ABSTRACT

WHEAT AS THE SOURCE OF PROTEIN FOR HUMAN ADULTS

EFFECT OF WHEAT PROTEIN ON NITROGEN BALANCE,

UREA, AND AMINO ACID METABOLISM

by Simin D. Bolourchi-Vaghefi

Normal young men were maintained in nitrogen equilibrium and good health when they were fed a diet in which white flour provided most of the protein with the remainder coming from fruits and vegetables. This was shown by a balance study carried out with 12 normal young men who were fed a control diet for 20 days. During that time, the diets provided 12.2 g of nitrogen per day from both plant and animal sources. For the next 50 days, they were fed a diet free of animal protein with 90 - 95% of the 11.8 g of nitrogen supplied by white flour. Throughout the study, the subjects maintained their body weights by consuming extra protein-free foods. Nitrogen equilibrium was maintained throughout the control phase. For the first 10 days of the wheat diet, the subjects were in negative nitrogen balance but for the remainder of the 50-day experimental phase, the subjects retained small amounts of nitrogen. The level of physical activity increased throughout the study with the result that 3300 calories were consumed in the control phase and 3800 in the experimental phase. Although fecal nitrogen values were constant throughout the study, the wet weights of the 24/hour fecal samples increased during the first part of the experimental phase to about twice that of the control value; thereafter, the weights decreased but did

not reach the control value.

When subjects were fed a diet providing 90 to 95% of the protein intake from wheat, their blood urea levels were half what they had been in the preceding control period. During the control period, the subjects consumed a mixed diet which was isonitrogenous with that for the experimental period. Practically all the protein in the wheat diets came from commercial white flour. The reduction in blood urea occurred in both men and women. In one study involving 12 men, the low blood urea levels continued for the 50 days the wheat diets were fed. The reduction in blood urea was practically completed within the first 48 hours after the initiation of the wheat diet. It was accompanied by a slower but equally pronounced reduction in the blood non-protein nitrogen level. No alteration in protein metabolism as evidenced by urinary urea, creatinine or uric acid excretion accompanied the reduction in blood urea. The results of this study indicate that the level of blood urea may be influenced to a marked extent by the nature of the dietary protein. The reduction in blood urea level seen when normal subjects consume a wheat diet is as dramatic as the change associated with a drastic alteration in the level of dietary protein. Analysis of the diets served in both periods indicated that percentage-wise, the greatest reduction in intake during the wheat period involved threonine and lysine, but even for these, the wheat diet provided more than the daily requirement. Urinary excretion of the essential amino acids in both control and experimental periods was closely related to dietary intakes. For such amino acids as isoleucine, leucine and valine, one percent or less of the intake was excreted, while for lysine and cystine,

about 10% was excreted.

Fasting plasma free amino acid levels were within normal limits throughout the study. There was a reduction in the levels of lysine and valine during the experimental phase. The reduction in valine level occurred despite the consistancy of its intake in both the control and experimental periods.

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INTRODUCTION

In many developing countries, the existing food supplies are not adequate to meet the nutritional requirements of the people. Estimates of per capita calories and available protein made by W.H.O. (1953) indicated that in such countries the average diets are insufficient in quantity and defective in quality. According to Borgstrom (1965), the average per capita caloric intake of the world population is 2200 and only 1/10 of that is of animal protein. World population, according to Borgstrom, consumes about 85 million metric tons of protein annually. Of this 2/3 is provided by plant products (about 61 million metric tons) such as grains, beans and pulses. Cereals provide 40 million metric tons of the protein while only 24 million metric tons of the protein are supplied from animal sources. (In terms of calories, however, nine-tenths of the human protein intake is derived from plant protein.)

Browne et al.(1961) stated that people of wheat growing countries in the Near and the Middle East receive 70% of their daily caloric intake in the form of bread and other wheat products. The average Iranian peasant consumes 1500-2500 calories per day. In some parts of this country, cereal and pulses provide anywhere from 52 to 98.7% of the protein intake and for the majority of the population, cereal and pulses provide between 80 and 90% of the protein intake. (Sen Gupta and Hedayat 1966)

As a low cost more readily available food, wheat, bread and flour occupy an important place in the diet of the Iranian peasants. With world food needs increasing and the adequacy of the available food supply already a serious problem in many areas of the world (Borgstrom 1965), it would appear justifiable to give more attention to the foods that might be economically feasible. Plants may be consumed as such or converted into animal protein prior to ingestion by the human. The conversion to animal protein would serve to elevate the cost, since the process is only about 20% efficient. Borgstrom (1965) has calculated that 5-8 calories of primary plant products are required to produce one calorie of animal protein. According to Lepkovsky (1944) only 5 to 10% of the feed fed to animals is recovered as meat and only 15 to 20% as milk or eggs. Thus, in view of the crucial aspects of the world feeding problem, it would be more appropriate to consider plants per se as protein sources in the diet.

Animal studies on the biological value of bread proteins suggest that amino acids therein are not adequate for maintaining nitrogen equilibrium in adult rats and are grossly deficient for normal growth of the young. (Mitchell and Carman 1924 and 1926).

Data from numerous animal studies suggest the proteins of bread are deficient in essential amino acids (for more complete discussion of these studies see the review of the literature).

Most of the studies in the literature center on the adequacy of bread proteins for growth or maintenance of rats. Studies in which the

primary purpose was to evaluate bread alone as the source of protein for human subjects are very scarce. The closest approach to this was the study of Widdowson and McCance (1954) with German orphans. In that study, girls and boys 8 to 13 years old received 51-73 grams of protein per day for a year. Of this 8-11 grams were of animal origin; the rest came from bread. The purpose of the study was to evaluate the nutritional quality of various types of flour that were used in the baking of bread. This study showed that there were no differences in growth or health among the groups of children who received bread made of flours of various extractions (including enriched). "The addition of 500 ml of reconstituted dried whole milk per day over the period of 6 months caused no apparent improvement in the growth or the health of the children."

Since most of the previous work emphasized the inadequacy of the proteins in bread, the first approach to the improvement of bread in this laboratory was to supplement its protein. For a review of these studies please refer to Bolourchi,(1963).

In order to determine how valuable animal data are in predicting the adequacy of bread for humans, we attempted to mathematically correlate the amino acid content of bread and the requirement. The amino acid intake of adult human beings, who might receive a diet of 2500 calories of which 70% of the calories are obtained from bread, was calculated. These calculations showed that all the essential amino acids would be

provided in more than adequate amounts, regardless of the remaining 30% of caloric intake. However, even when weanling rats were fed a ration containing 90% dried bread, they would not secure an adequate intake of the essential amino acids.

On the basis of these calculations, bread made with wheat flour should not require any fortification with proteins or amino acids to maintain nitrogen equilibrium in adult human subjects.

A comparison of the requirements of the growing rat for amino acids, and those supplied by bread, indicated lysine to be the most limiting amino acid.

Chapter One

REVIEW OF LITERATURE

A. Bread as a Constituent of Human Diets

Cereal products occupy a primary position in meeting the demand of a large and ever increasing group of the world population. According to Wirths (1966) cereals provide 53% of the total protein, 15% of the fats, 70% of all carbohydrates, and 55% of the total calories of the world population. He indicated that many civilized nations meet two-thirds of their carbohydrate intake and one-third of their protein needs from cereal, while much higher figures are estimated for peoples of developing countries. The highest consumption of wheat and bread is among Eastern Europe, Near and Middle Eastern countries. The following is a list of annual per capita wheat consumption in some of these countries: Iran 110.9 kg, Israel 118.3 kg, Jordan 112.9 kg, Lebanon 150.5 kg, Turkey 155.9 kg, India 22.4 kg, Japan 25.5 kg, Thailand 1.2 kg, Burma 1.4 kg (Hand 1962). Guggenheim (1957) showed that the new settlers of Israel consume about 380 grams of bread and cereal per day.

According to Bennett (1942) the per capita consumption of bread in USSR was 123.0 kg per year. In some countries, while the per capita consumption is still high, the intake of this foodstuff is in a decline. The consumption of wheat and bread dropped from 150 kg per year per man in 1934-38 to 92 kg in 1945 in Switzerland (Rosen 1947). In Germany at the beginning of the 20th century cereal products supplied 35% of the

total calories. By 1962-63 this percentage had dropped to 26%. In that year per capita consumption in Germany was 53 kg of wheat flour, 20 kg rye flour, 5 kg of other cereals and grains supplying 25% of their protein intake (Wirths 1966).

According to Mitchell and Carman (1926), in the United States 30% of the protein (nitrogen) in the average American diet was provided by white flour. Friend and Clark (1959) report that 20% of the total protein intake of the average American came from flour and cereal, which would be approximately 70 kg/year/man. Wheat consumption in the United States was about 73 kg/year/man in 1966 (The World Food Budget 1970).

Hodges (1966) indicated that the consumption of bread and cereal and other high carbohydrate foods increases with the decrease or scarcity of the total calories. In other words, the percentage of cereal or bread in the diet has an inverse relationship with the degree of affluence of the society. Westerman et al. (1949) consider a high cereal diet, one consumed by people with "poor food habits" or with low income. They believe that these groups are most likely to include considerable quantities of cereals in their diets, either from habit or by economic necessity, since such foods represent cheaper sources of energy.

According to Parpia and Bains (1966) a major portion of the diet in India is composed of cereal grains and legumes. The per capita consumption of cereal in India averages 375 g/day (which amounts to 137 kg/

year). This level of cereal in the diet would provide 86% of the protein intake of people which for a reported intake of 2060 calories per day (World Food Budget 1970) would amount to 50.4 grams of wheat.

Sen Gupta and Hedayat (1966) give average figures of 600 to 700 grams of cereal and bread consumption in the villages of Iran. For a daily caloric intake of 1500-2500, cereal would provide 66-98% of the calories.

Tekeli (1966) states that the principal cereal used in Turkey for human consumption is wheat, which is prepared and consumed in various ways. Lapman (1966) agrees with Hodges (1966) that the nations developmental state influences cereal consumption. He emphasizes the contribution of wheat as a source of protein for countries struggling for survival, and the low fat, cholesterol inhibiting effects of diet high in wheat foods. Hegsted (1962) points out that low fat diets are automatically high cereal, high carbohydrate diets.

The U.S. Department of Agriculture in their reports on the World Food Budget, and the projection of the food budget for 1970, based on the data collected in the preceding decade, present the data from which Table 1 is taken. This table shows the percentage of caloric intake of people of the sub-regions of the world from selected food groups. These are averages for 1959-61.

Table 1. Caloric intake per capita and percent of calories derived from selected food groups.

	No. of Calories	CHO % of Cal.	Wheat % of Cal.	Other Grains % of Cal.	Animal Protein % of Cal.
United States	3,190	40	17.4	3.4	30.4
Canada	3,100	42	18.8	2.5	36.1
Oceania	3,260	43	25.2	1.9	36.5
Northern Europe	3,060	48	23.4	4.6	27.7
Southern Europe	2,720	60	40.1	6.2	12.7
Eastern Europe	3,000	66	32.1	17.5	18.5
West Asia	2,350	72	48.0	13.0	8.2
North Africa	2,210	73	26.4	34.3	9.1
South Africa	2,670	72	14.0	42.7	18.8
India	2,060	74	11.3	52.1	6.4
Japan	2,360	78	11.7	51.5	7.3
USSR	3,040	73	35.7	17.7	14.7
Communist Asia	1,790	87	12.2	62.4	2.4

B. Proteins and Amino Acids in Wheat, Flour and Bread

I. Introduction

a. Biological Value of Wheat and its Products

Osborne and Mendel (1914) showed that wheat proteins are generally of poor biological value as compared with animal proteins. The studies of Munaver and Harper (1959), Howard et al. (1958), Block and Mandl (1958) and Bender (1958) suggest that the protein content of wheat flour is inferior to animal protein for growth and maintenance of rats. There are numerous reports of animal studies on the biological value of wheat, flour and bread proteins which support the above facts. These studies have shown that lysine is the primary amino acid deficiency as far as growth of rats is concerned (Mitchell and Block 1946, Block and Weiss 1956, Hepburn et al. 1957, Kon and Markuze 1931, Mitchell and Smuts 1932).

Jonick and Kawalizyk (1965) showed in several samples of wheat and rye bread that lysine and methionine levels control the biological value of wheat while methionine and isoleucine limit that of rye. They also showed that although the protein content of rye was less than that of wheat, its biological value was higher probably because of its lysine content.

Csonka (1937) analyzed whole wheat flour and patent flour for their amino acid content. He found that cystine and tryptophane were higher

in patent flour, while tyrosine and dibasic amino acids were higher in whole wheat flour. Simmonds (1962), on the other hand, found that amino acids were parallel in every extraction that he examined. Guggenheim and Freedmann (1960) showed that the nutritional value of bread protein diets increased as the percentage of extraction of flour arose. They attributed this to an increased lysine content, since the lysine content increased per gram of nitrogen by increase in extraction rate.

b. Relative Concentration of Protein in Different Cereals

According to Jacobs (1951) the protein concentration of corn is between 8 to 11% varying among products of this grain. Corn meal is said to contain 9.1% protein. Rice contains between 6.5 to 10.5% protein. Whole oat varies in protein content from 9 to 20%. Its protein content averages between 11.5 to 14.0%. Proteins of rye comprise 9.0% of its weight. Barley has a protein content of 8-20% in different samples. Wheat and wheat products contain 8-12% protein with an average of 10.5%.

With the development of the new technique of "air classification" which separates the protein particles by an air current, yielding a higher protein flour, wheat flours with protein content of as high as 20% are obtained (Wrigley 1963).

c. Comparison of Biological Value of Wheat with other Cereals

Mitchell and Block (1946) found a biological value of 70 for whole wheat bread. They showed that soybean (heated) had a biological value of 75, rolled oats and white rice, a biological value of 66, and corn one of 60. The biological value of white flour was 52. They showed that the greater the deficiency of the amino acid in a protein, the lower the biological value would be.

Sure (1947) showed that rats gained 2.06 g per gram of protein intake from rolled oats while an only 0.80 g gain was obtained from each gram of protein from wheat flour. He found that the proteins in polished rice were three times as efficient biologically as those in white flour, when fed on the same level of protein intake. Prior to this Kon and Markuze (1931) obtained results indicating the superiority of rye bread with respect to the biological value of the protein. (0.61 g gain per gram of protein from white wheat bread and 1.14 g gain per g protein from rye bread)

In general rice proteins have a higher biological value among the cereals (with the exception of whole wheat grain and soybean) and they appear to be fairly well balanced. The most limiting amino acid in rice is lysine (Mitchell and Block 1946). Jones et al. (1948) showed that rice with a 2.22 protein utilization ratio was superior to wheat (1.72 PUR) and barley (1.55 PUR) and corn (1.42 PUR) in the biological

value of its proteins. They consider oats (2.23 PUR) similar to rice as far as biological value of protein is concerned. Rye has a higher lysine content than most of the cereals. However, its protein utilization ratio is 1.83 which is lower than that of rice and oats. Proteins of corn are deficient in lysine, cystine and tryptophane. The biological value of corn is similar to that of wheat. Barley also resembles wheat in its biological value (Jones et al.1948). Lamb et al. (1966) showed that sorghum proteins were nutritionally inferior to wheat proteins in rat growth and reproduction assays.

d. Major Amino Acid Deficiencies

Wheat, flour and bread are shown to be deficient in lysine. When lysine was added to the wheat diet, there was a large increase in the growth promoting value of wheat (Mitchell and Smuts 1932, Hutchinson et al. 1958, Rosenberg and Rohdenburg 1952, Jahnke and Schuck 1957, Flodin 1956).

While lysine is the most limiting amino acid in wheat and wheat products, additional amino acids have been shown to be limiting. The addition of threonine along with lysine further increased the quality of wheat protein when the level of protein in the rat ration was 9.5% or less. Above this level, the addition of threonine had no effect (Desphande et al. 1957, Sure 1954, Rosenberg et al. 1954). According to the studies of Sure (1952, 1954) the most deficient amino acid in

whole wheat is lysine, followed by valine and threonine. He showed that in milled wheat flour, however, the sequence of most limiting amino acids varies as follows: lysine, threonine, and valine (Sure 1953 and 1955). Bender (1957 and 1958) showed that when a diet of bread fed to the rats for 10 days at 1.5% nitrogen level, the first and second limiting amino acids were lysine and threonine, but methionine proved to be the third limiting amino acid. There is general agreement between investigators that the most limiting amino acid in wheat and wheat products is lysine. However, depending on the type of product; whole grain, wheat flour, or bread, the order of the second and the third limiting amino acid will vary between valine, methionine and threonine.

e. Losses of Lysine Due to Baking

Horn et al. (1958) accounted quantitatively for 11 amino acids of whole wheat, when the extraction products; flour, bran, and shorts were analyzed microbiologically. No destruction of amino acids happened during fermentation. Losses of cystine, lysine, and methionine during baking were significant. Most of the loss occurred in the crust (browning reaction).

Jansen et al. (1964) measured the nutritional losses of added lysine during baking, by rat assays. They showed that 30% of lysine became unavailable nutritionally when the time of baking was increased

from zero to 50 minutes. At 20 minutes baking time no loss was observed at 425°F. This was confirmed by rat assay but not by microbiological assay in which 18% loss was detected at 20 minutes baking time.

McGarr et al.(1964), Ericson and Larson (1961) have shown that lysine is lost in bread baking, therefore the biological value of bread is lower than its unbaked ingredients. Hepburn et al. (1957) showed that in the process of milling and baking, lysine is the first amino acid that has an appreciable loss. Then arginine and aspartic acid are also lost to a large extent. Larson (1966) suggests that protein bound and free lysine can be made nutritionally unavailable by the browning reaction which is a reaction between the ε group of lysine and carbohydrates or other compounds containing aldehyde groups.

II. Supplementation of Bread with Protein and Amino Acids

a. Animal Studies

Many attempts have been made to improve the nutritional value of bread by different supplements. Lysine addition to wheat flour proteins was first shown by Osborn and Mendel (1914) to improve the growth of the rats. Mitchell and Smuts (1932) showed that addition of lysine to 8 and 10% wheat protein diet increased the growth of rats. Hutchinson et al. (1958), Rosenberg and Rohdenburg (1952), Jahnke and Shuck (1957) and Flodin (1956) have all confirmed the beneficial

effects of fortification of bread with amino acids and protein.

