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Evaluation of selected biochemical indices  
of protein-calorie malnutrition in rats

by

Josefa Sevilla Eusebio

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## INTRODUCTION

Children of developing countries today continue to be afflicted with protein-calorie malnutrition (PCM). This deficiency disease usually is chronic and occurs in some vaguely classifiable forms. Historically, the word kwashiorkor (k wäsh ē 'or ker) was introduced into the nutritional and medical vocabulary by Dr. Cicely Williams in 1933 when she was working in the Gold Coast (now Ghana) of West Africa (Hathcock and Eusebio, 1977). To the Ga people with whom Dr. Williams worked, kwashiorkor was a specific name given to a specific disease of the displaced baby when the next one is born. The symptoms of the disease are delayed growth, swelling of the feet and legs, irritability, gross apathy, changes in hair color, severe and rather characteristic skin disorders, enlarged livers, and in many cases, diarrhea and eventual death. The disease was associated with protein deficiency, since patients had diets with substantial amounts of energy but little protein. Epidemiological data in many other countries also show that kwashiorkor is common among children fed low protein and high carbohydrate diets.

Marasmus is the term used to describe a severe growth retardation and wasting of the muscle mass and subcutaneous fat. Marasmic children appear literally to be "skin and bones" except for a bloated abdomen. At the same time, these children may show almost no change in blood composition. In severe cases, the visible muscle of the arms and legs may nearly disappear. Often the marasmic child is too weak to be irritable or cry. The symptoms of both kwashiorkor and marasmus can occur simultaneously in the same child, otherwise known as the

marasmic-kwashiorkor syndrome. Kwashiorkor, associated with a relatively normal calorie intake, is at one end of the spectrum; marasmus, associated with suboptimal calorie intake, is at the other end. Marasmic-kwashiorkor lies at the center of the spectrum. These forms of PCM are often easily diagnosed clinically through overt signs without using biochemical information.

Gopalan (1975) reported in longitudinal studies that there are no striking qualitative differences in the dietary pattern or on the protein-calorie ratio of diets between cases of marasmus and kwashiorkor, on the one hand, and the undernourished children in the community, on the other. Among communities of poor children subsisting on almost uniform protein-calorie deficient diets, kwashiorkor, marasmus, and nutritional dwarfism are often found to coexist. Approximately 1-3% of the children below five years of age show signs of either kwashiorkor or marasmus. Not only do marasmus and kwashiorkor coexist in the same community but they also can be seen in one child who feeds on the same diet at different points in time. Marasmic children may eventually develop kwashiorkor (marasmic-kwashiorkor) and children with kwashiorkor may look marasmic if they survive long enough for the edema to subside. Gopalan (1968) had originally proposed that kwashiorkor and marasmus result, not from a difference in the diet, but in the capacity of the child to adapt.

It is difficult to recognize the malnutrition condition during the subclinical and marginal stages of the deficiency disease. Growth failure, low weight compared to standards for age, may occur without clinical signs. A nutritional dwarf can be easily mistaken for a healthy younger child. Reliable biochemical information would be very useful,

especially during these early stages of malnutrition, to prevent many nutritionally deficient children from developing the severe symptoms; such methods could help assess those who are on their way to recovery.

Depletion of nutrients during nutrient deprivation will generally be reflected in the low nutrient levels in the stores and the tissues. As the deprivation state continues, the tissue damage and disturbances in function may be accompanied by biochemical lesions. These are characterized by the appearance of metabolites or changes in nutrient or enzyme levels. The biochemical response in PCM, by and large, would therefore depend on the relative proportions of protein and calories in the diet, and even on the source of the protein and calories. If the intake of calories is inadequate for energy needs, the diversion of protein as an energy source may deprive the body of essential amino acids required for the enzyme systems. If the deficiency is caused by poor quality protein, the metabolic changes in the body depend on the amino acid pattern of the protein in the diet. One particular pattern may allow the synthesis of some enzymes but not the others. Secondary deficiencies can also arise because a deficiency of one nutrient may lead to disturbances in the metabolism of another.

So far, no laboratory has been able to produce a completely satisfactory set of biochemical tests for PCM that is suitable for use in all parts of the world (Sauberlich, 1974). The use of several biochemical measurements, carefully interpreted, can provide a reasonable basis for proper diagnosis when used in conjunction with clinical and anthropometric assessments. One goal of biochemical tests is to achieve measurements that reflect the total metabolic pool. The levels of the various

nutrients should then be a true reflection of the nutritional status of the organism. Furthermore, such biochemical demonstration of metabolic changes during the development of PCM can be useful as clues to the fundamental lesions that develop later on.

