF), full fat whole kernel cottonseed (FFCSF) and defatted cottonseed (DFCSF) flours. Two other groups were fed diets containing a commercial yeast bread (Proteina, Mrs. Bairds Bakery, Dallas), containing cottonseed whole kernels. The commercial bread was fed at two levels of protein in the diet in order to evaluate a possible supplementary effect (Table 4). The whole breads were ground, and then dried overnight at 60° C. A sample from each dried bread was analyzed for available lysine.

A control group was fed a diet containing 9.34 % protein supplied by ANRC casein. Another group was fed a protein free diet in which corn starch replaced the casein (Table 5).

At the end of the first two weeks of the experiment the protein free group was sacrificed, and the food balance for each animal was calculated in order to determine the Protein Retention Efficiency (PRE). The algebraic difference in weight between the test group and the protein free group was calculated, and then divided by the amount of protein consumed by the test group; the value obtained was the Net Protein Ratio (NPR). The PRE was computed as: NPRx16

Table 4.- Treatment administered to each group of experimental rats in the PER study of yeast breads

Group	Protein by calculation	Protein source[b]	Protein by analysis[a]
1	0	----	0.25
2	10	Casein	9.44
3	10	HGWF Bread	10.06
4	10	DFCS Bread	9.31
5	10	FFCS Bread	8.75
6	10	PROTEINA	9.37
7	13	PROTEINA	11.94

[a] Conversion factor 6.25.

[b] HGWF = High Gluten Wheat Flour
DFCS = Defatted Cottonseed
FFCS = Full Fat Cottonseed
PROTEINA = Commercial cottonseed bread

Table 5.- Ingredients (%) in the diets

Group [a]	1	2	3	4	5	6	7
Moisture	5.00	5.00	5.00	2.29	2.80	5.00	5.00
Breads	0	0	67.61	45.23	45.91	36.68	47.69
Casein	0	10.00	0	0	0	0	0
Corn oil	8.00	8.00	4.34	4.45	2.59	6.09	5.52
Cellulose	1.00	1.00	0.57	0.57	0.66	0.53	0.38
Min.mix	5.00	5.00	2.99	3.19	3.44	3.33	2.83
Choline	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Vit.mix	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Corn starch	79.80	69.80	18.29	44.07	43.40	47.17	37.38

[a]
1= Protein free
2= Casein control
3= High Gluten Wheat Bread
4= Defatted Cottonseed Bread
5= Full Fat Cottonseed Bread
6= Commercial Bread (Proteina)
7= Commercial Bread (Proteina) (11.93 % protein).

$$PRE = \frac{\text{Weight gained(g) by a test group in first 2 wks} + \text{Mean weight loss(g) of the protein free diet group}}{\text{Protein intake (g) of a test group in the first two wks}} \times 16$$

Since there has never been general agreement in the use of the factor to find the protein content from nitrogen analyses, it was desirable to report Nitrogen Efficiency Ratio (NER) which is the quotient of the increase in weight over the nitrogen consumed during the 28 day experimental period. NER was used as a means to compare this bread study with others in the literature (71). NER was calculated as follows:

$$NER = \frac{\text{Weight gained (g)}}{\text{Nitrogen consumed(g)}}$$

The available lysine in bread was estimated by the Carpenter method as modified by Booth (72). The FDNB reactive lysine in ANRC casein was determined in a 500 mg sample, and in breads it was determined in a 2,000 mg sample. The ethanol was evaporated using a rotavapor, care being taken to avoid reflux during the first five minutes of evaporation. Epsilon DNP-lysine (Sigma Lab., St.Louis, Mo.) was used as an external standard to build up the calibration curve for lysine quantitation.