Banks et al. (1964) fed rats diets containing, as dietary protein, wheat gluten in agar gel, wheat gluten supplemented with lysine (4.7 g/100 g protein) and wheat gluten plus casein (2.1: 1) or egg albumin, and also a protein free diet as control. Nitrogen growth index for wheat gluten was 8.6, casein 20.2 and egg albumin 27.0. Supplementing wheat gluten with either lysine or casein improved the nitrogen growth index to a value of 16.7. Thus they showed that the value of wheat gluten as a dietary protein source for weight gain of rats can be improved equally by either type of supplementation. Gates and Kennedy (1964) showed that supplementation of bread with 0.25% lysine HCl or 3, 6 and 12% dried milk solids improved growth protein efficiency ratio in rats. This has been repeatedly shown by different investigators. Ehle and Jansen (1965) measured growth and PER in rats for several wheat products when supplemented with lysine and threonine. The PER was proportional to the amount of lysine supplement up to 4.7 or 5.3 grams lysine per 16 grams nitrogen, when 85% and 60% of the protein, respectively, was supplied by wheat, flour, white bread and wheat gluten. Bains et al. (1964) used lysine (0.25 grams per 10 grams protein), whole milk (7%) or non-fat dry milk (3%) to supplement the basal diet of wheat macaroni for weanling rats. They demonstrated that the nutritive value of wheat macaroni was improved by the addition of lysine, as was shown by an

increase weight gain of the rats. Milk protein increased the protein content of the product, the PER and the body weight gain of the rats.

McCollum et al.(1921) showed that milk is an effective supplement to wheat, with respect to protein, calcium and vitamin A. Fairbanks (1938) reported that addition of 6% dried milk solids to bread formula increased the nutritive value of the bread. Addition of 12% dried milk solids produced a better gain in weight and animals with longer bones, even when the rats were pair fed. The weight gains were the same when rats were fed diets containing 6% dried milk solids, but the rats receiving 12% supplement had more ash in their carcasses. Mitchell and co-workers (1943) showed that enrichment of bread with dried milk solids promoted a better growth and bone calcification than the bread that was enriched with equal quantities of calcium and thiamin present in dried milk solids. Parks et al. (1954) showed that addition of milk improves the amino acid composition of white bread. (Dried milk solids contain 7.5% lysine and 4.5% valine.) Light et al. (1943) stated that addition of 6% dried milk solids to the bread formula produces a bread which is equal to whole wheat bread in so far as growth of rat is concerned.

Commercial white bread is baked with 4% dried milk solids added to improve the baking quality.

The amino acid deficiency of bread as shown by animal bioassays has been the basis of many studies undertaken by investigators to show

the necessity of supplementation of bread for human consumption.

b. Human Studies

1. Adults

As early as 1907 Chittenden showed that nitrogen equilibrium could be maintained in human adult subjects when the protein in the diet (a mixture of plant and animal protein) was as low as 35 to 40 grams per day, the exact amount varying among individuals. He concluded that the dietary standards existing at that time were one to two times higher than the actual requirements of adults for protein depending on the source of protein used.

Almost a century ago Karl Thomas in Berlin showed that he could maintain nitrogen equilibrium when he consumed sufficient bread to provide, in his diet, 13.1 grams of nitrogen per day from a bread diet. (Sahyun 1948)

Hegsted et al.(1946) postulated that if healthy adults consume diets "relatively high" in cereal, they should remain in protein equilibrium.

Clark et al.(1963) (personal communications) showed that a woman could maintain nitrogen equilibrium consuming 242 grams of wheat flour without any supplementary amino acids, as the sole source of protein.

Hoffman and McNeil (1949) demonstrated that supplementation of wheat gluten with 4% lysine improved the nitrogen balance index for

adult human subjects suffering from protein malnutrition. However in this study it was shown that in patients with chronic protein deficiency, even unenriched gluten was capable of producing positive nitrogen balance with the intake of 35 g gluten per day. The authors of this paper expressed their surprise at the ability of "such a poor protein" to maintain nitrogen balance in adults but they attributed it to the fact that the subjects were suffering from undernutrition thus their requirements for protein were lower.

In most of the studies in which the bread proteins were supplemented with amino acids for human consumption, the emphasis has been on the "improved" nitrogen retention of adult subjects due to supplementation of bread with lysine. One of these studies is that of Rice et al. (1960). In this study bread was the primary source of the protein for adults. They reported, in an abstract, that supplementation of bread with lysine increased nitrogen retention, not emphasizing the fact that subjects were in nitrogen equilibrium when they consumed only unsupplemented bread. This bread was made with 4% dried milk solids and provided the subjects with 95% of their protein intake.

Watts et al.(1964) studied the nitrogen balance of 6 men who received the FAO amino acid reference pattern as compared to the wheat flour pattern, a diet made of amino acids with the same proportion in wheat flour. The wheat flour pattern was fed at a level so that it provided an equal amount of lysine as that in the FAO reference pattern.

The mean nitrogen retention for each diet was equal; +0.42 and + 0.41 g per day for FAO and wheat pattern respectively. Thus they concluded that the level of lysine in bread is the crucial factor in nitrogen balance studies using wheat bread as the sole source of protein for adults.

Clark et al.(1963) showed that only 2 of the 4 adult subjects consuming 4.5 grams of nitrogen 3.27 g of which came from wheat maintained nitrogen balance. The other 2 subjects remained in negative nitrogen balance even when as much as 1500 mg lysine were given, showing that individuals differ in their minimal total protein requirements. The intake of calories was constant for each subject and adequate to maintain weight.

2. Studies with infants and children

Bressani et al. (1960) fed wheat flour diets to 1 to 5 year old children to study the effects on growth of supplementation of wheat flour with amino acids. These children were just recovering from severe protein malnutrition. A 2-day adaptation period was used before each three 3-day balance period for each diet combination. The basal diet used contained wheat flour 85%, wheat gluten 7%, glycine 3%, and cornstarch 5%. Vitamin and mineral capsules were given daily. The amino acids were substituted for corn starch and when this happened the glycine was reduced so that all diets remained iso-caloric and iso-nitrogenous. These diets provided 2 grams of protein per kg per day, with a calorie intake of 80-100 calories per kg per day. When lysine

was added to the wheat diet, the nitrogen retention of the children was increased to that obtained with a milk diet. The basal diet usually had 1/3 to 1/2 the value of nitrogen retention obtained on the milk diet. They showed that the addition to the basal diet of other amino acids to bring their level up to those of the FAO pattern produced more consistent results.

Albanese et al.(1949) showed that a lysine supplemented wheat gluten diet, used as the sole source of protein supported a nutritional state in infants comparable to that afforded by an evaporated milk formula.

Krut et al. (1961) in South Africa fed a diet in which bread provided 89% of the protein and 54% of the calories to children 2-4 years old. One group received bread that was supplemented with lysine. The control group received the same bread but an isonitrogenous amount of glycine was used in place of lysine. Children receiving lysine supplemented bread showed a "better growth", ($P<0.02$). Serum albumin of all the children dropped as a result of a change to the bread diet, but the children who were receiving lysine supplemented bread had a lesser drop in their serum albumin. The magnitude of changes in the body weight of the children, although considered by the authors significant, is very little, less than 1/2 kg. There are no data on nitrogen retention or loss by the children. In this paper the beneficial effects of lysine supplementation is also emphasized;

although many of the children had to be withdrawn from both the diets since they were adversely affected by the diet. They concluded that bread of low protein content (6.4%) was unsuitable as the principal source of protein for children of 2-4 years old unless wheat of sufficiently high protein content is used.

King et al. (1963) showed that addition of lysine to the bread eaten by "undernourished" Haitian school children improved their growth and weight gain "not impressively but significantly." These children were from rural villages who received most of their protein from vegetable sources. These children were given the bread in addition to their daily meal that they received at their homes. No data on the daily total nutrient intake of children are given. The investigators of this study, however found marked weight gain in children in which supplementary bread without added lysine was given. It appears that the extra calories and protein in the form of 150 grams of bread with 9 grams of jam daily was what caused the latter weight gain in both groups of school children vs. the third school group who did not receive any bread. The authors again emphasized the "not impressive but significant" increase in weight gain in the group receiving lysine supplemented bread.

No reports could be found in the literature of a study carried out to assay the adequacy of the proteins in flour or bread prepared only with white flour, a leavening agent, salt, sugar and fat and water for

human adults. According to the calculations made in this laboratory, a diet in which 70% of 2500 calories and 95% of protein are provided by flour and bread, all the essential amino acids are available to the subjects well above the requirements suggested by Rose et al. (1955).

Similar calculations were made by Hegsted (1962).On the basis of these, he suggested that if all the calories in the diet came from 80% extraction wheat flour, the protein therein would provide all the amino acids required by both adults and growing children.

Chapter 2.

WHEAT FLOUR AS A SOURCE OF PROTEIN FOR ADULT HUMAN SUBJECTS

A. Nitrogen Balances

Estimates of the nutritional value of wheat flour and bread are based largely on animal experiments. One of the earliest of these studies was that of Osborne and Mendel (1914). This and other work with rats (Howard et al. 1958, Bender 1958) suggested that the protein in wheat is inferior to animal protein for both growth and maintenance. These studies showed that lysine is the primary amino acid deficiency in wheat (Mitchell and Block 1946, Hepburn et al. 1957) followed by threonine and then either valine or methionine (Deshpande et al. 1957, Sure 1952). On the basis of such work, many animal studies were carried out to determine the effect of fortifying bread with amino acids and/or proteins. (Osborn and Mendle 1914, McCollum et al. 1921)

The spate of reports describing the deficiencies of wheat protein almost obliterated a paper by Thomas which appeared in 1911. Therein he showed that he could maintain nitrogen equilibrium when he consumed sufficient bread to provide, in his diet, 13.1 g of nitrogen per day. Since then a number of studies with infants and children (Bressani et al. 1960, King et al. 1963) and adults (Hoffman and McNeil 1949) have demonstrated the beneficial effects of fortifying bread with proteins and amino acids. Rice et al. (1960) found that college students showed an increased nitrogen retention when lysine was added to the bread which provided 95% of their protein intake. The bread in that

study was made with 4% skim milk powder. There are indications that the unsupplemented bread maintained nitrogen balance in both normal subjects and those recovering from malnutrition. The studies of Widdowson and McCance (1954) showed that children grew at a normal rate when the diet provided 50 to 70 g of protein from bread plus 8 to 11 g from animal sources.

Since many people in Iran consume diets composed primarily of bread, it appeared desirable to determine how the biological value of this food could be improved. To this end, weanling rats were fed a ration containing 90% dried bread purchased from a bakery in Tehran. This was supplemented so as to provide an adequate intake of minerals and vitamins. The addition of 7 or 15% dried lentils or 4% skim milk powder to the bread increased the weight gains of the rats by as much as two- to three-fold.

Calculations indicated that the basal bread diet did not contain an adequate concentration of essential amino acids for the growing rat (Table 2). Similar calculations showed that a 2500 kcalorie diet high in bread would provide human adults with more than an adequate amount of all essential amino acids. This would be true if 70% of the calories came from white flour and was independent of the composition of the other 30% of the diet (Table 2). Similar calculations were made by Hegsted in 1962. On the basis of these, he suggested that if

all the calories in the diet came from 80% extraction wheat flour, the protein therein would provide all the amino acids required by both adults and growing children. However, to our knowledge, this was not experimentally tested.

No nitrogen equilibrium data could be located which involved diets with 90% or more of the protein from wheat with the other 10% secured from plant sources. For this reason, a study was undertaken to determine the effect produced by feeding young men a diet in which 90 to 95% of the protein came from bread with the remainder from other plant sources.

B. Experimental Procedures

I. Subjects

Twelve male college students, 19 to 27 years of age, free of gross signs of thyroid, chest, cardiac, neurological and muscular diseases, were chosen as subjects for the study. Besides passing the physical examination, each subject had to be within 10% of his standard weight (Society of Actuaries 1959). The Minnesota Multiphasic Personality Inventory Test was used as an aid in choosing the subjects.

II. Schedule

The study consisted of a control phase of 20 days which began on Monday, March 30, 1964. This was followed by the experimental phase of 50 days, which ended Sunday, June 7.

Twenty-four hour urine and stool samples were collected throughout the study. Blood samples (about 50 ml. on each occasion) were drawn at the beginning and end of the control phase and, at the midpoint and end of the experimental phase.

Both the control and experimental phases were divided into 5-day balance periods. Seven daily menus were used in both the control and experimental phases. This permitted a bag lunch for Sunday evenings whereby both the subjects and cook-dietitians had a little freedom. Apart from Sunday evenings, all meals were served in the diet kitchen.

III. Diets

During the control phase, the subjects received a normal-type diet which provided an average of 72 g protein (12.2 g nitrogen) per day. From 43 to 45% of the protein in those diets were of animal sources. These diets contained small amounts of milk, eggs and meat. Although the amount of protein in these rations is smaller than the 97 g intake of the average adult in the United States (Leverton 1959), it is approximately the same as that recommended by the Food and Nutrition Board of NRC (1964). The level of protein in the diets served during the control phase was designed to be isonitrogenous with that of the diets in the experimental phase.

The diets served during the experimental phase provided an average of 11.8 g of nitrogen per day. Of this, 90 to 95% was derived from

commercial wheat flour used largely in preparing bread and rolls. These were baked in the laboratory to insure that the same formula was followed throughout the study. The bread and rolls for both the control and experimental phases were made from: 4790 g white flour, 200 g vegetable shortening, 150 g sucrose, 85 g salt and 49 g dry yeast.

The diets supplied the recommended nutritive allowances with the exception of calcium. Calcium was sufficient in the control diet, however during the experimental regimen the diets supplied an average of 250 mg of calcium per day. In order to compensate for this deficiency, calcium lactate pills which supplied 650 mg of calcium were given to the subjects daily.

Throughout the study the same brands of foods were used. The butter for the experimental phase had been melted and washed free of whey. The resulting oil was reconstituted with sodium chloride and water to a consistency similar to that of butter. Kjeldahl analysis of a number of samples of the whey-free butter indicated the absence of any detectable nitrogen. For this reason, the subjects were permitted enough butter to maintain their body weights.

Throughout the study, the subjects were required to consume the "core" diet which was composed of the protein-containing foods. Other foods, such as butter, jam, tea and coffee were permitted in unrestricted amounts but the quantities consumed were recorded for each subject. Known amounts of "Instant" coffee and tea were provided at regular

intervals. The nitrogen content of the tea and coffee consumed were used in correcting the nitrogen balance figures.

IV. Sample Collections

Twenty-four hour urine samples were collected in polyethylene bottles containing toluene as a preservative. Stools were collected directly in one quart, wide-mouth cylindrical cartons which had snugly-fitting covers. Each container had some dry ice in it and additional dry ice was available at all times. A plastic carrying case was provided in which the subject could keep his urine and stool containers.

Blood was withdrawn from the antecubital vein using Vacutainers, some of which contained heparin and others nothing. On the day the blood sample was collected, the subjects reported to the laboratory where a small amount of blood was secured by a finger prick for hemoglobin and hematocrit determinations.

At every meal, an extra serving was prepared and weighed in the same way as that for the subjects. At the end of the day, the combined extra servings were weighed. When water had to be added to the latter to facilitate hemogenization, the volume thereof was recorded and the weight of the sample corrected accordingly.

V. Analytical Procedures

a. Urine

As soon as the urine samples were brought into the laboratory,

the volume, specific gravity and pH were determined. Approximately 100 ml of each sample was saved for urea nitrogen determination. Exactly one-half the urine volume was used in preparing the 5-day composite sample for that metabolic period. These composite samples were saved for determination of total nitrogen (digestion with sulfuric acid in a heating block, followed by Nesslerization) and creatinine (by the Folin picric acid method).

b. Feces

The stools for each metabolic period were weighed and placed in a covered jar containing a measured volume of 10% hydrochloric acid. As soon as the last sample was received, the entire mass was homogenized. in a blender. Approximately 50 ml was saved for the determination of nitrogen by the macro-Kjeldahl method.

c. Diets

The nitrogen content of the diet samples was determined by the macro-Kjeldahl method. Fat was extracted in the Goldfisch apparatus using diethyl ether as the solvent. The calorie content of the diets was measured by means of the Parr Bomb Calorimeter. Carbohydrates were calculated using the USDA Handbook No. 8 (1963).

C. Results and Discussion

The "core" diets, which each subject had to consume, provided comparable amounts of nitrogen in both the control and experimental phases (Table 3). The protein values for the two phases differ to a greater extent than the nitrogen values. This difference arises from the use of the factors 6.25 and 5.70 for animal and plant proteins respectively.

The increased calorie content of the experimental diets was necessitated by the increased physical activity of the subjects. The control diet initially was designed on the assumption that 3000 kcalories per day should maintain college men in weight equilibrium. During the control phase, the "core" diet had to be supplemented with extra protein-free calories to provide an average intake of 3346 kcalories. For this reason, the experimental "core" diets were designed to provide approximately 3300 kcalories per day.

During the experimental phase the subjects continued to increase their activity. To maintain body weight, extra protein-free calories raised the intake to 3835 kcalories.

At first glance, one might conclude that the very high calorie intake of the subjects resulted in a high intake of all essential nutrients. The experimental diets contained the same amount of white flour as the diet for which the calculations in Table 1 were made. That

2500 kcalorie diet with 70% of the calories from white flour required an intake of 500 g of flour per day. The diets served during the experimental phase provided 515 g of flour per day.

The diet used in the experimental phase provides no more protein than that in diets used by large numbers of people throughout much of the Middle East (Browne et al.1961). For this reason, the data collected in the present study should have some relevance in evaluating the contribution that might be made by wheat in the diets of large groups of human adults.

I. Nitrogen Balance

A week before the control phase started, the subjects restricted their intake of such foods as milk, cheese, meat and poultry. This was done since the ordinary eating habits of the subjects indicated a daily protein intake of 90 to 120 g.

During the first metabolic period of the control phase, the subjects showed an average loss of 0.23 g nitrogen per day (Table 4). The rate of loss varied slightly for the remainder of the control phase, but essentially, the subjects were in nitrogen equilibrium. This is especially true if the criteria proposed by Leverton and Steel (1962) are accepted. They stated that a daily variation of $\pm$0.5 g in the nitrogen balance was compatible with an equilibrium state.

The first two metabolic periods of the experimental phase resulted in a definite negative nitrogen balance (Table 4). The subjects lost

an average of almost 2 g of nitrogen per day during that 10-day period. This was followed by a retention of nitrogen which continued for the remainder of the study. The overall change in body nitrogen content for the experimental phase was a retention of 20.5 g of nitrogen equivalent to about 512 g of lean body tissue. The latter would be too small to detect as a change in body weight. It was so small that other losses (e.g. hair, skin, saliva, etc.) probably accounted for most of this apparent nitrogen retention (Mitchell and Edman 1962).

The data in Table 4 show that these normal young men maintained nitrogen balance when fed a diet in which white flour provided 90 to 95% of the daily protein intake. The remaining protein in the diets were of plant origin. Consequently, for normal young men, the amino acids in wheat provide adequate amounts of all essential amino acids and, when the calorie intake is adequate, these amino acids can be utilized effectively by the body.

If the experimental phase had been limited to the first two balance periods, which covered 10 days, the conclusion might be that wheat protein could not maintain nitrogen equilibrium. The negative balance during those two periods was so great as to justify the conclusion that the protein was inadequate. However, after that, the nitrogen balance became positive and remained so throughout the rest of the study.

The initial negative nitrogen balance during the experimental phase probably resulted from an inadequacy of body enzymes required for

the proper utilization of the amino acid mixture present in white flour. Validation of such a suggestion is hard to come by. Actual analyses of the experimental and control rations for the essential amino acids show some variations. However, the differences between the two sets of diets are smaller than one might have assumed (Table 5). Threonine showed the greatest difference, being present in the control diets to an extent which was 2.2 times that in the experimental diets. At the other extreme, the amount of methionine in the control diets was only 0.68 times that in the experimental diets. It seems reasonable to assume that most enzymes involved in normal metabolic reactions should be capable of a two- to three-fold range in activity. Under such circumstances, it becomes difficult to visualize how the difference in the amounts of the essential amino acids between the control and experimental diets could require drastic alterations in the enzyme patterns of the body.

The metabolic adjustment to a high wheat diet exhibited by human subjects is similar to that shown by rats. When adult rats that had been reared on a natural grain (stock) ration were transferred to a ration containing 90% of a high protein, white flour, they were in negative nitrogen balance for at least the first three days. After that they started to retain nitrogen. The rats that were transferred from the grain to a semi-purified ration containing casein as the protein, did not show this initial loss of nitrogen (Nitsan et al. 1967).

II. Body Weights

The body weights of the subjects showed some fluctuations throughout the study (Fig. 1) but for the group as a whole, there was no great difference between the initial and final weights. The differences in body weights become even less when the weight at the end of the control phase (71.3 kg) is compared with that at the end of the experimental phase (71.0 kg).

The slight reduction in body weights of the subjects during the study may reflect their improved physical condition. At the beginning of the study, each subject ran for 10 minutes, on a motor-driven treadmill at a rate of 6 miles per hour. The pulse rates for this run were lower at the end of the experimental phase than at the beginning of the control phase (Fig. 2). The improvement in physical condition represented by the lower pulse rates during and following the run on the treadmill are not attributable to the wheat diet. However, if the wheat diet could not support normal body functions, such an improvement in physical performance would not have occurred over such an extended period as 50 days.

Since physical activity varied throughout the study and consequently the intake of non-protein foods, no evaluation can be made of the caloric efficiency of the control and wheat diets. The closest approach to this is a comparison of changes in the lean body mass of individual subjects (based on changes in nitrogen metabolism) and their changes in body

weights. The control phase was treated as a unit since no prominent changes occurred at that time. The first two metabolic periods during the experimental phase were separated out since the average nitrogen balances at that time were negative. The changes in body weight were plotted against the changes in lean body mass. When this was done, the points were so randomly scattered that nothing could be concluded therefrom. The absence of any correlation between these two parameters probably stems from the fact that in most cases the changes along both axes of the figure were small. Under such circumstances, an accumulation of small errors could produce a marked deviation from a straight line relationship. The loss of nitrogen through hair, skin, nails, etc., might be a major factor responsible for an "accumulated error." Furthermore, the increased activity of the subjects as the study progressed could have resulted in body fat changes which might account for some of the apparent increases in lean body mass associated with a loss of body weight.

III. Protein Digestibility

The digestibility of the protein in the control and experimental diets was the same as evidenced by fecal nitrogen excretion. During the control phase, the fecal nitrogen excretion averaged 1.35 ± 0.23 g per day and during the experimental phase, it averaged 1.30 ± 0.17 g. After the first two metabolic periods in the control phase, the fecal

nitrogen excretion was very constant (Fig. 3). The slight increase in fecal nitrogen during the control period occurred despite the absence of any significant change in nitrogen balance values (Table 4).

An unexpected finding was the marked increase in weight of the stools during the first half of the experimental phase (Fig. 3). In the control phase, the average stool weight was 84 ± 1.5 g while in the experimental phase, it was 125 ± 6.2 ($P<0.001$). In the experimental phase, the weight of the stools increased progressively until the eighth metabolic period, when the average was 155 g. From then on, the weight of feces began to return to the values observed during the control phase.

Since the stools were analyzed only for nitrogen, it is difficult to identify the substance responsible for their increased weight. Had the increase in weight of the stools during the experimental phase been attributable to fat, the percentage thereof would have approached 70%. Such a level of fat would have changed the nature of the stools in a very obvious way. Since there was no change in the appearance of the stool samples throughout the study, it would seem that fat, per se, could not account for the increased weight. Minerals and water either together or individually could not account for the marked change in stool weight. On this basis, carbohydrate would appear to be the primary stool component that increased during the experimental phase. The carbohydrate might have come from the mucopolysaccharides in the

intestinal tract. Until this suggestion can be checked by actual analyses, it must remain in the realm of theory.

Relatively little has been done on the variation in weight or composition of stools from normal individuals. The work of Toscani and Whedon (1951) indicated a variation in stool nitrogen from 0.91 to 2.22 g per day when an intake of 90 g protein was maintained for 18-20 weeks. No satisfactory explanation was available for these fluctuations in stool nitrogens. Analyses of stools indicated the absence of glucose, galactose and lactose, with the occasional presence of some pentoses and hexoses from fruits and plants (Gryboski et al.1964). Further evidence that very little carbohydrate is normally present in feces comes from the observation that its total caloric content can be accounted for on the basis of fat and protein (Watts et al. 1963). The dry matter in each day's stool sample can be influenced by the type of diet even when the change appears to be as minor as that represented by the isocaloric substitution of a liter of whole milk in a diet devoid of dairy products. This and other studies (Wallaeger et al.1947) indicate that under normal circumstances, the wet weight of the stools parallels their dry matter content.

The initial increase in the stool weight during the experimental phase followed by a return toward normal is somewhat analogous to the changes in the fecal flora of animals whose ration is supplemented with antibiotics. The addition of antibiotics to the rations of a number of

animal species frequently produces a marked initial alteration in the microflora of the intestinal tract. If the antibiotic-supplemented ration is continued over a number of weeks, the microflora gradually returns to what it had been before antibiotic feeding was instituted (Gant et al. 1943).

IV. Urine Volume

The urine volume of the subjects was the same in both control and experimental phases. The average 24-hour volume in the control phase was 1077±24 ml, while in the experimental phase it was 1070±65 ml. Not only was there no difference in volume for the two phases of the study, but there was no trend. There were no changes in urinary pH or specific gravity throughout the study.

intake calculated from food consumption of commercial white bread plus 5% corn oil, salts and minerals.

Table 2

The extent to which wheat flour (white or patent) provides the amino acids required by adult human beings and by growing rats.

Amino Acid	Conc. in flour[1]	For adult men (g/day)		For weanling rats (g/day)	
	g/16 g N	Requirement[2]	From flour[3]	Requirement[4]	From flour[5]
Histidine	2.2	---	1.21	0.080	0.019
Isoleucine	4.2	0.70	2.31	0.090	0.030
Leucine	7.0	1.10	3.85	0.138	0.037
Lysine	1.9	0.80	1.04	0.138	0.022
Methionine + Cystine	1.5	1.10	1.86	0.090	0.015
Phenylalanine	5.5	1.10	3.02	0.138	0.035
Threonine	2.7	0.50	1.48	0.080	0.018
Tryptophan	0.8	0.25	0.44	0.045	0.062
Valine	4.1	0.80	2.25	0.115	0.029

[1] From Block and Bolling, p. 488.

[2] From Rose.

[3] The amino acid intake was calculated on the basis of a diet providing 2500 kcal per day with 70% of the calories coming from white or patent flour. The amino acids listed represent only the amounts that would be provided by the white flour in such a diet.

[4] From Albritton. The values represent the amounts of the essential amino acids that a 50 g rat requires for growth.

[5] Intake calculated from feed consumption records of weanling rats fed a ration composed of 90% commercial white bread plus 5% corn oil, salts and minerals.

Table 3

Composition of the "core" diets. Average amounts provided per day by the "core" diets are given. Each subject had to eat all food included in these diets. Extra calories, required to maintain body weight, came from whey-free butter, hard candies, jelly, jam and honey

Diets	Nitrogen (g)	Protein (g)	Fat (g)	CHO (g)	Calories
Control	12.2	72.4	101	446	2983
Experimental	11.8	67.3	80	559	3320

Table 4. Nitrogen retained or lost per day by the individual subjects.

Each metabolic period was 5 days.

Values are expressed as grams per day

	Control Phase				Experimental Phase									
Subj.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	-0.19	-0.64	-0.75	-1.28	-1.13	-1.90	-0.42	+2.31	+1.74	+2.05	+1.75	+0.97	+1.82	+2.35
2	-0.89	-0.49	-0.54	-0.25	-2.81	-1.49	-2.16	+0.65	-2.71	+0.97	-0.24	+0.30	-0.95	+1.30
3	-1.18	-0.08	-1.42	-1.74	-2.47	-1.20	+0.61	+0.41	+0.74	+1.71	+1.16	+0.12	+1.10	+1.78
4	+0.02	-0.58	+0.68	-2.02	-2.95	-2.04	0.00	+0.72	+1.53	+1.53	+0.37	+0.53	+0.63	+0.79
5	+0.45	-0.89	+0.63	-0.39	+0.89	-2.49	+2.79	+1.42	+0.83	+1.91	+0.85	+2.51	+0.76	+1.52
6	+0.21	-0.92	-1.14	-0.84	-3.39	-1.51	+0.15	-0.22	-0.28	+1.03	-0.31	+0.28	+1.04	+0.99
7	-0.36	+0.14	-0.85	-2.03	-2.49	-3.71	+0.70	+2.17	+0.72	+1.05	+0.75	+1.37	-1.03	+1.69
8	+0.23	-0.02	-0.29	-0.56	-1.11	-4.77	+0.09	+1.57	+1.08	+0.86	+0.55	+0.04	+0.56	+0.90
9	+0.24	-0.67	+0.65	+1.42	+0.07	-4.58	+1.98	+0.92	+1.70	+4.36	+4.49	+1.16	+4.77	+1.49
10	-1.44	-1.33	-1.48	-0.18	-0.99	-3.44	+2.65	+0.96	+0.94	+2.8	-1.53	-2.83	+0.35	+1.03
11	-1.36	-1.53	+1.06	+1.59	-2.65	-0.19	+1.11	-0.47	+0.46	+2.62	-0.43	+0.50	+0.37	+1.39
12	+1.47	+0.60	+0.95	-4.22	-0.34	+2.25	+2.31	+2.03	-0.24	+1.70	+0.30	+1.58	+2.15	+2.09
Per Av.	-0.23	-0.53	-0.21	-0.84	-1.61	-2.09	+0.82	+1.04	+0.54	+1.88	+0.64	+0.54	+0.96	+1.40
Phase Av.				-0.45										+0.41

Table 5

The average daily amino acid intake (in g) of subjects during control and experimental phases of the study. Each subject received the same "core" diet which contained all the protein. These values represent the results of microbiological assays of separate samples of each of seven menus used in both phases of the study

Phases	Lysine	Isoleucine	Leucine	Methionine	Cystine	Threonine	Phenylalanine	Valine	Tryosine	Tryto-phan
Control	2.03	5.50	5.11	1.94	1.15	3.62	2.24	4.71	1.42	0.71
Experimental	1.27	5.43	4.35	2.85	1.48	1.64	1.84	4.07	1.14	0.81
Control / Experimental	1.6	1.01	1.27	0.68	0.78	2.21	1.21	1.16	1.25	0.88

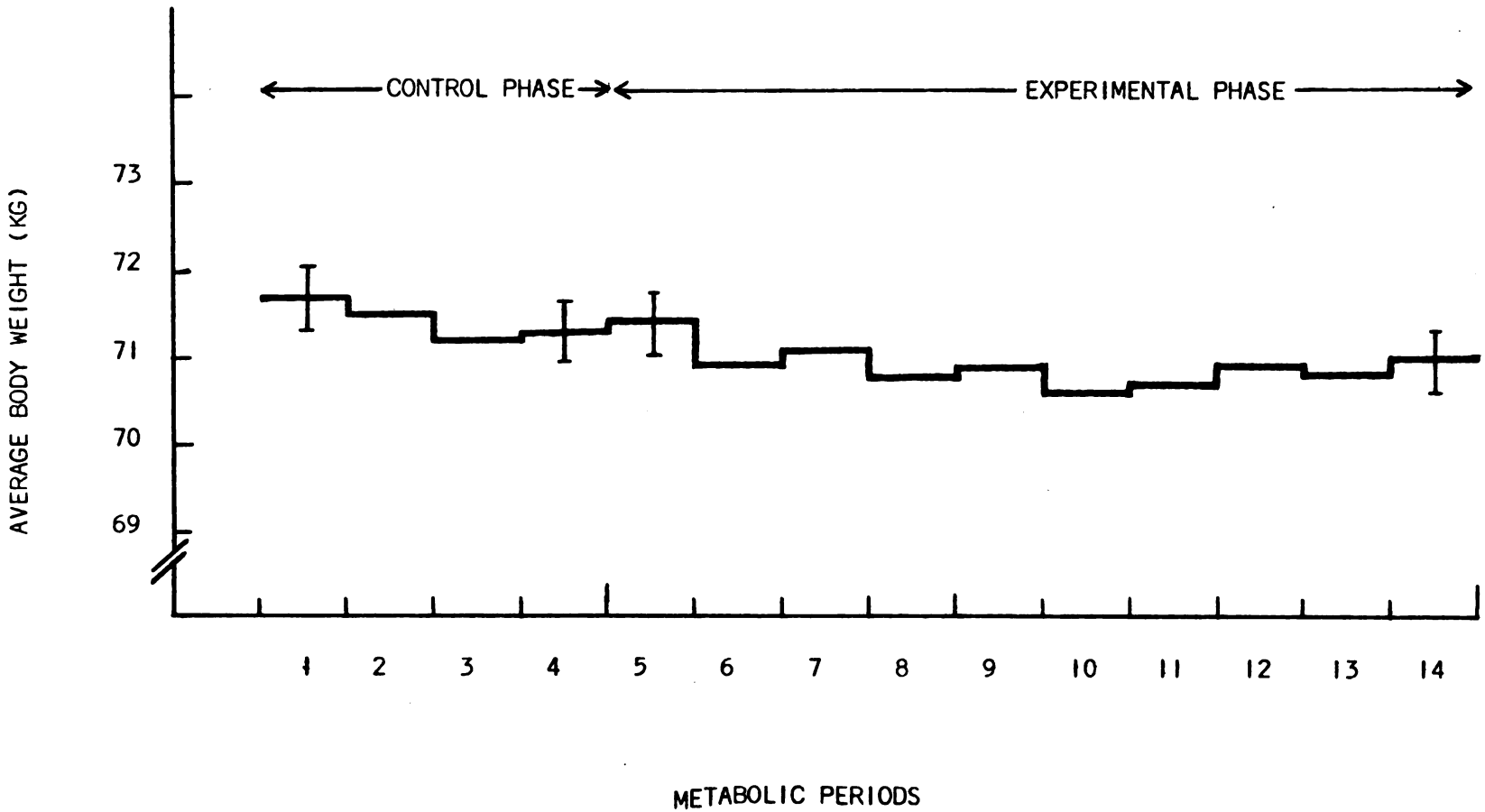

Fig. 1 Average body weights for each 5-day metabolic period (in kg) for the subjects. The standard deviations are given for the metabolic periods at the beginning and end of the control and experimental phases; the variation in the intervening periods was the same as that indicated on the figure.

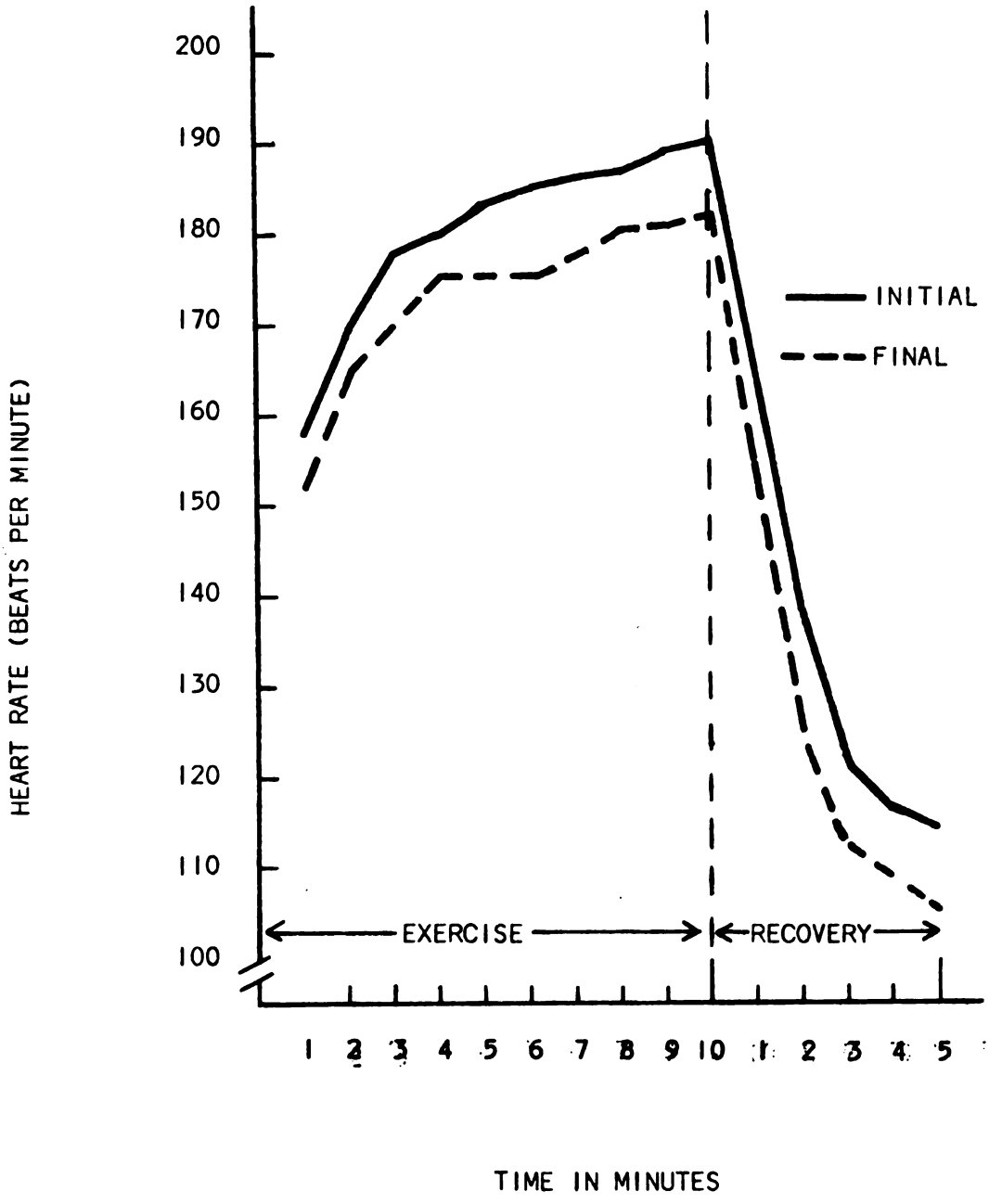

Fig. 2 Average heart rates during a 10-minute run of the subjects on a motor-driven treadmill and for the first five minutes of the recovery period. The treadmill was set for a speed of 6 mph and zero grade. This test of physical fitness was made at the beginning of the control and at the end of the experimental phase.