In order to correlate the availability of lysine in

breads with the results of the biological assay, a new ratio was defined as Lysine Efficiency Ratio (LER):

$$LER = \frac{\text{Weight gained (g)}}{\text{Estimated available lysine consumed (g)}}$$

The rats were anesthetized with ether and blood was removed through heart puncture. Plasma and sera obtained were kept in the freezer. Afterwards, plasma and sera were deproteinized using sulfosalicilic acid (0.2 mol/liter). Blood lysine concentration was determined using DNP derivatives (73). The following substances were added to a 20 milliliter scintillation vial:200 microliters of deproteinized serum or plasma, 5 milliliters of sodium bicarbonate (0.24 mol/ 1 of water), and 10 milliliters of absolute ethanol containing 2.50 milliliters of FDNB (Fisher Chemicals, Dallas) per liter. The reaction was allowed to proceed for 3 hours at 40°C in an incubator. Afterwards the reaction mixture was acidified with 500 microliters of formic acid, then evaporated using a rotavapor. The residues were dissolved in 1 milliliter of acetonitrile:formic acid (80:20). The chromatographic system consisted of a Waters Associates Model 6000A pump, U6K injector, and a UV-Visible variable wavelength detector (Varichrom, Varian). The wavelength used to detect

di-DNP-lysine was 360 nm. The separation was accomplished on a Spherisorb C18 column (4.6 mm id x 25 cm), with a mobile phase of 46% acetonitrile and 54% solvent A (containing 2 milliliters of pyridine, 5 milliliters of formic acid and 0.4 milliliters of isopropanol in a liter of bidistilled water).

With a flow rate of 0.8 ml/min, di-DNP-lysine eluted in 7-8 minutes. Injection size was 10 microliters. For peak identification, di-DNP-lysine (Sigma Lab., St.Louis, Mo.) was used, and for quantitation of lysine di-DNP-lysine derived from l-lysine HCl was used. The last reagent peak was considered as the internal standard. To improve the quantitation, a new internal standard was investigated and dihydroxyphenylalanine (DOPA) was found to be adequate. Nevertheless, DOPA was not used as the internal standard, because the standard curve using the last reagent peak as the internal standard was sufficient. For the statistical analyses of the studies on protein combinations omega squared for t-test was run considering 2-way analysis of variance (ANOVA). The graph was obtained by the subprogram SCATTERGRAM from the Statistical Package for the Social Sciences (SPSS) system , which prints a two-dimensional plot of data points represented by asterisks when a single case falls into a printing position. To analyze the data from

the PER studies of breads, one way ANOVA, Student-Newman-Keuls and Scheffe tests were used from the SPSS system.

CHAPTER IV

RESULTS AND DISCUSSION

The animal response observed in studies 1 and 2 indicated that both studies may be combined since the variance of groups within studies (sigma squared for factor A=0.865) was significantly greater than variance between studies (sigma squared for factor B=0.018). Therefore, it was possible to integrate the studies.

The distribution of PER values showed a peak at 95% cottonseed flour : 5% wheat flour ratio (Figure 1); however, it was not sharp enough to be considered as an independent value with respect to the other groups. Therefore, the values were computed as having a straight line relation, and the line was found to have the following equation:

$$y=0.6301 +0.0185(x)$$

In complementation studies, Bressani (29) has classified this response as type IV, in which both protein sources have a common limiting amino acid and are rich sources of the same amino acids, although to different extent. There was no combination showing a synergistic effect for a PER value. A synergistic effect was difficult to establish from these

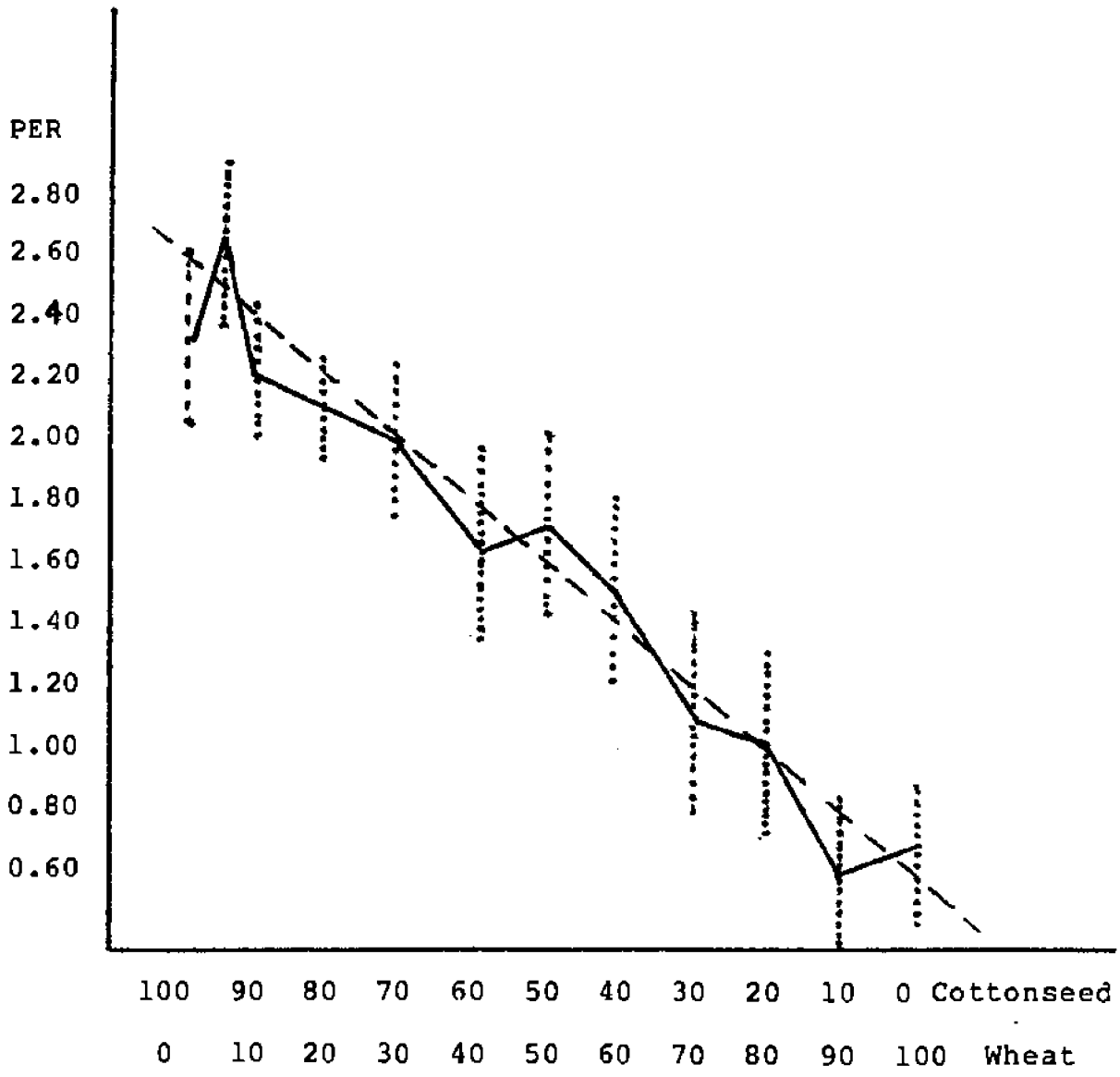

Figure 1

Distribution of PER values (combination of study 1 and study 2)

Table 6.- PER of various combinations (%) of whole kernel cottonseed flour (FFCS) and high gluten wheat flour

FFCS	No of animals	PER
100	10	2.32
95	9	2.65
90	10	2.21
80	10	2.08
70	10	2.00
60	5	1.59
50	10	1.65
40	10	1.52
30	10	1.07
20	10	0.95
10	5	0.64
0	10	0.71

data due to the standard deviation, especially at the 95% cottonseed flour level. The 95% cottonseed flour level was introduced in the second study in order to clarify the suspected curvature of the PER response line which was suspected on the basis of data at the 90 and 100% levels in study 1 (Table 6). The protein quality of wheat flour was improved from 0.71 to 2.65 for addition of 0% and 95% cottonseed flour, respectively; which was probably due to an increased concentration of lysine, as has been suggested by Jarquin et al. (74).

No significant difference in PER values was found among the bread treatments. The adjusted ratios ranged from 0.72 to 0.91 (Table 7), which is the interval that is usually reported in the literature for wheat bread protein (51). Therefore, the supplementation of wheat bread with cottonseed flour did not improve the PER of bread. Cottonseed fat content did not make any significant difference in the PER.

There is not a single publication showing that wheat proteins have PER as high as animal protein. However, increasing the lysine concentration resulted in increased PER for the flour mixtures. Therefore, the baked mixtures should have had similar increases in PER.