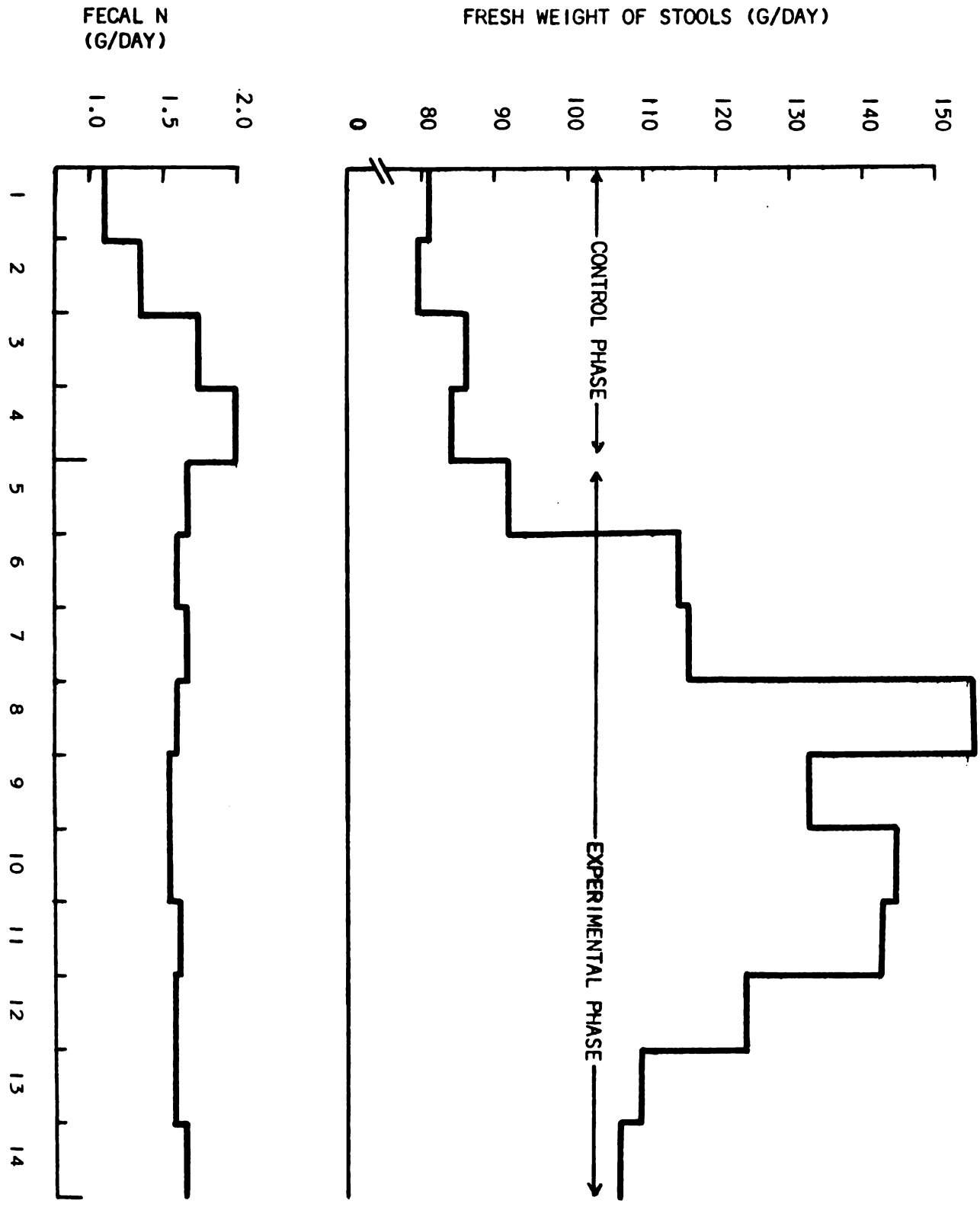

Fig. 3 Average fecal weights and fecal nitrogen content expressed as grams per day, for four metabolic periods of the control and ten metabolic periods for the experimental phase. Each metabolic period represents five days. The fecal weights represent the fresh weight of the stools as they were received in the laboratory.

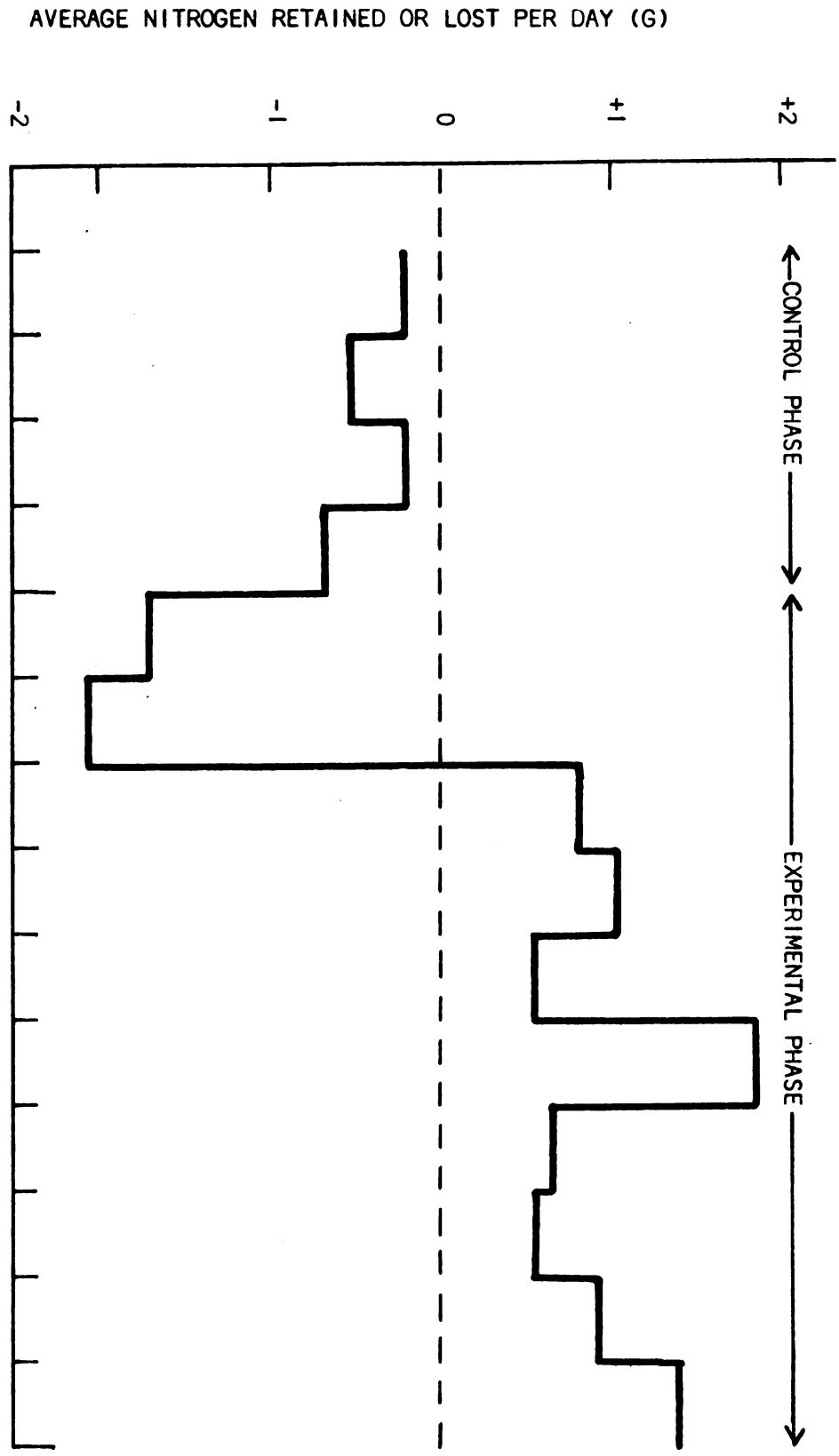

Fig. 4. Average retention or loss of nitrogen by the subjects throughout the study. All values are based on analyses carried out on diets, urine and stool samples composited for each 5-day metabolic balance period

Chapter 3

INFLUENCE OF WHEAT FLOUR ON BLOOD UREA CONCENTRATION AND UREA METABOLISM OF ADULT HUMAN SUBJECTS

In normal individuals, the blood urea level has been related primarily to the level of dietary protein. This was shown by Addis and co-workers (1917, 1947) who gave 10 medical students diets which provided 0.5, 1.5 and 2.5 g of protein per kg of body weights in successive 5 or 6-day periods. At the end of each dietary period, the subjects had blood urea levels of 19, 39 and 45 mg per 100 ml. The correlation between dietary protein intake and blood urea occurs not only in adults, but also in infants (Barnett and Vesterdal 1953, Edelmann et al. 1960). Even premature infants (provided they weighed more than 1500 g) responded to protein intakes ranging from 1.1 to 4.9 g per kg of body weight with increases in blood urea levels (Williams 1963). The change in blood urea levels of infants was not associated with alterations in serum protein levels, Fomon (1961).

Dogs also respond to an increased protein intake with an increase in blood urea levels (Shannon et al. 1932, Pitts 1932, Deuel et al. 1928). There is an indication from work done on rats for another purpose that this species also shows an alteration in blood urea levels that is related to protein intake (Wergedal and Harper 1964).

A possible explanation for the relation between protein intake and urea levels in the blood stems from the work of a number of investigators

during the past few years. They have shown that when rats are fed high protein rations there is an increase in activity of those enzymes involved in protein metabolism (Schimke 1962) as well as those associated with urea formation (Schimke 1963).

The reduction in blood urea levels and the concomitant lowered protein metabolism associated with a low protein diet was the basis for advocating a reduced protein intake for the treatment of a variety of renal disorders. Although this practice is still followed to a limited extent, the patient that is most likely to benefit from such diet therapy is the one with acute renal failure (Kark 1964). An illustration of the change that has occurred in the dietary treatment of diseases involving the kidneys can be seen in the history of the Kempner diet. Initially, the beneficial effects of that regimen were attributed partially to rice protein (Kempner 1949). Later work suggested that the reduction in blood pressure associated with the use of the Kempner regimen occurred when other diets were prescribed provided the sodium intake was markedly restricted (Hatch et al. 1954).

Kempner's work (1948, 1949) and that of other investigators (Watkins et al. 1950) indicated that nitrogen equilibrium can be achieved with the low protein intakes provided by the rice diet (20 g per day). From 4 to 14 weeks were required for the attainment of the equilibrium state. Regardless of whether an equilibrium state was

achieved, the patients showed a reduction in both non-protein nitrogen and blood urea levels. These reductions were attributed to the low protein intake.

This report presents evidence that the type of protein apart from the quantity of protein in the diet has an influence on the blood urea nitrogen (BUN) level of adult human subjects. Low BUN levels were observed in the subjects while consuming a diet in which 90 percent of the protein was provided by wheat, with the remainder from other plant sources. The low BUN levels were maintained for the 50 days when the high wheat flour diet was consumed. During that period, the subjects were in nitrogen equilibrium or storing small amounts of protein.

A. Experimental

I. Effect of a High Bread Diet on BUN

Twelve normal young men 19 to 27 years of age were fed a "normal" diet for 20 days. During this 3-week control phase, the diets provided 72.4 g of protein (12.2 g nitrogen per day per subject) of which 43 to 45 percent was of animal origin. This was followed by the experimental phase of 50 days when the subjects received diets which contained no animal protein. Over 90 percent of the protein in the diets during the experimental phase came primarily from commercial white flour or such other wheat products as spaghetti; the remaining protein came from fruits and vegetables. The protein in the diets fed during the 50-day experimental phase provided 67.3 g protein (11.8 g of nitrogen).

Urinary urea nitrogen was determined for each subject on every 24-hour urine sample collected in both the control and experimental phases. The method used in determining urea involved Nesslerization of the ammonia formed by the action of urease on the urea. Creatinine was measured each day on the same urine samples by the picric acid method of Folin (Hawk et al. 1947).

Blood samples were secured from the antecubital vein at the beginning and end of the control phase as well as at the mid-point and end of the experimental phase. These samples were analyzed for serum urea by a method similar to that used for the urine samples; total protein by the method of Caraway (1960); protein fractions by paper electrophoresis; SGOT and SGPT by the method described by Sigma Chemicals; hemoglobin by the cyanmethemoglobin procedure; hematocrit by means of the capillary tube technique as described by Hawk, et al. (1947).

II. Reduction in BUN Levels after Initiating a High Bread Diet

Two men and three women consumed a high bread diet after a 5-day preliminary period when they restricted their ordinary diet to about 70 g of protein per day. During this preliminary period, no attempt was made to limit the kind of protein in the diet. The high bread diet provided about 67 g of protein per day. The same breakfast, lunch and dinner were served on each of the four experimental days. These meals were patterned after those served the subjects in the preceding section. After this 4-day experimental period, the subjects again resumed their

self-selected patterns of eating.

Blood samples were secured from the antecubital vein of each subject 48 hours and one hour before the start of the bread diet. Additional samples were taken 48 and 96 hours after the start of the bread diet. Blood samples were also secured 48 and 96 hours after the subjects returned to their ordinary diets. All blood samples were taken before the subjects ate breakfast.

Throughout the study, 24-hour urine samples were collected from each subject on every alternate day. The urine collections started the morning when the blood samples were taken.

On the blood samples, the following analyses were run: BUN, total serum protein, NPN and creatinine. Each urine sample was analyzed for total nitrogen, creatinine and uric acid. The analytical methods for both blood and urine were those described for the first study.

B. Results

I. Effect of a High Bread Diet on BUN

The serum urea nitrogen was lower for each of the 12 subjects during the experimental than during the control phase. The average BUN increased from 13.0 mg per 100 ml at the start of the study to 16.9 mg at the end of the control phase. Both of these values are within normal limits. There was nothing to suggest that this slight increase was of any physiological significance.

At the mid-point of the experimental phase, the average value for the BUN was 6.6 mg and at the 50th day of that period, it was 6.4 mg. On both of these occasions, the highest BUN value for any subject was below the lowest value seen during the control phase (Table 6).

There is no evidence from the urinary urea data that the changes in BUN levels were reflected in compensatory excretions. If anything, just the opposite appeared to be true. While the average BUN level increased in the control phase, the urinary urea increased slightly (Table 7). During the experimental phase, when the BUN was at low levels, the urinary excretion also decreased slightly.

Although there was some change during the control phase in the relative proportion of total urinary nitrogen excreted as urea, there was a pronounced change during the experimental phase. In the control phase, the urea ranged from 80 to 99 percent of the total nitrogen in the urine. The change in urea nitrogen excretion in the experimental phase became prominent only during the last half of that phase. At that time, urea constituted from 64 to 78 percent of the total urinary nitrogen (Fig. 5).

During the control phase and the first half of the experimental phase, there was considerable variation in the range of urea excretion (Table 7). Although the individual variation in urea excretion appeared to be smaller during the second half of the experimental phase, that was more apparent than real. Actually, the standard deviations represented

almost the same percentage of their respective averages throughout the entire study. There was no consistent individual pattern since some subjects, e.g. No. 11, excreted the largest amount in certain metabolic periods (3 and 4) but then in other periods (2) was among the lowest.

Fecal nitrogen excretion throughout the entire study showed no change. For this reason, it would be difficult to explain the changes in urinary urea excretion on the basis of alterations in fecal nitrogen. If there is any correlation between urinary urea excretion values and body weight changes, it is very tenuous. There were no consistent changes in body weights, yet, there was a marked reduction in urea nitrogen excretion from an average of 10.7 g per day for the control phase to 6.4 g for the second half of the experimental phase (Table 7).

The change in the blood urea levels during the period when the subjects consumed wheat protein was not associated with any alterations either in total serum proteins or the levels of albumin and globulin (Table 8).

II. Reduction in BUN Levels after Initiating a High Bread Diet

The reduction in BUN levels observed among the 12 subjects in the preceding study was so dramatic and unexpected that the short-term study was carried out.

While the five normal adults ate self-restricted diets which provided about 70 g of mixed protein per day, their BUN levels showed relatively little day-to-day change (Table 9). The last blood sample

taken just before the breakfast which initiated the high bread diet showed BUN levels of 13.0±1.1 mg per 100 ml of serum. The next blood sample secured 48 hours after the start of the bread diet showed BUN levels of 8.1±1.3 mg. The second sample during the high bread diet showed that the low BUN levels were maintained and that the non-protein nitrogen constituents in the blood were also decreasing.

Within 48 hours after the subjects ceased eating the high bread diet and returned to their normal diets, the BUN levels had increased to the values seen in the control period (Table 9). A partial explanation for the rapid increase in BUN levels may reside in the fact that the subjects did not restrict their protein intake in the post-control period. Both the slightly elevated protein level plus the change from a predominantly wheat protein (no animal protein) diet may have contributed to the increase.

Despite the rapid increase in the BUN level with a return to normal dietary pattern, the non-protein nitrogen in the serum increased much more slowly. Even 4 days after the termination of the high-bread diet, the NPN in the serum was only 57 percent of the average pre-control value.

Throughout the entire 10-day period, the variation each day in both the BUN and NPN values was approximately the same when the standard deviations were expressed as a percentage of the average values. There were two occasions (blood samples 4 and 6) when the standard

deviations for the BUNs were remarkably small.

Again, the reduction in BUN levels occurred in the absence of any change in serum protein levels. Throughout the 10 days of the study, the serum protein levels showed relatively little inter- and intra-individual variation as evidenced by the small standard deviations (Table 9).

The urinary excretion of nitrogen showed some reduction during the initial self-selected dietary period (Table 10). This reduction probably stemmed from the fact that the protein content of the diet prior to the start of the study was higher than the 70 g provided during the high bread period. After that first high value, there was relatively little change in the total nitrogen excretion throughout the remainder of the study. These values for total urinary nitrogen agree very well with those for the 12 subjects during the period when their diets provided 70 g of protein per day.

Urinary urea excretion was lower on the fourth day of the high bread diet than on any of the other days (Table 10). The change in urea excretion both as an absolute value and as a percentage of total nitrogen followed somewhat the same course as it did for the first 12 subjects. The time intervals were, however, much shorter. Urea nitrogen represented 71 percent of the total nitrogen in the first urine sample. For the next two, the values were 88 and 96 percent respectively. The third urine sample collected after 3 days of the high bread diet

contained 78 percent of the total nitrogen as urea.

The creatinine excretion showed no essential change throughout the entire study (Table 10). Except for an increase in ammonia excretion during the second 24-hour urine collection (in the self-selected diet period), there was no marked change in this urinary constituent. Urinary pHs were not measured, so it is impossible to relate the change in ammonia excretion to an alteration in the excretion of either acid or base.

Uric acid excretion fluctuated to a slight extend throughout the study with no consistent change occuring in either the self-selection or the high bread period.

C. Discussion

The ingestion of a diet free of animal protein and high in wheat protein by adults produced a rapid drop in the BUN level. This reduction appeared within less than 48 hours after the "bread diet" was started (Table 9) and continued at the lower level to the end of the study. In the first trial, the BUN was one half the control level for the 50 days of the "bread diet"(Table 6).

The rapidity of the change in BUN of the subjects in this study was comparable to that seen when other normal young men were fed diets containing very different levels of protein. This was evident in a study where the daily protein intake was reduced from 150 to 25 g. The decrease in BUN levels was completed in less than a week. The reduction

in BUN levels was seen in both groups of subjects who consumed the high bread diet. These two studies were carried out about one year apart, so if some environmental factor other than diet were responsible for the change, it was operative both years.

The average reduction in BUN for the first 12 subjects was over 50 percent. The first blood sample secured from these subjects after the initiation of the bread diet was on the 25th day. The first comparable blood sample from the second group of subjects was secured on the 2nd day of the bread diet. The reduction in BUN for the samples in the latter study was 40 percent of the control values. The differences in the BUN levels for these two groups may be due either to the shortness of the period (more than 4 days may be required for the complete lowering of the BUN level when a high bread diet is consumed) or to the incompleteness of dietary control in the second study. The only reason for suggesting the latter is that some of the women found it difficult to consume the total allotment of 550 g of bread each day.

The reduction in BUN levels occurred despite the intake of a constant amount of protein. This was especially true of the first study where the subjects received weighed amounts of food. As an additional check, one portion of everything served the subjects was saved for nitrogen analyses. In that study, the daily diets during the control phase contained an average of 12.2 g of nitrogen while the "bread diets" in the experimental phase contained 11.8 g. Not only was the nitrogen

intake constant throughout that study, but so also was the digestibility of the protein. This was based on the fact that fecal nitrogen excretion was the same in both the control and experimental phases. These facts should effectively preclude any suggestion that the reduction in BUN was attributable to a decrease in the amino acids available to the tissue cells.

The lowering of the BUN levels when a "bread diet" is consumed occurs in both men and women. The subjects for the second study were three women and two men (Table 10). The serum values for both the men and women in that study showed the same responses. All of these subjects were mature adults (ages 27 to 52 years) who were presumably in nitrogen equilibrium. On these bases, it would appear that neither sex nor age about 19 years (the lowest age of the first 12 subjects) has any effect on the reduction in BUN levels associated with a high bread diet.

Whether a similar reduction in BUN will be seen in children fed the same type of diet is problematical. As a first postulate, it is doubtful whether it will. This suggestion is based on the observation that adult rats respond to a white flour ration with a reduction in BUN levels, whereas weanling rats do not (unpublished data). Whether the difference in reaction of young and mature rats is due entirely to the inability of the weanling rat to even maintain its body weight when fed the "bread ration" has not been determined.

The significant finding in this study is that the non-protein nitrogen,as well as the BUN levels were reduced while the subjects ate the bread diet (Table 9). This fact eliminates the suggestion that the lowered urea level was associated with an increase in some other nitrogenous constituent. Furthermore, the NPN levels remained low for at least four days after the subjects returned to their regular self-selected diets. By that time, the BUN levels had returned almost to "normal" but since the NPN was still low, the urea represented a larger fraction of the NPN than in the pre-bread period.

It is likely that the reduction in BUN level was brought about by an increase in urea excretion. In the second study, the lowered BUN level was seen within 48 hours after the start of the bread diet. During that two-day period, the subjects should have lost an average of 3.9 g urea N per day to account for the reduction in BUN levels. This figure was calculated from the average weight of the subjects (62 kg) and the assumption that their bodies contained 60% water. The first 24-hour urine sample collected after the start of the bread diet showed a slightly elevated urea content. However, the total nitrogen excretion was not increased. From the behavior of urinary nitrogen and urea excretion observed in the first study, it may be difficult to detect any increased urea excretion unless isotopically labelled compounds are used.

During the 50-day period when 12 subjects were fed the bread diet, their BUN levels remained low despite rather marked changes in urinary

urea excretion and drastic alterations in nitrogen balance. It is assumed that these subjects showed the same rapid reduction in BUN levels as did the five adults. If that had been so, and there is nothing to contradict this suggestion, then the lower BUN occurred while the subjects were in negative nitrogen balance (page 41). The first blood sample during the experimental phase coincided with a state of nitrogen equilibrium. The second blood sample, taken at the end of the 50-day experimental phase, came at the time when the subjects were retaining some nitrogen. The urinary urea showed both an absolute and a relative change (as a fraction of total nitrogen) during the period when the low BUN levels were seen. These alterations in urinary constituents occurred after the reduction in BUN had been completed and so cannot be considered a causative factor.

That the type of diet may influence BUN levels is suggested by studies of West African adults. A group of these people in Nigeria had plasma urea levels of 13.9 mg per 100 ml compared with 26.7 in a group of Europeans (Phillips and Kenney 1952). When a similar study was carried out with West African male students who had lived in London for an average of 2-$\frac{1}{2}$ years, their mean blood urea level was 24.8 mg per 100 ml while that in a comparable group of English students was 28.0. However,blood secured from Nigerian university students in Ibadan contained 16.6 mg urea per 100 ml. It was reported that the Ibadan students "ate meat daily, and most of them took beans several days a week and

some of them eggs and milk occasionally; these were the only important sources of protein in their diets" (Barnicot and Sai 1954). Although there were no data on the quantitative protein intake, the implication is made that the lower BUN levels among the students in Ibadan was the result of a low protein intake. There is still the possibility that some peculiarity of the Nigerian diet per se might be responsible for the reduction in BUN. This should not be ruled out until the daily protein intake is checked by determining the 24-hour urinary nitrogen excretion.

Most of the early work on the effect of different levels of dietary protein on the metabolism of that nutrient was focused on BUN levels. However, some thought was given to the possible repercussions of these alterations in BUN levels on the kidneys. This was evident when Addis and Drury in 1923 stated "There are many other factors than blood urea concentration which influence the rate of urea excretion." Nothing more was said about it at that time. They did show, however, that in the fasting state, urea excretion is directly related to BUN levels. The ratio of urinary to blood urea may vary slightly from person to person but for the same individual, the ratio is fairly constant over a wide range of BUN levels (Addis and Drury 1923).

Although there is still some confusion as to the effect of dietary protein level on urea clearance especially in the upper ranges of intake, there appears to be agreement that a diet low in protein consumed

for a week or more results in a reduction in urea clearance (Schmidt-Nielsen 1958). A suggestion for such a relationship was made by Jolliffe and Smith (1931) when they stated that "The diet upon which the dogs are maintained...is a very important factor in the urea clearance even when this test is made 18 hours after the last meal." Shortly thereafter, evidence to support this statement came from the same laboratory. This consisted in showing that when dogs were fed a "cracker meal" diet (100 g cracker meal, 30 g sucrose, 30 g lard) their urea clearance was about 28 ml per minute. After the animals were fed meat for a number of days, the urea clearance increased to values as high as 88 ml (Jolliffe and Smith 1931).

About the same time, work with human subjects indicated that a diet providing 40 g of protein reduced the urea clearance from an average of 100 ml per minute to 70 to 80 ml (Cope 1933). Thereafter, a number of investigators provided data to support a reduction in urea clearance when the protein content of the diet was reduced to levels of 40 g or less per day (for tabulation of these papers see Pullman et al. (1954). There is some evidence that the reduction in urea clearance in individuals maintained on a low protein diet is even greater when the urine volume is low (Murdaugh et al. 1958).

Despite the reduction in urea clearance in subjects consuming a low protein diet, the glomerular filtration rate showed no change (Murdaugh et al. 1958, Pullman et al. 1954). In one of these studies, the dietary

protein was reduced from 150 g per day to 25 g and maintained at that level for 6 weeks. The change in protein intake was associated with a small reduction in glomerular filtration rate for some subjects, whereas most of them showed no change (Murdaugh et al. 1958). These two extremes in protein intake had no influence on urine volume.

The renal tubule is the part of the kidney that has been implicated as being altered by the ingestion of a low protein diet. The emphasis on this part of the kidney has resulted entirely from a process of elimination. The mechanism whereby the tubules control the excretion of urea in urine "is a regulated active process that involves the counter current principle" (Murdaugh et al. 1958). This explanation, however, provides no indication as to how that regulatory process in the tubules is influenced by a reduction in dietary protein intake.

The present finding that the type of protein in the diet influences the urea level in the blood will require additional studies of kidney function to elucidate the mechanism involved in this change. Research in that area is fraught with more potential problems than that previously done on the relation of dietary protein level to kidney function. The new dimension introduced by the current finding is that the BUN level is drastically reduced with no change in urinary urea excretion. On that basis, it would appear that the ingestion of a high wheat or flour diet doubles the urea clearance.

Table 6

Serum urea levels in the subjects, expressed as mg N per 100 ml.

Subject	Control Phase		Experimental Phase	
No.	Start	End	Mid-point	End
1	14.4	16.1	7.7	6.6
2	17.8	13.9	8.8	8.2
3	13.9	12.2	8.3	9.0
4	11.1	13.3	5.4	9.1
5	11.1	12.8	3.8	4.6
6	15.6	19.5	9.8	10.3
7	15.6	21.1	5.7	4.4
8	11.7	19.5	8.8	3.9
9	11.7	20.0	6.8	7.9
10	11.1	17.5	3.3	4.4
11	11.7	18.4	4.4	3.1
12	11.1	18.9	6.1	4.7
Averages	13.0 ± 2*	16.9 ± 3	6.6 ± 2	6.4 ± 2

*Standard deviations are given for each average.

Table 7

Urinary urea excretion of the individual subjects expressed as g of nitrogen per 24 hours.

Each value is the average for a 5-day metabolic perid.

Met. Per.	Control Phase				Experimental Phase									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Subj. 1	8.9	8.9	9.8	10.2	8.9	9.5	7.9	5.5	7.1	5.9	5.4	6.8	5.6	5.9
2	8.5	8.4	10.5	10.9	10.5	7.8	7.6	8.9	7.7	7.7	6.2	5.6	6.0	6.8
3	8.9	9.1	9.1	10.0	8.3	8.4	7.4	7.8	8.4	5.5	5.0	8.4	5.9	6.1
4	8.7	9.2	9.9	9.8	8.8	8.9	8.4	8.3	8.4	6.3	6.0	6.2	5.9	6.8
5	8.9	9.4	11.5	10.8	10.7	10.0	8.2	8.1	7.5	6.3	4.3	5.6	5.4	5.8
6	8.9	12.9	10.5	12.3	10.9	9.7	8.1	8.9	8.4	6.9	6.3	7.3	7.6	7.4
7	8.1	12.2	10.4	10.9	9.6	9.5	8.0	7.6	7.1	6.1	6.0	6.5	7.6	6.6
8	8.9	8.5	9.9	9.9	8.8	9.7	7.8	9.0	7.6	6.7	6.4	8.2	7.4	8.0
9	9.2	10.6	11.2	9.8	9.3	9.9	6.7	8.3	7.2	5.0	4.1	6.0	4.1	6.9
10	10.1	12.1	12.2	13.3	12.1	14.0	11.0	11.1	8.3	6.0	6.9	7.3	7.1	6.1
11	10.2	9.2	15.2	16.9	13.2	13.1	7.3	8.8	8.0	6.2	6.0	6.5	6.7	6.4
12	7.8	11.5	11.6	16.1	8.7	8.2	10.4	8.2	8.0	6.7	7.5	7.7	7.1	6.6
Period Average	8.9	10.1	10.8	13.1	10.0	10.0	8.5	8.3	7.9	6.3	5.8	6.8	6.4	6.6
Std. Dev.	0.7	1.6	1.5	2.5	1.5	1.8	1.2	1.3	0.5	0.7	0.9	0.9	1.1	0.6
Phase Average				10.7										8.9

Table 8

Total serum proteins and albumin to globulin values in blood samples taken from the anti-cubital vein after an overnight fast. Averages and Standard deviations are given.

	Control Phase		Experimental Phase	
	Begin.	End	Begin.	End
Protein (g/100 ml)	7.05 ± .70	7.06 ± .67	6.84 ± .73	7.26 ± .67
Albumen (%)	77.6 ± 6.7	76.6 ± 5.6	76.5 ± 6.8	77.4 ± 6.1
Globulin (%)	22.3 ± 6.3	23.8 ± 5.8	23.7 ± 6.9	22.5 ± 6.1

Table 9

Serum values for 5 normal adults (three women and two men) before, during and after a four-day period when a high bread diet was consumed.

Diet	Self-Selected		High-Bread		Self-Selected	
Blood Samples	1	2	3	4	5	6
BUN mg%	13.9 ± 1.9	13.0 ± 1.1	8.1 ± 1.3	9.2 ± 0.3	13.5 ± 2.8	13.8 ± 0.2
NPN mg%	29.0 ± 5.6	31.8 ± 5.2	25.0 ± 3.5	15.0 ± 2.3	17.0 ± 1.5	17.4 ± 2.3
Serum Protein %	7.2 ± 0.3	7.5 ± 0.3	7.8 ± 0.2	7.4 ± 0.6	7.3 ± 0.7	7.4 ± 0.6

All values given as averages ± standard deviations.

Blood samples were taken before breakfast every alternate day with the second sample on the morning when the high bread diet started.

Table 10

Urinary nitrogen constituents of the 5 subjects before, during and after the high bread diet. The 24-hour urine samples were collected on alternate days. The urine collections started the morning the blood samples were taken (see Table 4). All values are expressed as g per day.

	Diet				
	Self-Selected		High-Bread		Self-Selected
Urine Sample	1	2	3	4	5
Urea N	8.2 ±1.7	8.4 ±1.6	9.3 ±1.4	7.3 ±0.4	10.1 ±1.3
Total N	11.8 ±0.5	9.6 ±2.3	9.7 ±0.8	9.4 ±0.3	10.7 ±1.2
Creatinine	1.4 ±0.5	1.3 ±0.3	1.1 ±0.2	1.3 ±0.1	1.3 ±0.1
Ammonia	0.3 ±0.06	0.8 ±0.05	0.2 ±0.05	0.2 ±0.08	
Uric Acid	0.3 ±0.1	0.1 ±0.02	0.4 ±0.07	0.2 ±0.05	0.2 ±0.01

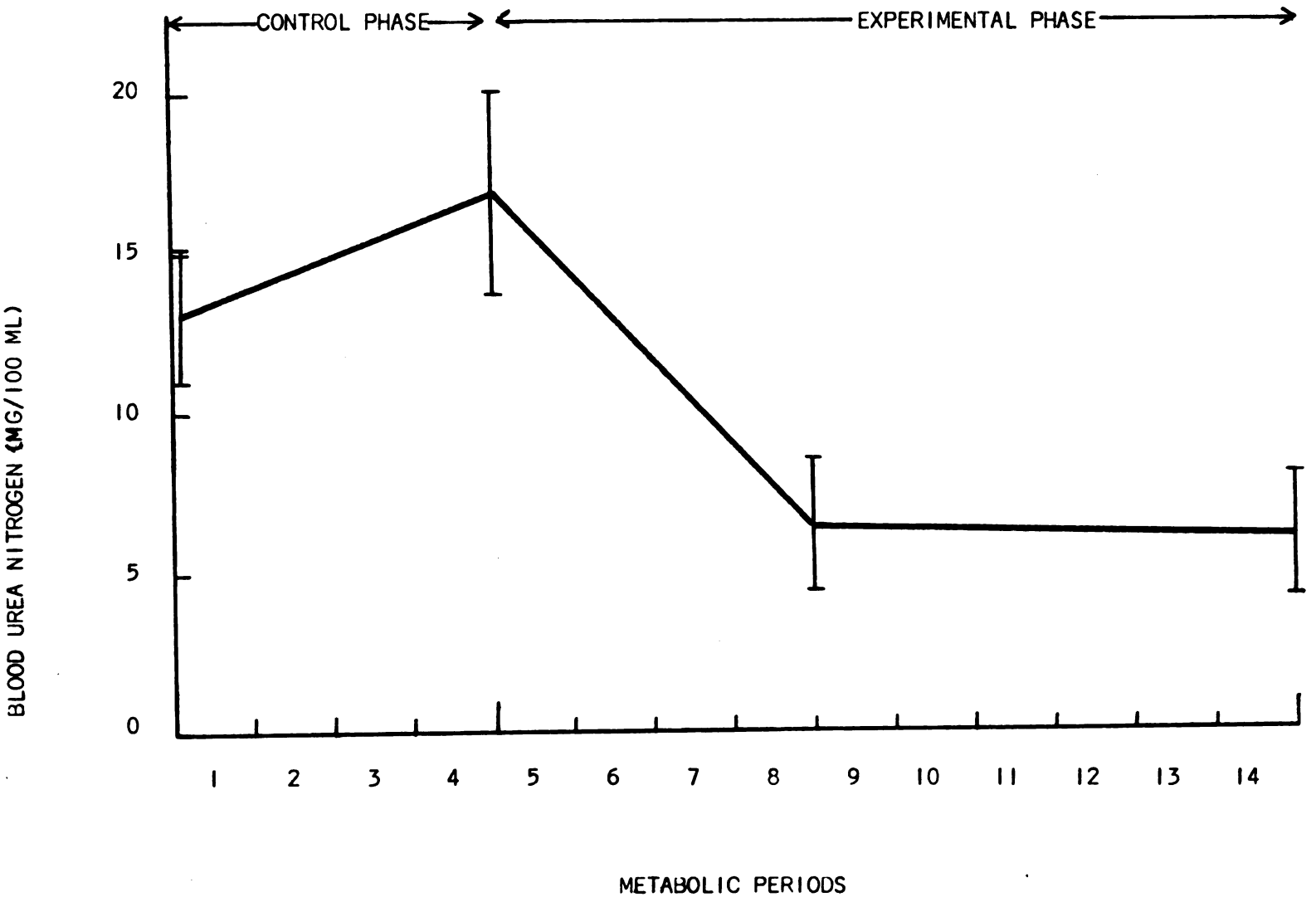

Fig. 5. Plasma urea nitrogen in blood samples secured at the beginning and end of the control phase and midpoint and end of the experimental phase. The horizontal line represents the average values; the vertical lines, the standard deviations.