Table 7.- Average values of weight gain, protein consumed, PER and PRE of different bread diets

Group	Weight gain g ± S.D.	Protein consumed g ± S.D.	PER	PRE ± S.D.
2	160.30 ±18.89	39.60±4.38	2.50	69.99 ±4.48
3	33.00 ± 9.35	25.10±4.48	0.84	[c] 40.65 ±9.48
4	[c] 41.60 ±10.13	29.36±4.66	0.91	[c] 41.13 ±6.76
5	[b] 21.67 ± 5.77	20.94±2.54	0.72	[c] 41.31 ±5.48
6	[a] 29.10 ± 2.28	24.70±2.84	0.74	[a] 34.17 ±5.56
7	32.33 ± 9.31	29.42±5.91	0.65	[b] 30.60 ±4.55

PER=adjusted PER= PER x 2.50/ANRC casein PER

Group 2= Casein
3= High Gluten Wheat
4= Defatted Cottonseed
5= Full Fat Cottonseed
6= Proteina 9.37%
7= Proteina 11.93%

[a] was significantly (P<0.05) lower than [c]

[b] was significantly (P<0.01) lower than [c]

Surprisingly, this was not true in this study. There are several reasons that could explain these results:

a) Animals had a low intake of feed, which limits the efficiency of the protein (75).

b) The mixtures were exposed to heat treatments that could greatly reduce the availability of nutrients, especially lysine (76).

c) Antinutritional factors existed in cottonseed flour that could have been enhanced by heat (77).

In spite of the similarity in PER, the body weight gained was not similar. The increase in body weight was significantly ($P<0.001$) higher for the DFCSF bread in comparison with the FFCSF bread, and also higher ($P<0.05$) in comparison with the whole kernel commercial bread (Proteina). In considering the ingredients in the diet of these groups the fat source was the main variable. Although it was not possible to find the fat composition of the commercial bread, it was possible to compare the FFCSF bread and the DFCSF bread because both of them had the same known recipe. In the FFCSF diet 67.6% of the total fat was supplied by the fat in the FFCSF bread and in the DFCSF diet only 44.4% of the total fat was from the fat in the DFCSF bread. Therefore, in the FFCSF diet 23.2% of the total fat

was the cottonseed fat supplied by the FFCSF flour and that fat was removed in the DFCSF flour. Based on these data, it could be concluded that the fat in whole kernel cottonseed could have a negative effect on growth of the animal. Moreover, this negative effect could cancel the benefits of increased lysine concentration.

The PRE of the test groups were significantly ($P<0.001$) lower in comparison to the casein control (Table 7). There was not a significant difference between the PRE of HGWF, DFCSF and FFCSF breads (40.65, 41.13 and 41.31). and the group fed commercial bread (9.37% protein) had significantly ($P<0.05$) lower PRE than the rest of the breads. Therefore, the PRE did not indicate any nutritional benefit for the groups fed a cottonseed supplemented bread.

The values for available lysine obtained by the FDNB method were ranked in the same order as the estimated total lysine in the recipe (14). The ANRC casein is usually reported to have a value of 80 mg of available lysine/g of protein (66), and in this study it was found to be 90 mg/g. The FDNB reactive lysine in wheat breads has been reported to be 2.00g/ 100g of protein (20), and in this study it was reported to be 2.53g/ 100g of protein. Also, in this study the DFCSF bread was found to have 4.59g of available lysine/100g of protein,the FFCSF bread 3.73g/100g and the

commercial bread 4.07g/100g of protein. Therefore, it could be concluded that the values reported here were slightly higher than was expected. These values could be overestimating the availability of lysine. Consequently, they should only be compared among themselves, so that the overestimation should be cancelled.

The correlation between lysine content and the nutritional value of bread protein was measured as Lysine Efficiency Ratio (LER). Casein and HGWF bread showed a significantly ($P<0.001$) higher LER than any of the cottonseed breads (Table 8).