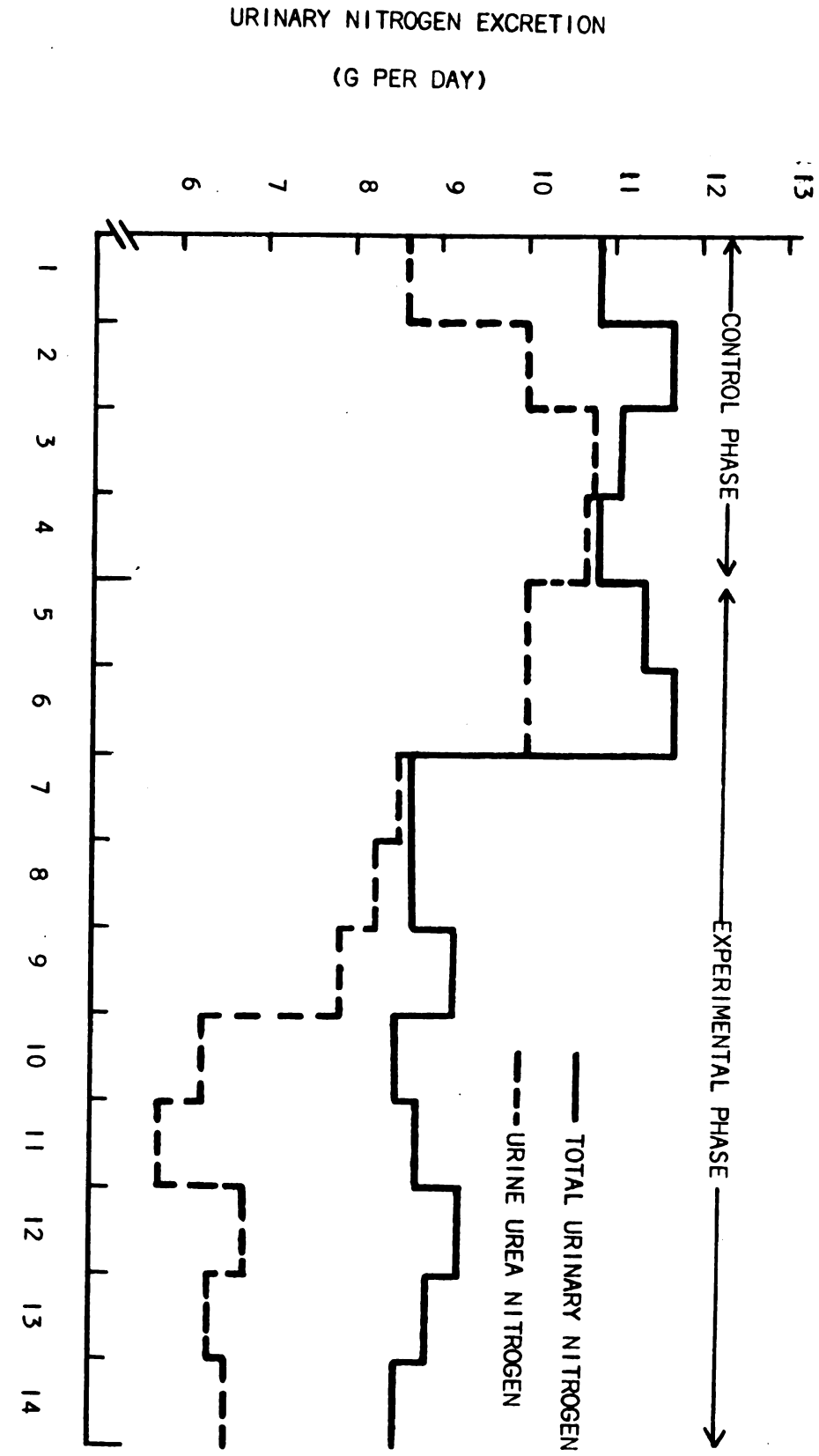

Fig. 6. Total and urea nitrogen excreted in the urine. Both curves represent the average excretion for the 12 subjects for each 5-day metabolic period.

Chapter 4.

EFFECT OF HIGH WHEAT DIET ON AMINO ACID METABOLISM

At first glance, the plasma amino acid levels should be related to the preceding protein intake. Attempts to evaluate the validity of this hypothesis had to await the development of analytical methods which could be applied to the relatively small volumes of blood available from experimental animals. With the advent of such techniques, a large number of papers have appeared which provide information about the changes in plasma amino acid levels following the ingestion of different proteins. About 1950 a number of reports indicated that the plasma levels of arginine, lysine, methionine, tryptophane and valine in the plasma of chicks were correlated with the level of these amino acids in the dietary protein(Charkey et al. 1950 and 1953, Richardson et al. 1953a and 1953b). In these studies, chicks were fed soybean meal, peanut meal, or other proteins for six weeks before the blood samples were taken.

In 1959, Longenecker and Hause observed that the plasma amino acid changes after a meal containing protein were directly related to the concentration of the amino acids in the ingested protein. This procedure required blood samples, cardiac or venous, 18-hours after the last meal and every hour for five hours after the test meal. The fasting amino acid concentration was subtracted from the corresponding average of the five post-feeding blood samples. This difference was expressed as a percentage of the day's requirement for that amino acid. By this

means they found that lysine showed the smallest change in plasma concentration when wheat gluten was fed, tryptophane when gelatine was fed, and arginine when casein was fed. A shortened version of this procedure was reported to provide comparable results (Longenecker and House 1961).

The results secured by recent investigators in this area suggest that the plasma level of threonine is not related to its concentration in the diet (Gray et al. 1960, Morrison et al. 1961, and Sanahuja and Harper 1963). There does, however, appear to be considerable evidence for an abnormally low levels of an amino acid in the plasma when the dietary protein is deficient in that amino acid (McLaughlan 1963).

Much of the work on plasma amino acid levels as related to dietary intake has been done with young animals. The levels of protein in the ration of these animals has usually been 10% or less, a level inadequate for normal growth rates (McLaughlan 1963). When similar studies were carried out with human subjects, the intake of protein at a single meal had to approach the equivalent of 19 g of nitrogen before meaningful results were secured. Such high intakes of protein at one meal, especially when low protein foods such as cereals were tested, produced nausea in the subjects. An abnormality manifested by overt symptoms of nausea might very well distort any plasma constituents, especially those dependent upon digestive and absorptive processes for their plasma concentrations.

In longer studies with human subjects, the emphasis in one laboratory has shifted from a specific plasma amino acid level to the ratio of the sum of the essential to the non-essential amino acids (Swendseid et al. 1963 and 1966). The change in the ratio increased as the diet was continued over a period of five weeks. Diets providing 3.5 g of nitrogen per day resulted in a ratio of 0.28, while a daily intake of protein providing 14 g of nitrogen resulted in a ratio of 0.43. In the present work, since normal young men were receiving a diet containing about 12 g of nitrogen per day with more than 90 percent from wheat flour, it seemed desirable to determine whether the plasma amino acids showed any deviations from normal. The results of the plasma and urinary amino acids are presented in this part.

A. Experimental

The subjects, the diet and the duration of the study is described in Chapter 2. Throughout the study one portion of the food served the subjects was saved each day. During the day, the can containing the servings for analysis was refrigerated. At the end of the day, the contents of the can were weighed and diluted with a known amount of water to facilitate homogenizations in a large Waring blender. A part of the homogenized sample was dried to constant weight in a vacuum oven. The dried sample was ground in a small Wiley mill. The fat was removed by petroleum ether extraction in a Goldfisch apparatus. Ten grams of the dry, defatted diet samples were hydrolyzed with acid and another

10 grams sample with alkali according to the procedure of Barton-Wright (1952).

Ten amino acids in the diet samples were determined by the microbiological procedure described by Horn et al.(1950). Analyses were carried out for these same ten amino acids in the urine samples for the last 5-day metabolic period in both the control and experimental phases.

Blood plasma samples were analyzed for ten free amino acids by the microbiological assay procedure of McLaughlan et al. (1961).

B. Results

As it has been shown previously (Hegsted 1962), a diet providing most of the protein from wheat should meet the requirements of adults for all the essential amino acids (Table 11). Even in the case of lysine, which has been recognized as the limiting amino acid in wheat, the experimental diets provided at least 1.6 times the daily requirement for that amino acid. The values for the amino acid as listed by Rose (1957) represent the largest amount of that amino acid required by any of his subjects for nitrogen equilibrium.

The experimental diets provided almost as much of the essential amino acids as the control diets. The major exceptions to this were threonine and lysine where the control diets provided considerably more than the requirement and the sulfur containing amino acids, where experimental diets provided more than the control (Table 11). The control diets were comparable to those served in American College dormitories as

far as variety was concerned, but slightly restricted in the amounts of meat, milk, cheese and other protein foods (Table 12). These diets provided sufficient protein according to the recommended allowances proposed by the Food and Nutrition Board of N.R.C.

The urinary excretion of these amino acids showed only slight differences for the last 5-days of both the control and the experimental phase. Although the reduction in excretion of tryptophane during the experimental phase appeared large. the difference was non-significant due to the large deviations. The only significant change ($P < 0.01$) was that for valine excretion.Even for this amino acid, the reduced excretion seen during the experimental phase resulted in a value that was still within normal limits as expressed by Altman (1961). These reductions in tryptophane and valine excretion occurred despite the absence of any or only a minor change in the intake of these amino acids during the control and experimental phase (Table 11).

The average level of free amino acids in the fasting blood samples secured from the subjects were all within the normal levels listed by Altman (1961). There were slight alterations in the levels of some plasma amino acids throughout the study, but except for lysine and valine, they were not significant (Table13). There was a significant reduction in the plasma levels of lysine and valine ($P<0.01$) during the experimental phase with the reduction for both completed by the twenty-fifth day. For the remaining twenty-five days of the experimental phase there was

no further change in the levels of these amino acids.

C. Discussion

A diet high in wheat and free of animal protein not only permits the establishment of nitrogen equilibrium in young men but also maintains normal plasma levels of the essential amino acids (Table 13). The establishment of nitrogen equilibrium can be explained on the basis of the amino acid content of the diets. Actual analyses of the diets indicated that the high wheat diet provided adequate amounts of all the essential amino acids required by adult men (Table 11).

The similarity in the amino acid composition of the two diets makes it difficult to explain why the subjects showed a marked negative nitrogen balance during the first ten days of the experimental phase (chapter 2). During that ten-day period, the subjects lost almost 2 g of nitrogen per day more than they consumed. There was undoubtedly some psychological disturbance associated with the consumption of a pound or more of bread and rolls each day in addition to such "starchy" foods as spaghetti, cookies, pies, etc. Such a psychological disturbance could hardly have been operative over the entire ten-day period since the subjects had been adequately forewarned prior to the start of the study as to the nature of the experimental diet. Regardless of the cause of the initial negative nitrogen balance, thereafter, the subjects rapidly came into equilibrium and retained enough nitrogen during the remainder of the experimental phase to compensate for the loss in the initial

stage of the experimental phase.

The plasma amino acid levels remained within normal limits even after the high wheat diet had been consumed for fifty days (Table 13). For the two amino acids (lysine and valine), the levels of which were lower in the experimental than in the control phase, the reduction occurred sometime within the first twenty-five days of the bread diet. There was no subsequent change in the levels of these amino acids during the last twenty-five days of the high bread diet period.

There was no relation between the changes in plasma amino acid levels and urinary excretion. This was apparent for lysine and valine (Table 13). In the case of valine, the reduction in plasma level occurred in association with a reduction of about 13 percent in the valine intake (Table 11). The reduction in urinary excretion of valine during the experimental phase was almost three times as great (36%) as the reduction in plasma level. For lysine, the reduction in plasma levels during the experimental phase was about 22 percent. This was associated with a proportionately greater reduction in lysine intake (Table 11). Despite the reduction in dietary intake and plasma level of lysine, there was no change in the urinary excretion of this amino acid.

If one were to argue that the reduction in plasma lysine level during the experimental phase was a reflection of the limiting amino acid in wheat protein, then an equally strong argument could be made

for valine as the second most limiting amino acid. Despite the fact that the plasma level of valine was reduced when the subjects consumed the high wheat diet, this reduction was not related to a dietary inadequacy of that amino acid. Both the control and experimental diets provided an adequate amount of this amino acid; for the experimental period, the diets contained about five times the daily requirement (Table 11).

For both the control and experimental periods, the urinary excretion of the essential amino acids showed considerable variation. This was true for the inter-individual variation as represented by the standard deviations and the variable fraction of the intake which was excreted in the urine. The standard deviations for the excretion of some amino acids (isoleucine, leucine, phenylalanine and tryptophan approached 50 percent of their averages. Although the variations for the other amino acids were less than that, there was still considerable fluctuation in their excretion.

The amino acid excretion was not related to the amount of nitrogen retained. Some subjects who retained the largest amount of nitrogen during the last metabolic period of the experimental phase excreted the same amounts of most of the amino acids as the subjects retaining the smallest amounts. The opposite was also true.

When the 24-hour urinary excretion of the amino acids was expressed as a percentage of the intake, there was only a slight difference between

the values for the control and experimental phases. A partial explanation for the similarity in urinary excretion in the control and experimental periods lies in the relative constancy of the amino acid intakes (Table 11). Each amino acid appeared to be excreted as a fairly uniform percentage of the intake but for the different amino acids, there was a 10 - 15 fold range in values. Isoleucine and leucine showed the smallest percentage (0.6 and 0.9%) of dietary intake appearing in the urine, and cystine and lysine the greatest (10%).

Changes in the blood levels of free amino acids offer at best only an approximate indication of the biological value of an ingested protein. This has been emphasized by McLaughlan and co-workers (1963) who found considerable variation in the plasma elevation of free methionine and threonine when fish, or fish plus butter or eggs was fed. Butter, starch and sucrose were the only non-protein nutrients studied by McLaughlan *et al.* (1963), and of these, only butter had any effect on the free amino acid levels in the plasma when these foods were ingested with proteins. This is an important consideration since in almost all situations, a protein food is consumed in combination with other foods, and the protein food itself usually contains large amounts of fat.

For these reasons, the level of plasma free amino acids was determined in the fasting state for the subjects in the present study. Any attempt to alter the dietary regimen as would be required for the

feeding of large amounts of an isolated protein would have defeated the main purpose of the study - to evaluate the ability of wheat protein in the absence of animal protein to maintain nitrogen equilibrium in normal young men. This the wheat protein did. The evidence presented in this part adds additional evidence for the nutritional adequacy of wheat as a source of protein for adult males.

Table 11

Essential amino acid intakes and urinary excretions compared with the requirements of normal male adults

The urinary excretions are the 24-hour averages for the pooled samples collected on the last five days of the control and experimental phases.

		Intake		Urinary Excretion			
Amino Acid	Requirement[1] g	Control[2] g	Experimental[2] g	Control[3] mg	Experimental[3] mg	Control[4] g	Experimental[4] g
Isoleucine	0.7	5.5	5.4	49.8 ± 9.8	32.7 ± 16.9	0.9	0.6
Leucine	1.1	5.1	4.3	32.7 ± 14.0	27.9 ± 9.6	0.6	0.6
Lysine	0.8	2.0	1.3	122.7 ± 35.0	136.4 ± 36.8	6.1	10.1
Cystine	*	1.2	1.5	118.9 ± 17.4	145.2 ± 16.9	9.9	9.7
Methionine	1.1	1.9	2.8	50.7 ± 15.5	67.6 ± 16.6	2.7	2.4
Phenylalanine	1.1	2.2	1.8	24.7 ± 6.4	26.1 ± 13.8	1.1	1.4
Threonine	0.5	3.6	1.6	67.8 ± 11.3	63.2 ± 17.7	1.9	3.9
Tryptophan	0.25	0.7	0.8	51.4 ± 35.8	29.4 ± 9.9	7.4	3.7
Tyrosine	**	1.4	1.1	28.2 ± 8.5	39.9 ± 13.6	2.0	3.6
Valine	0.8	4.7	4.1	46.7 ± 14.4	29.9 ± 5.1	1.0	0.7

[1] From Rose, as g per day.

[2] From microbiological assays of diets -- expressed as g per day per subject.

[3] Average values for a 5-day metabolic period expressed as mg per day per subject.

[4] Expressed as % of intake.

* Value listed for methionine includes that for cystine.

** Value listed for phenylalanine includes that for tyrosine.

Table 12

Typical foods served on two of every seven days of the control phase

Diet III	g	Diet VII	g
Grapefruit juice	185	Cube steak	50
Cereal: shredded wheat	22	Ham	40
Beef patty	40	Baked Potato	100
Roast chicken	40	Potato chips	20
French fries	50	Vegetables: whole canned carrots 75; lettuce 40; radish 10	125
Steamed rice	100		
Stewed tomatoes (canned)	100	Fruit: orange 190; apple 230; strawberries 100	520
Lettuce	30		
Green pepper	20	Bread: plain 50; hot rolls 100; sweet roll 130	200
Pickles (dill 30; catsup 15; mustard 10	55	Crunchy cookies	75
		White cake	100
Apricots (canned)	120	Milk	240
Bread: toast 50; bun 38; hot rolls 50	138	Butter 40: jelly 10	50
Cupcake, white 50; icing 30	80		
Lemon meringue pie	160		
Refrigerator cookies	50		
Milk	240		
Butter 50; jelly 20	70		

Table 13

Plasma free amino acid levels in blood samples secured from the subjects 14 hours after the last meal

Amino Acid	Control Phase		Experimental Phase	
	Start	End	Midpoint	End
Isoleucine	0.5 ±0.1[1]	0.7 ±0.2[1]	0.8 ±0.7[1]	0.7 ±0.1[1]
Leucine	1.8 ±0.3	1.6 ±0.2	1.4 ±0.3	1.7 ±0.3
Lysine	2.7 ±0.4	2.5 ±0.4	1.9 ±0.5	2.0 ±0.5
Cystine	0.4 ±0.10	0.5 ±0.06	0.5 ±0.05	0.5 ±0.04
Methionine	0.3 ±0.06	0.3 ±0.06	0.4 ±0.02	0.4 ±0.01
Phenylalanine	0.8 ±0.2	0.8 ±0.2	0.8 ±0.2	0.9 ±0.1
Threonine	2.0 ±0.6	2.0 ±0.5	2.2 ±0.2	2.0 ±0.2
Tryptophan	1.0 ±0.1	1.0 ±0.2	0.8 ±0.2	1.0 ±0.1
Tyrosine	0.7 ±0.2	0.7 ±0.1	0.7 ±0.2	0.7 ±0.2
Valine	2.5 ±0.6	2.6 ±0.3	2.0 ±0.3	1.9 ±0.2

[1]Averages and standard deviations for 12 subjects.
All values expressed as mg per 100 ml plasma.

SUMMARY AND CONCLUSIONS

A long term metabolic study was undertaken with human subjects in order to investigate bread and flour as the sole source of protein for human adults.

Twelve healthy male college students were selected from forty volunteers. They were chosen on the basis of age, approximation of body weights to the values listed in standard height-weight tables, freedom from disease as evidenced by a general physical examination, and a normal psychological profile as measured by the Minnesota Multiphasic Personality Inventory.

The study consisted of a 20-day control phase during which subjects received a normal diet which provided 12.2 grams of nitrogen per day. Small amounts of milk, eggs and meat were used in this phase. The control phase was immediately followed by a 50-day experimental diet which provided 11.8 grams of nitrogen per day, with 95% of this coming from wheat bread and flour, and the remainder from fruits and vegetables. An attempt was made to maintain weight by adjusting the calorie intake while keeping the protein intake constant.

The breads and rolls were baked fresh every day in the laboratory, from all-purpose flour, vegetable shortening, sugar, salt, yeast, and water. Each day of the study an extra meal was prepared which was an exact duplicate of what the subjects received. These were homogenized

and used for analyses.

Throughout the study, 24-hour urine and stool samples were collected. Volume of the urine and the weight of the stools were recorded. For each 5-day metabolic period, total nitrogen was determined in both stool and urine and nitrogen balances were calculated. Urinary urea nitrogen was determined on every 24-hour urine sample. Creatinine and 10 amino acids were determined in one 5-day composite sample of urine for control and one for experimental phase.

Venous fasting blood samples were drawn from the antecubital vein of each subject at the beginning and end of the control phase as well as the midpoint and end of the experimental phase. Serum total protein (Biuret), protein components (paper electrophoresis), urea, hemoglobin, hematocrit, free iron, glutamic-oxalacetic transaminase and glutamic-pyruvate transaminase and 10 free amino acids were determined.

During the control phase, subjects were more or less in nitrogen equilibrium. The first two 5-day metabolic periods of the experimental phase resulted in a definite negative nitrogen balance with subjects losing an average of 2 grams nitrogen per day. At this point almost all of the subjects went into the equilibrium and for the rest of the experimental phase they were retaining an average of 1 gram of nitrogen per day.

The retention or loss of nitrogen by the subjects was not reflected in their body weight changes. The changes in body fluids or body fat

content may have covered any change in body weight due to nitrogen retained or lost. The physical condition of the subjects improved towards the end of the study as evidenced by changes observed in the pulse rate and the rate of recovery of the subjects when they ran on a treadmill for 10 minutes at the beginning as compared with the same parameters measured at the end of the study. At the end of the study the standard exercise on the treadmill was accomplished with a lower pulse rate and more rapid rate of recovery for all subjects. This may be the result of increased outdoor activity of the subjects with the advent of spring weather.

The strong negative nitrogen balance during the first 10 days of the experimental phase may be the result of some over-all adaptation processes. The subjects apparently adapted to a different plasma amino acid pattern induced by the bread diet compared with the control diet since nitrogen intake in both periods was essentially identical.

The apparent digestibility of the proteins was the same for both diets. The wet weight of the feces was increased during the first half of the experimental phase. The change in the digestive tract organisms and the fiber content of the diet are suggested to be the cause. Wet weight of stools was decreased towards the end of the experimental phase.

The amino acid intake of subjects during both the control and the experimental phase was well above the minimum daily requirements of

adult men. The free amino acid pattern of the blood for 8 of the 10 amino acids determined did not change in the experimental period compared with the control. However, there was a significant reduction in the plasma level of lysine and valine during the experimental phase.

The urinary amino acid excretion varied widely among subjects but these variations were not significant for all the amino acids except for valine which was reduced during the experimental phase.

The data on the blood analysis showed that the level of hemoglobin and serum free iron and the hematocrit remained the same throughout the study for all subjects.

The two serum enzymes, glutamic-oxalacetic transaminase and glutamic-pyruvic transaminase remained the same throughout the study. At the end of the study, however, S.G.P.T. was increased significantly but the values were within the normal range.

Serum total proteins and the components albumin and globulins showed no significant change during the study.

Daily creatinine excretion by the subjects remained constant during both the control and the experimental phases. Excretion of xanthurenic acid after oral administration of 2 grams tryptophane during the experimental phase did not increase. This ruled out any pyridoxine deficiency due to high intake of bread.

Serum urea nitrogen was decreased from 13.0 and 16.9 during the control phase to 6.8 and 6.3 during the experimental phase. These low

levels persisted throughout the experimental phase. A follow-up study showed that when a high bread diet devoid of animal protein is consumed by adult subjects, reduction in BUN occurs as early as 48 hours after the change of the diet, and it occurs in both men and women. This reduction in BUN was not due to lowered intake of protein since both diets were isonitrogenous. It was due to change in the source of protein from a mixture of animal plus plant protein to one of solely plant origin. The reduction in BUN was not compensated for by increased urinary urea excretion. Towards the end of the experimental phase, the excretion of urea nitrogen was also decreased. Enzymatic changes or improved urea clearance due to high bread diet is suggested as a cause for decreased BUN.

It can be concluded, therefore, that for normal young men, the amino acids in wheat provide adequate amounts of all essential amino acids and, when the caloric intake is adequate, these amino acids can be utilized to maintain N balance. It is also important to note that a short term balance study may produce erroneous results when studying the adequacy of a protein for human subjects. Since in this study a period of about 10 days was necessary for adaptations of the subject to their new diet and to reach equilibrium.

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A P P E N D I X

Calendar of events for Bread Study

Date (1964)	Events
3/31	First balance period starts. Blood samples secured.
4/3	Underwater weighing. Skin fold measurements. Evaluation of Physical Fitness.
4/5 to 4/9	Second balance period.
4/10 to 4/14	Third balance period.
4/15 to 4/19	Fourth balance period. End of control diet.
4/20 to 4/24	Fifth balance period. Start of experimental diet. Blood samples secured. Underwater weighing. Skin fold measurements.
4/25 to 4/29	Sixth balance period.
4/30 to 5/4	Seventh balance period.
5/5 to 5/9	Eighth balance period.
5/10 to 5/14	Ninth balance period. Blood samples were secured on 13th.
5/15 to 5/19	Tenth balance period. Underwater weighing. Skin fold measurements on 15th.
5/20 to 5/24	Eleventh balance period.
5/25 to 5/29	Twelfth balance period.
5/30 to 6/3	Thirteenth balance period.

Calendar of events for Bread Study (continued)

Date	Events
6/4 to 6/8	Fourteenth balance period. Blood samples on 8th. Tryptophan load test on fifth. Underwater weighing, skin fold and physical fitness measurements on 5th.
6/8 to 6/12	15th balance period. Control diet was given. Complete physical and MMPI tests.

Description of subjects with result of their physical examinations made at the end of the study

Subject Name	No.	Major Field	Age	Blood Pressure (mm. Hg)		Pulse (Rest.)	Urine Anal.		Hematology		
									White	Hb	Hemot.
			years	Dias.	Systol.	Per mm.	Sp. Gr.	pH	Cells	gm/100 ml	%
Atekwana J.	1	Agriculture	27	118	72	72	1.022	Ac[1]		15.2	42.0
Berry A J.	2	Biochemistry	22	122	76	74	1.012	Al	6,850	14.9	45.0
Bruin W. J.	3	Chemistry	22	124	80	74	1.026	Al	5,750	13.8	42.5
Bolock L. K.	4	Forestry	22	128	82	78	1.027	Ac	8,850	15.5	48.0
Ettah A. E	5	Forestry	25	122	74	70	1.020	Al	6,700	14.0	42.0
Haile Mariam S.N.	6	Horticulture	27	118	72	70	1.027	Ac	5,050	14.3	42.0
Hess J. L.	7	Biochemistry	24	122	76	74	1.017	Ac	5,000	13.9	41.5
Judy J.	8	Sociology	23	120	74	78	1.016	Al	8,000	13.8	43.5
Koelsch K. D.	9	German	19	128	82	78	1.022	Ac	8,550	14.6	45.0
Luhring D. L.	10	Vet. Med.	21	128	82	74	1.016	Al	8,400	13.8	42.0
Martz D. L.	11	Non-Pref.	19	122	78	78	1.027	Ac	5,800	13.8	42.0
Stone J. A.	12	Biochemistry	22	126	78	74	1.010	Al	7,450	14.8	42.0

[1] Ac = Acid
Al = Alkaline

This information was provided by the Olin Health Center Laboratory.

Table ii

Height and body weights of the subjects at the start of study with deviations from standard.

Subj. No.	Ht.	Wt.	Dev. from "Std."
	Cm.	Kg.	%
1	165	70.45	+ 8
2	170	65.91	+ 1
3	170	74.14	0
4	173	75.00	+ 5
5	175	72.73	+ 3
6	178	77.27	0
7	167	68.82	+ 6
8	179	71.45	+ 1
9	180	71.45	-5
10	183	79.54	0
11	178	70.00	0
12	173	68.18	+ 7

Table iii

Average body weights (in kg.) for each subject for each metabolic period. These values are for the control phase when the "normal diet" was served.

Subjects	1	2	3	4
1	70.45	70.22	70.16	69.78
2	65.91	67.04	67.14	66.94
3	74.14	74.46	74.29	74.08
4	75.00	74.91	74.37	74.26
5	72.73	69.81	69.88	69.92
6	77.27	76.61	76.74	76.60
7	68.82	68.35	67.62	67.36
8	71.45	68.96	66.96	69.14
9	71.45	71.29	71.59	71.36
10	79.54	77.72	76.97	76.97
11	70.00	70.44	70.43	69.98
12	68.18	68.10	68.27	68.80
Average	72.07	71.57	71.20	71.26
Stand. Dev.	± 3.48	± 3.58	± 3.60	± 3.41

Table iv

Average body weights (in kg) for each subject for each metabolic period. These values are for the experimental phase when the "Bread Diet" was served.

Subject	5	6	7	8	9	10	11	12	13	14
1	70.06	70.00	70.07	69.78	69.64	69.60	69.46	69.28	69.26	69.40
2	67.59	66.94	67.17	66.82	67.18	67.06	67.10	67.32	67.58	67.85
3	74.44	73.96	74.36	73.78	73.86	73.34	73.27	73.29	73.11	72.98
4	74.38	74.05	73.98	73.57	74.22	73.81	73.96	74.20	74.28	74.58
5	69.40	68.34	68.96	69.10	69.10	69.00	69.22	69.52	69.20	69.00
6	76.87	76.61	77.37	76.11	76.34	75.90	76.28	76.67	76.45	76.00
7	67.49	66.80	66.94	66.67	67.00	66.54	66.60	67.12	67.18	67.65
8	69.72	69.14	68.96	68.60	68.90	68.18	68.70	68.72	68.50	68.62
9	71.15	70.62	70.31	70.31	70.72	70.31	70.11	70.12	69.90	70.75
10	77.16	76.46	76.77	76.88	76.73	76.00	76.68	76.45	75.92	76.33
11	69.72	69.27	68.82	68.62	68.67	68.95	68.98	69.63	69.37	69.51
12	69.23	69.15	69.19	69.82	69.00	68.87	68.75	68.44	68.67	68.61
Average	71.42	70.95	71.04	70.84	70.95	70.60	70.76	70.81	70.81	70.94
Stand. Dev.	±3.39	±3.45	±3.54	±3.43	±3.43	±3.16	±3.67	±3.78	±3.31	±3.71

Table v

Total serum protein

Grams percent

Subject	Control		Experimental	
No.	Begin	End	Midpoint	End
1	6.10	6.90	5.66	6.70
2	6.91	6.90	6.90	6.75
3	7.80	7.38	7.22	7.64
4	6.56	6.90	5.39	6.23
5	7.53	7.42		6.60
6	7.14	7.22	5.31	7.77
7	6.98	7.53	7.22	7.75
8	6.74	7.56	7.22	8.19
9	6.46	6.88	7.06	6.57
10	8.08	8.16	6.59	7.92
11	6.79	5.42	6.58	7.06
12	6.18	6.47		7.94
Average	7.05	7.06	6.84	7.26
S.D.	±.70	±.67	±.73	±.67

Total serum protein determination was carried out using the Biuret method described by Wendell T. Caraway,Ph.D. in "Micro chemical methods for blood analysis" and modified to be read by the Coleman spectrophotemeter as follows:

1. .1 ml of serum was pipeted into a 12 x 75 mm Cuvet.
2. 8 ml of Biuret reagent were added and mixed well by tapping and kept for 30 minutes.
3. The tubes were read at 550 mu. Total protein was determined by multiplying optical density by a total protein factor which was obtained by using standard control serum (normal clinical chemistry control serum) Hyland Laboratories, Los Angeles 39, California.

Table vi

Serum protein components

Subject No.	% Albumin				% Globulins			
	Control		Experimental		Control		Experimental	
	Begin	End	Mid-point	End	Begin	End	Mid-point	End
1	75.0	69.7	75.5	76.8	25.0	30.2	26.9	23.2
2	73.8	78.6	76.2	74.1	26.2	21.5	23.6	25.9
3	84.5	81.0	85.0	78.4	15.4	19.1	14.9	21.5
4	83.3	80.4	75.0	88.1	17.2	19.6	24.9	11.8
5	62.3	62.4	58.9	64.8	37.7	37.6	41.0	35.2
6	83.5	79.1	76.4	75.8	17.0	20.9	23.5	24.1
7	80.7	74.5	83.8	85.9	19.2	25.5	16.2	14.0
8	85.0	76.3	81.4	77.3	15.0	23.6	18.5	22.7
9	77.4	81.6	75.3	79.0	22.6	18.3	24.1	20.1
10	74.5	77.9	78.2	73.6	25.5	22.0	22.4	25.8
11	77.7	81.8	81.5	81.0	22.3	18.1	18.5	19.0
12	74.4	76.5	71.3	73.4	25.4	29.0	28.6	26.6
Ave.	77.6	76.6	76.5	77.4	22.3	23.8	23.7	22.5
S.D.	± 6.7	± 5.6	± 6.8	± 6.1	± 6.3	± 5.8	± 6.9	± 6.1

Table vii

Level of two enzymes in serum of subjects

	Serum glutamic-pyruvic-transaminase				Serum glutamic-pyruvic-transaminase			
	Control		Experimental		Control		Experimental	
No.	Begin	End	Mid-point	End	Begin	End	Mid-point	End
1	34*	54	42	50	16	12	20	38
2	38	61	48	46	31	22	35	43
3	23	35	48	46	10	10	21	33
4	44	58	120	130	44	48	74	210
5	25	31	48	22	15	12	20	27
6	35	20	44	62	19	13	22	36
7	28	29	20	38	10	13	20	28
8	34	49	34	90	10	20	33	43
9	36	46	28	54	20	8	25	43
10	28	40	22	32	12	15	20	24
11	34	49	44	48	13	11	30	34
12	32	68	58	48	19	36	30	33
Ave.	32.5	45.0	56.3	55.3	18.2	18.3	29.1	42.5
S.D.	± 4.83	±10.48	± 12.0	± 17.4	± 7.7	± 7.9	± 5.8	± 6.1

* The values are expressed in sigma Frankel Unit.