FDNB reactive lysine may not be a good measure to estimate the nutritional value of supplemented breads. Several factors could possibly interfere, e.g., the difference in lysine utilization of the different breads. There may be an overestimation of available lysine by the FDNB technique in samples containing high concentration of carbohydrates that have been exposed to high temperatures for a long time (e.g., 30 minutes). This is due to the formation of color compounds that can be spectrophotometrically read at the same wavelength as epsilon-DNP-lysine (436 nm). Therefore, there is an obvious need for techniques that would isolate the epsilon-DNP-lysine to avoid the color compounds formed

Table 8.- Average values of NER,LER and blood lysine

Group[a]	NER	LER	Blood lysine mg / 100 ml±S.D.
2	24.63	43.80	10.50±2.39
3	8.22	51.97	1.95±0.72
4	8.86	30.87	1.47±0.47
5	6.47	27.67	1.39±0.32
6	7.36	28.73	1.15±0.25
7	6.87	26.80	2.00±1.02

[a]
2- Casein control
3- High Gluten Wheat Bread
4- Defatted Cottonseed Bread
5- Full Fat Cottonseed Bread
6- Commercial Bread (Proteina)
7- Commercial Bread (Proteina) (11.93 % of protein).

during baking. The same is true for the color compounds formed during derivatization and hydrolysis of the sample which can also interfere with the spectrophotometric measurement. Among the techniques proposed can be mentioned thin layer chromatography (78) and high pressure liquid chromatography (79).

The blood lysine level in the experimental groups was significantly ($P<0.0001$) lower than in the casein control (Table 8). This could be a reflection of the deficiency of lysine in the experimental groups diet. There was not a significant difference among the bread groups in spite of the different levels of lysine in the diets. Therefore, blood lysine could not be considered as a good parameter to measure moderate differences in protein quality. Perhaps, it could not even be considered for big differences as was reported by Fujita et al.(80). To understand the significance of blood lysine requires some understanding of the correlation between regulatory metabolic changes that take place in different tissues, and protein in the diet. The response of free amino acids in blood may also be different in adult and growing rats as compared with humans (80). In deficiency states the free amino acids in blood may not reflect the amino acid pattern in the diet, but the change in muscles and tissues (80).

In conclusion,there was no significant difference between the growth of the animals fed supplemented yeast, wheat breads and those fed the control yeast, wheat bread. The content of lysine in bread was higher for supplemented yeast, wheat breads, but the PER, PRE and body weight gained were not increased proportionally to the protein supplementation. The supplementary lysine in cottonseed breads did not appear to be as efficiently utilized as in casein. Therefore, there appears to be no improvement in the protein quality as a result of supplementing wheat bread with cottonseed flours or whole kernel cottonseeds. Future research should consider the development of new recipes which would increase the availability of lysine and improve its utilization.

CHAPTER V

SUMMARY

The purpose of this study was to evaluate the protein supplementary effect of cottonseed flour (CSF). PER, PRE, FDNB reactive lysine and blood lysine were considered as criteria for this evaluation. Mixtures of whole kernel CSF and high gluten wheat flour (HGWF) were fed at 10% of protein in the PER diet. The PER values were distributed in a straight line. Mixtures of HGWF and CSF (85:15) were baked as yeast breads. PER and PRE were not significantly different among the experimental breads. However, the increase in weight of defatted cottonseed bread group was significantly ($P<0.001$) higher than full fat cottonseed bread group and significantly ($P<0.05$) higher than commercial bread group. Available lysine in the experimental bread estimated as FDNB reactive lysine was higher in CSF breads ($P<0.001$) than in HGWF bread. The blood concentration of di-DNP-lysine showed a lysine deficiency in the experimental bread groups vs casein control group. There was no correlation between the biological and the chemical tests. The supplementary lysine in cottonseed breads was not efficiently utilized.

APPENDICES

Appendix 1 .- Analysis (%) of experimental breads(%solids)

	HGWF	DFCSF	FFCSF	PROTEINA
Protein	14.29	22.11	21.78	27.26
Fat	5.42	7.85	11.79	5.21
Fiber	0.65	0.95	0.74	1.29
Ash	2.98	4.01	3.61	4.54
Nitrogen free extract	76.16	65.07	62.07	61.70

Appendix 2 .- Proximate analysis (%) of whole kernel cottonseed flour[a]

Moisture & volatiles	6.20
Ash	4.30
Oil	35.45
Protein	39.13
Crude fiber	1.48
Gossypol (free)	0.037
Gossypol (total)	0.042
Free fatty acid	0.80
Lead,ppm	1.50
Arsenic,ppm	0.10
Heavy metals,ppm	10.00
Salmonella	negative
Aflatoxin,ppb	0

[a] Analyses performed at the Food Protein Research and Development Center , Texas A & M University; and Pope Testing Laboratories Inc., Dallas.