Table viii

Hemoglobin and hemotocrit of the subjects

	Control		Experimental		Control		Experimental	
No.	Begin	End	Mid-point	End	Begin	End	Mid-point	End
1	15.2	14.6	15.5	15.2	39.7	41.0	42.0	42.0
2	14.5	14.0	14.7	14.9	41.2	44.5	44.0	44.5
3	13.5	13.7	14.1	13.8	42.5	43.0	45.0	45.5
4	15.6	13.9	15.9	15.5	45.0	48.0	42.7	45.5
5	14.6	14.2	13.3	14.0	42.0	41.0	38.0	40.7
6	15.5	14.1	15.1	14.3	42.0	43.0	45.0	44.0
7	12.9	14.9	13.8	13.9	42.7	42.0	43.0	43.0
8	14.0	13.4	13.3	13.8	43.2	41.5	45.0	45.0
9	13.3	13.7	13.7	13.4	43.7	43.0	44.0	46.0
10	14.9	14.2	14.2	13.8	44.0	41.0	39.0	37.5
11	14.3	14.5	14.6	13.8	44.0	39.0	42.0	41.5
12	14.8	15.6	15.8	14.8	41.2	40.0	39.5	41.2
Ave.	14.4	14.6	14.5	14.2	42.6	42.2	42.4	43.0
S.D.	± 2.6	± 1.04	± 0.89	± 1.61	± 1.48	± 2.44	± 2.54	± 2.64

Table ix

Serum "free" iron

(Micrograms/100 ml)

Subject No.	Control		Experimental	
	Beginning	End	Beginning	End
1	162	118	124	122
2	190	82	94	90
3	176	184	166	124
4	146	190	158	84
5	152	186	178	210
6	142	108	128	192
7	158	142	156	156
8	168	174	178	144
9	186	172	186	172
10	162	176	172	200
11	168	110	190	144
12	200	106	196	162
Ave.	167.6±17.8	145.6±38.8	160.5±30.7	150.0± 40.5

The method of W.N.M. Ramsay, The determination of Iron in Blood Plasma or Serum, Clinical Chemical Acta 2, 214 (1957) was used.

Table x

Daily urinary creatinine excretion as measured on 5-day pooled urine samples. For the control period, the urine covers the second metabolic period while the experimental period sample represents the sixth metabolic period. The values are grams of creatinine excreted per 24-hours.

Subject No.	Control Grams Apr. 8	Experimental Grams May 20
1	1.66	1.71
2	1.84	1.51
3	1.71	1.74
4	1.86	1.64
5	2.07	1.72
6	1.58	1.70
7	1.34	1.67
8	1.54	1.63
9	1.72	1.01
10	2.1	2.02
11	2.67	1.69
12	1.68	1.62
Average	1.81±.052	1.64±.072

The excretion of creatinine by the subjects was measured in composits of urine samples secured during the second 5-day metabolic period of control part and the sixth 5-day metabolic period of experimental part.

Table xi

Twenty-four hour xanthurenic acid excretion before and after feeding 2 gms of tryptophane

No.	Before	After[1]
1	40.2	56.9
2	86.9	134.1
3	66.6	48.2
4	46.4	56.4
5	36.4	58.8
6	68.0	53.0
7	90.9	69.9
8	53.4	67.5
9	69.9	54.0
10	90.2	57.0
11	61.2	81.9
12	81.6	72.6
Ave.	66.3± 18.2	68.9 ±23.0

In order to rule out any deficiency of pyrodoxin or B_6 due to a high bread intake and absence of animal products from the diet a tryptophane load test was performed.

[1] The tryptophane was given in orange juice at 8 A.M. of the day the second urine sample was collected.

Table xii

Determined amino acids in diet samples (mg/day)

Days	Lysine	Isoleucine	Leucine	Methionine	Threonine	Phenyl-alanine	Valine	Cystine	Tyrosine	Tryptophane
					Control					
1	2089.4	6791.9	5433.7	2346.0	4489.3	2122.6	5218.1	1047.6	1521.8	759.6
2	1894.2	5969.8	6843.8	2274.7	4511.2	2870.4	5676.0	1302.5	1725.5	820.9
3	1463.7	5061.7	4369.3	1559.7	3399.8	2097.6	4318.0	1152.2	1032.8	865.0
4	1895.8	3788.1	5410.0	1237.5	2413.6	2053.8	4189.5	1237.5	1220.5	778.1
5	1076.6	6218.1	4651.6	1849.1	2669.4	1873.8	4103.7	1114.4	1286.8	686.0
6	3222.2	4589.0	4189.7	2021.1	3052.5	2312.8	4147.4	1058.4	1563.6	932.0
7	3214.7	3247.8	3528.3	1959.8	3271.9	2034.9	4198.1	1073.6	1595.8	854.1
Ave.	2122.4	5094.9	4932.3	1906.8	3401.1	2195.0	3185.1	1140.9	1420.9	813.7
					Experimental					
1	1718.3	5483.4	4466.7	2179.7	1592.2	2353.7	4559.8	1295.8	1056.1	633.9
2	1009.1	5635.5	4107.4	2685.4	1193.8	2172.3	4250.8	1364.9	1166.8	895.8
3	905.2	5488.3	4089.8	3196.8	1852.9	2128.6	4092.6	1499.4	1135.8	530.1
4	1308.5	5663.1	4328.2	3068.2	1985.2	1275.4	3739.4	1458.8	1086.7	632.6
5	1477.4	6467.5	4828.9	3131.3	1908.4	1243.5	3995.3	1669.8	1357.4	622.5
6	1276.4	4828.4	3834.6	2621.4	1807.9	1196.9	4079.2	1705.4	1127.8	846.6
7	1190.5	5007.3	5019.6	3185.2	934.6	2905.1	3714.1	1268.7	1035.6	883.2
Ave.	1269.3	5510.5	4381.5	2886.1	1610.6	1896.5	4068.6	1466.1	1138.0	720.7

Table xiii

Amino acid level of blood plasma

All values in mg per 100 ml plasma

Tyrosine in blood				
Sub-	Control		Experimental	
ject	Beginning	End	Midpoint	End
1	.56	.60	.98	.93
2	.51	.66	.84	.60
3	.47	.47	.45	.39
4	.78	.80	.80	.41
5	.75	.80	.18	.84
6	.85	.77	.36	.39
7	.33	.47	.87	.44
8	.54	.85	.75	.51
9	.75	.86	.51	.66
10	.80	.48	.54	.84
11	.78	.72	.72	.70
12	.78	.66	.70	.90
Ave.	.69±.2	.68±.1	.71±.2	.74±.2
Normal Range	.81 - 1.45			

Tryptophane in blood				
Sub-	Control		Experimental	
ject	Beginning	End	Midpoint	End
1	1.20	1.23	0.75	1.02
2	1.08	1.08	0.96	0.93
3	1.08	0.69	0.96	0.96
4	1.14	1.14	1.14	0.95
5	1.17	0.71	0.96	0.90
6	0.99	1.25	1.14	1.06
7	0.93	0.60	0.72	1.02
8	1.05	0.99	1.14	0.96
9	1.02	0.75	0.69	1.08
10	0.69	0.78	1.09	1.05
11	0.96	0.96	0.84	0.96
12	1.05	1.02	0.96	0.69
Ave.	1.03±.1	0.956±.2	0.85±.2	0.96±.1
Normal Range	.7 - 1.5			

Amino acid level of blood plasma

All values in mg per 100 ml plasma

Valine in blood				
Subject	Beginning	End	Midpoint	End
1	2.02	2.70	1.87	2.05
2	2.46	1.90	2.37	2.13
3	1.95	1.99	1.66	2.07
4	2.77	2.52	1.68	1.45
5	2.23	2.22	2.53	1.84
6	3.66	1.81	1.80	1.86
7	3.39	2.49	1.90	1.62
8	2.02	2.49	1.90	1.62
9	2.87	1.99	1.92	1.69
10	2.37	1.56	2.26	1.71
11	2.19	2.04	2.16	1.96
12	1.93	2.23	1.92	1.96
Ave.	$2.49 \pm .6$	$2.60 \pm .3$	$*1.99 \pm .3$	$*1.86 \pm .2$
Normal Range		2.37 - 3.71		

Cystine in blood				
Subject	Beginning	End	Midpoint	End
1	.330	.563	.465	.450
2	.530	.533	.405	.457
3	.290	.450	.570	.450
4	.580	.427	.420	.412
5	.600	.420	.420	.412
6	.562	.427	.465	.413
7	.375	.420	.420	.405
8	.563	.465	.420	.562
9	.310	.420	.465	.450
10	.330	.360	.450	.460
11	.375	.420	.547	.487
12	.532	.577	.540	.450
Ave.	$.42 \pm .1$	$.46 \pm .06$	$.47 \pm .05$	$.45 \pm .04$
Normal Range		.3 - .7		

* Statistically significant $P<0.01$

Amino acid level of blood plasma

All values in mg per 100 ml plasma

Threonine in blood				
Subject	Control		Experimental	
	Beginning	End	Midpoint	End
1	2.06	2.30	2.30	2.25
2	2.81	2.30	2.30	2.30
3	1.72	0.74	2.25	2.06
4	0.97	2.06	2.25	2.25
5	2.81	2.81	2.25	2.06
6	1.72	2.06	2.25	2.25
7	0.75	2.06	1.65	1.65
8	2.32	1.72	2.06	2.17
9	2.31	1.95	2.06	2.40
10	2.31	1.72	2.06	2.25
11	2.28	1.72	2.25	2.17
12	1.75	2.06	2.30	2.06
Ave.	$1.985^{\pm}.6$	$1.96^{\pm}.5$	$2.16^{\pm}.2$	$2.02^{\pm}.2$
Normal Range		1.21 - 1.72		

Phenylalanine in blood				
Subject	Control		Experimental	
	Beginning	End	Midpoint	End
1	.60	.84	.90	1.14
2	.93	.90	.44	.81
3	.81	.81	.39	.92
4	.59	.93	.96	.96
5	.56	.45	.89	1.04
6	.62	.81	.59	.81
7	.60	.32	.39	.96
8	.53	.33	.45	.77
9	1.11	.39	.53	.96
10	1.08	.76	.77	.87
11	.83	.82	.77	.77
12	.83	.78	.81	.96
Ave.	$.76^{\pm}.2$	$.83^{\pm}.2$	$.77^{\pm}.2$	$*.914^{\pm}.1$
Normal Range		.69 - .95		

* Statistically significant $P < 0.05$.

Plasma amino acid level of blood secured at intervals throughout the study.

All values in mg per 100 ml plasma

Lysine in blood				
Sub-	Control		Experimental	
ject	Beginning	End	Midpoint	End
1	2.83	3.12	1.98	1.30
2	3.10	2.07	2.74	1.74
3	2.28	2.94	1.32	2.22
4	2.38	2.94	1.44	1.43
5	3.12	2.92	1.26	2.53
6	3.10	1.80	2.04	1.89
7	3.18	2.32	2.04	2.22
8	3.05	2.37	1.74	2.43
9	2.43	2.49	1.38	1.62
10	2.19	2.37	2.16	2.65
11	2.70	2.53	2.48	2.74
12	2.04	1.86	1.95	1.59
Av.	2.70±0.4	2.48±.4	*1.88±.5	*2.03±.5
Normal Range**	2.7			

Isoleucine in blood				
Sub-	Control		Experimental	
ject	Beginning	End	Midpoint	End
1	.48	.54	.92	.45
2	.42	1.40	.81	.53
3	.54	.84	.54	.53
4	.78	.72	.51	.72
5	.51	.50	.72	.74
6	.57	.47	.72	.83
7	.51	.61	.27	.74
8	.42	.42	.66	.83
9	.42	.54	.26	.72
10	.40	.96	.78	.72
111	.47	.60	1.29	.96
12	.81	.72	.77	.89
Ave.	.53±.1	.69±.2	.77±.7	.72±.1
Normal Range	.69 - 1.28			

* Statistically significant $P<0.05$

** D. S. Dittmer Biological Handbook, Blood and other Body Fluids, FASEB, Washington, D.C.

Plasma amino acid level of blood secured at intervals throughout the study.

All values in mg per 100 ml plasma

Lysine in blood				
Sub-	Control		Experimental	
ject	Beginning	End	Midpoint	End
1	2.83	3.12	1.98	1.30
2	3.10	2.07	2.74	1.74
3	2.28	2.94	1.32	2.22
4	2.38	2.94	1.44	1.43
5	3.12	2.92	1.26	2.53
6	3.10	1.80	2.04	1.89
7	3.18	2.32	2.04	2.22
8	3.05	2.37	1.74	2.43
9	2.43	2.49	1.38	1.62
10	2.19	2.37	2.16	2.65
11	2.70	2.53	2.48	2.74
12	2.04	1.86	1.95	1.59
Av.	2.70±0.4	2.48±.4	*1.88±.5	*2.03±.5
Normal Range**	2.7			

Isoleucine in blood				
Sub-	Control		Experimental	
ject	Beginning	End	Midpoint	End
1	.48	.54	.92	.45
2	.42	1.40	.81	.53
3	.54	.84	.54	.53
4	.78	.72	.51	.72
5	.51	.50	.72	.74
6	.57	.47	.72	.83
7	.51	.61	.27	.74
8	.42	.42	.66	.83
9	.42	.54	.26	.72
10	.40	.96	.78	.72
111	.47	.60	1.29	.96
12	.81	.72	.77	.89
Ave.	.53±.1	.69±.2	.77±.7	.72±.1
Normal Range	.69 - 1.28			

* Statistically significant $P<0.05$

** D. S. Dittmer Biological Handbook, Blood and other Body Fluids, FASEB, Washington, D.C.

Amino acid level of blood plasma

All values in mg per 100 ml plasma

Lysine in blood				
Sub-	Control		Experimental	
ject	Beginning	End	Midpoint	End
1	1.66	1.82	1.53	1.37
2	1.83	1.82	1.81	1.40
3	1.61	1.86	1.05	1.79
4	1.53	1.70	1.50	1.99
5	1.68	1.59	0.99	2.02
6	1.81	1.97	1.06	1.65
7	2.06	1.78	1.97	1.46
8	2.15	1.90	1.44	2.04
9	1.23	1.84	1.29	1.51
10	1.14	1.55	1.48	1.90
11	2.05	1.10	0.91	1.34
12	2.33	1.96	1.27	1.46
Ave.	$1.76 \pm .3$	$1.58 \pm .2$	$*1.36 \pm .3$	$1.66 \pm .3$
Normal Range	1.42 - 2.30			

Isoleucine in blood				
Sub-	Control		Experimental	
ject	Beginning	End	Midpoint	End
1	.30	.38	.43	.36
2	.27	.38	.38	.31
3	.27	.38	.33	.35
4	.26	.35	.33	.35
5	.27	.35	.33	.35
6	.20	.26	.36	.38
7	.26	.24	.32	.37
8	.41	.19	.42	.35
9	.40	.27	.41	.39
10	.39	.35	.34	.39
11	.35	.35	.41	.35
12	.35	.35	.41	.39
Ave.	$.31 \pm 0.06$	$.32 \pm .06$	$*.37 \pm .02$	$*.36 \pm .01$
Normal Range	.33 - .42			

* Statistically significant $P < 0.05$.

Table xiv

Urinary amino acid excretion of subjects during control and experimental phases. The control sample was for the pooled urine samples collected during the 4th metabolic period; the experimental sample was for the 14th metabolic period. All values are expressed as mg per day.

Sub-	Cystine		Isoleucine		Leucine		Lysine		Methionine	
jects	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
1	90.0	122.8	31.3	21.6	29.3	---**	96.6	114.8	52.2	26.6
2	131.4	170.7	35.6	28.9	37.2	30.2	179.6	177.3	50.1	84.1
3	112.5	145.1	15.0	28.6	33.6	29.6	109.7	105.3	59.8	65.4
4	93.7	166.0	31.5	28.2	30.4	24.5	92.4	110.0	53.5	65.3
5	114.7	140.8	47.0	19.0	33.5	22.8	104.9	110.0	36.2	60.4
6	136	143.8	44.0	19.0	32.8	23.0	103.8	100.9	72.5	59.2
7	141.3	148.6	28.3	20.6	14.0	28.8	89.2	119.3	42.1	63.3
8	142.9	157.2	22.3	68.1	30.1	23.0	106.4	109.2	31.7	66.1
9	103.1	118.1	26.4	20.9	33.7	20.9	97.0	153.4	26.4	82.8
10	119.9	162.5	42.3	28.1	70.1	35.7	158.6	219.6	63.0	75.6
11	123.6	138.9	35.3	59.9	34.2	37.9	179.9	159.7	77.1	83.8
12	118.0	127.5	37.5	50.2	13.5	30.5	157.2	157.0	53.9	87.3
Average	118.9	145.16	49.76	32.75	32.7	27.90	122.7	136.4	50.7	67.57
Std.Dev.	±17.4	±16.9***	±9.3	±16.9	±14.0	±9.6	±35.0	±36.8	±15.5	±16.6***
Normal Values*	119 (70 - 182)		14 (7 - 21)		21 (15.4 - 31.4)		56 (33.6-119)		98 (70 - 199)	

* D. S Dittmer Biological Handbook, Blood and other Body Fluids. FASEB, Washington, D.C.

** Sample lost.

*** Statistically Significant $P < 0.02$.

Urinary amino acid excretion of subjects during control and experimental phases (continued)

Sub-jects	Phenylalanine		Threonine		Tryptophane		Tyrosine		Valine	
	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
1	22.8	12.8	59.6	64.6	17.0	10.6	24.0	44.2	34.7	33.6
2	31.1	22.0	62.1	80.6	24.0	38.6	25.2	35.0	39.9	35.9
3	22.4	37.5	89.0	105.1	33.8	40.6	26.0	61.0	37.3	31.1
4	26.5	21.4	66.4	66.4	24.2	36.0	27.7	34.1	33.2	26.9
5	18.0	19.6	49.0	58.5	21.1	20.6	23.8	21.2	76.6	21.9
6	22.3	13.9	80.8	38.4	22.6	24.6	24.8	42.7	62.4	32.0
7	21.8	17.0	73.2	50.4	20.2	24.6	20.4	31.8	48.2	29.7
8	34.6	24.3	66.7	57.4	33.1	45.3	37.1	54.3	44.4	34.9
9	29.1	24.0	71.1	57.5	106.4	33.0	30.5	38.5	50.5	24.9
10	15.8	36.8	68.1	43.0	46.5	32.7	47.1	63.1	23.7	36.3
11	34.0	62.8	77.4	62.8	38.0	24.9	36.8	27.7	56.1	30.7
12	17.7	20.8	53.4	74.0	127.0	21.8	15.2	25.7	53.9	21.5
Average	24.67	26.07	67.8	63.2	51.4	29.44	28.22	39.9	46.7	29.9
Std.Dev.	±6.4	±13.8	±11.3	±17.2	±35.8	±9.9	±8.5	±13.6***	±14.4	±5.1***
Normal values	21 (14.7 -37)		35(25.2 - 182)		28 (16 - 15)		50.8 (30.8 - 57)		21.0 (11.9-42.0)	

***Statistically Significant $P < 0.02$.