Appendix 3 .- Proximate analysis (%) of high gluten wheat flour[a].

Moisture	10.50
Protein	13.88
Fat	0.56
Fiber	0.70
Ash	0.55
Nitrogen free extract	73.81

[a] Pope Testing Laboratories, Inc., Dallas TX

Appendix 4 .- Proximate analysis (%) of high gluten wheat bread[a].

Moisture	38.20
Protein	9.14
Fat	3.35
Fiber	0.40
Ash	1.84
Nitrogen free extract	47.07

[a] Pope Testing Laboratories , Inc., Dallas, TX.

Appendix 5 .- Proximate analysis (%) of defatted cottonseed bread [a]

Moisture	6.00
Protein	20.78
Fat	7.38
Fiber	0.90
Ash	3.77
Nitrogen free extract	61.17

[a] Pope Testing Laboratories , Inc.,Dallas,TX

Appendix 6 .- Proximate analysis (%) of full fat cottonseed bread[a]

Moisture	4.80
Protein	20.75
Fat	11.22
Fiber	0.70
Ash	3.44
Nitrogen free extract	59.09

[a] Pope Testing Laboratories ,Inc., Dallas, TX

Appendix 7.- Proximate analysis of commercial cottonseed bread (Proteina)[a]

Moisture	37.90
Protein	16.93
Fat	3.24
Fiber	0.80
Ash	2.82
Nitrogen free extract	38.31

[a] Pope Testing Laboratories , Inc., Dallas,TX

Appendix 8 .- Data from rats fed 10% casein diet

RAT	WEIGHT GAINED (g)	PROTEIN CONSUMED (g)	PER	PRE	BLOOD[a] LYSINE (mg/100ml)
1	145	35.87	4.04	68.46	11.25s
2	151	38.14	3.96	75.14	10.67s
3	175	42.67	4.10	62.01	9.30p
4	134	36.91	3.63	71.58	9.65s
5	132	32.47	4.06	73.03	11.45s
6	171	42.29	4.04	72.24	9.82s
7	173	44.93	3.85	70.51	7.71s
8	168	43.52	3.86	64.34	9.92s
9	165	---	--	---	8.70p
10	189	---	--	---	16.48p
MEAN	160.30	39.60	3.94	69.66	10.50
S.D.	18.89	4.38	0.16	4.48	2.39

[a] p = plasma, s = serum

Appendix 9.- Data from rats fed high gluten wheat protein

RAT	WEIGHT GAINED (g)	PROTEIN CONSUMED (g)	PER	PRE	BLOOD [a] LYSINE (mg/100ml)
1	37	25.25	1.45	38.90	1.84p
2	23	18.71	1.23	31.81	3.24p
3	52	25.65	2.03	60.46	1.61s
4	39	30.18	1.29	37.29	1.61p
5	28	21.03	1.33	47.87	1.08p
6	26	28.27	0.92	32.16	1.61s
7	23	19.72	1.17	40.54	1.49p
8	38	31.69	1.20	36.15	1.58p
9	26	---	--	---	2.38s
10	38	---	--	---	3.38p
MEAN	33.00	25.10	1.33	40.65	1.95
S.D.	9.35	4.48	0.32	9.48	0.72

[a] p = plasma, s = serum

Appendix 10 .- Data from rats fed defatted cottonseed protein

RAT	WEIGHT GAINED (g)	PROTEIN CONSUMED (g)	PER	PRE	BLOOD[a] LYSINE (mg/100ml)
1	31	24.21	1.28	37.02	1.25s
2	55	27.93	1.97	54.08	7.75p
3	44	38.73	1.14	35.70	1.17p
4	54	29.89	1.81	46.00	1.19s
5	39	28.95	1.35	39.20	1.96s
6	49	33.05	1.48	38.81	2.34s
7	28	26.16	1.07	36.03	8.98p
8	35	25.97	1.35	42.23	1.06s
9	31	---	--	---	1.15s
10	50	---	--	---	1.64s
MEAN	41.60	29.36	1.43	41.13	1.47
S.D.	10.13	4.66	0.31	6.76	0.47

[a] p = plasma, s = serum

Appendix 11 .- Data from rats fed full fat cottonseed protein

RAT	WEIGHT GAINED (g)	PROTEIN CONSUMED (g)	PER	PRE	BLOOD[a] LYSINE (mg/100ml)
1	23	19.60	1.17	37.51	1.44s
2	22	25.38	0.87	44.24	1.87s
3	29	18.55	1.56	49.71	0.82s
4	25	22.40	1.12	40.63	1.09p
5	15	18.38	0.82	33.50	1.13s
6	24	22.31	1.08	38.27	1.59s
7	27	19.95	1.35	45.34	1.73s
8	19	---	--	---	1.49s
9	11	---	--	---	1.41s
MEAN	21.61	20.94	1.14	41.31	1.39
S.D.	5.77	2.54	0.26	5.48	0.32

[a] p = plasma, s = serum

Appendix 12 .- Data from rats fed Proteina bread protein (9.37%)

RAT	WEIGHT GAINED (g)	PROTEIN CONSUMED (g)	PER	PRE	BLOOD[a] LYSINE (mg/100ml)
1	30	25.23	1.19	37.53	1.34s
2	26	21.76	1.19	43.98	0.87s
3	27	21.95	1.23	40.22	1.13p
4	40	26.92	1.49	37.91	1.17s
5	29	23.07	1.26	41.04	1.07s
6	25	29.08	0.86	27.29	1.51s
7	33	27.39	1.20	40.08	0.76s
8	21	22.39	0.94	30.90	1.40p
9	21	---	--	---	0.91s
10	33	---	--	---	0.91p
MEAN	29.10	24.70	1.17	34.17	1.15
S.D.	5.28	2.84	0.19	5.56	0.25

a
p =plasma, s = serum

Appendix 13 .- Data from rats fed PROTEINA (11.93%)

RAT	WEIGHT GAINED (g)	PROTEIN CONSUMED (g)	PER	PRE	BLOOD[a] LYSINE (mg/100ml)
1	30	31.28	0.96	29.18	2.28s
2	46	37.01	1.24	35.62	3.61s
3	20	18.87	1.06	23.93	1.04p
4	26	30.45	0.85	32.68	1.23s
5	24	24.84	0.97	25.28	1.05s
6	29	30.33	0.96	33.50	1.42s
7	36	33.19	1.08	34.02	3.54s
8	47	---	--	---	1.43p
9	33	---	--	---	2.38p
MEAN	32.33	29.42	1.02	30.60	2.00
S.D.	9.31	5.91	0.12	4.55	1.02

[a] p = plasma, s = serum

Appendix 14.- Recipe for experimental yeast breads

Ingredients	Quantity
High gluten wheat flour	200 g
Milk	148 g
Margarine	14 g
Sugar, granulated	12 g
Yeast, active dry	3.5 g
Salt	1/2 tsp

Variations and symbols

HGW- Control 100% High gluten wheat flour	200 g
FFCS-Full-fat cottonseed flour (15%)	30 g
High gluten wheat flour (85%)	170 g
DFCS-Defatted cottonseed flour (15%)	30 g
High gluten wheat flour (85%)	170 g

Method

Heat milk to lukewarm temperature. Add yeast and 1/2 teaspoon sugar to 1/2 of the milk. Combine remainder of milk with sugar, salt and melted margarine . Add softened yeast mixture to the other mixture. Add flour, one cup at a time. Beat well after each addition. Add last cup of the flour a little at a time until dough begins to leave the sides of the bowl. Turn dough onto lightly floured pastry board.

Knead 100 strokes avoiding heavy pressure. Place dough in an oiled bowl. Cover with a cloth and allow to rise 90 minutes in a cres-cor proofing oven with a setting of 3.5 . Shape dough into loaves. Return to proofing oven for an additional 45 minutes . Bake at 375 F for 20 minutes.

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