In the laboratory where experimental conditions can be controlled, biochemical determinations play a major role in the evaluation of nutritional status. In nutrition surveys, the situation is far more complicated and the value of biochemical tests as indices of nutritional status can be very uncertain, if not properly interpreted. Interrelationships between nutrients and the multiple nature of deficiencies in many persons complicate and cloud the biochemical picture. Depletion of several nutrients may be taking place at different rates and the body may be making attempts to adapt its functions to these deficiencies. For instance, it is often difficult to collect biochemical information from a malnourished child during the severe states when treatment is urgently needed. Changes during the stages of recovery may also be altered by medical treatment.

In order to study biochemical indices, it is essential to produce experimental PCM in animals. Such studies would contribute to the understanding of the clinical symptoms and, more importantly, the subclinical changes which precede them. If the relationships among diet, cellular functions, and blood/urine biochemistry can be established, actual detection of certain biochemical abnormalities can be related to changes in body functions as a consequence of malnutrition. Controlled nutritional experiments with animals should facilitate such insights.

Choice of the biochemical tests depends on several factors. Tests to be used for research purposes could be highly specific and perhaps complex. Tests to be used for clinical screening would require less refined measurements. Biochemical tests designed to use easily obtainable materials, such as blood and urine which can be sampled without undue trauma, are particularly important and useful in nutrition surveys of populations in developing countries. Those which can be performed by semiskilled technicians with minimum apparatus are most practical and are, of course, limited by the facilities and resources of the laboratory.

Although biochemical tests seem to have, at the present time, limited use in the evaluation of PCM, they should not be abandoned. In fact, a more exact clinical and laboratory definition of the early stages and minor syndromes of PCM is needed to provide greater precision, objectivity, and facility in identifying affected children and in monitoring susceptible groups in areas of low protein and/or calorie intakes. Further research may produce the needed reliable tests. Proving and testing the validity of some recommended biochemical measurements should therefore be considered of prime importance at the present time in the field of nutritional biochemistry. In addition, other biochemical indicators should be investigated.

These experiments were conducted with rats to evaluate simultaneously the validity of several biochemical indices of protein-calorie malnutrition during systematic variations of dietary energy and protein. Intermediate quality protein was chosen because of the

similarity to the average protein intake in human populations having high incidences of PCM.

The clinical condition of experimental PCM was produced in rats fed wheat gluten diets. The experiments were carried out with young rats as growth potential is essential to any reasonable model of infant malnutrition. Alterations in protein, albumin, and nonessential/essential (N/E) amino acid ratios in the blood were assessed, as well as levels of hydroxyproline, urea, and creatinine in the urine, to determine how they were affected by the quantity of wheat gluten protein in the diet and by modifications in the energy intake by replacement of cornstarch by cellulose or restriction of the total food intake. Comparisons also were made of the effects on the biochemical indicators during PCM of the protein source, wheat gluten, with those of lactalbumin and gelatin.

## REVIEW OF LITERATURE

Although at the present no single biochemical indicator can be used satisfactorily to evaluate PCM in communities of children, there are many analytical procedures that have been suggested (Sauberlich, 1974; Robson and Larkin, 1972). Included among these are serum total protein and albumin, serum amino acid ratios, urinary urea/creatinine ratio, and hydroxyproline/creatinine ratio. However, the value of these indices is still unproven and subject to much controversy.

The most commonly used tests at the present and in the past are serum total protein and albumin. In principle, such tests should reflect the nutritional status of the subject at the time of sample collection. Unfortunately, this is not always true.

Whitehead (1965, 1967, 1969) earlier proposed the use of plasma amino acid ratios, serum albumin, and hydroxyproline index to also differentiate between calorie and protein malnutrition in children. Arroyave et al. (1970) had proposed the ratios of several essential amino acids, individually, to glycine as positively correlated with nutritional history and protein status. These workers felt that these ratios or indices are useful for the recognition of marginal or pre-clinical cases of protein malnutrition. In marasmus, however, these do not serve as a gauge of severity.

Said-Akila et al. (1973) used immuno-electrophoresis in estimating the pattern of urine proteins among malnourished Egyptian children, and found an increasing tendency for proteinuria and albuminuria as kwashiorkor progresses. On the other hand, in severe marasmus the



immuno pattern was variable and associated with a number of other components.

Anthony and Edozien (1975) found lowered DNA and RNA content and RNA activity, and a decreased size distribution and amino acid-incorporating activities of liver ribosomes in protein-calorie malnutrition in rats. Sauberlich (1974) also had included sulfur-creatinine ratio as an indicator of protein intake and quality. Determinations of enzyme activities such as those of leucocyte pyruvic kinase, plasma, and urine RNase have been suggested. Serum transferrin levels have been reported to be associated with the severity of kwashiorkor (Ismadi et al., 1971; Gabr et al., 1971; Reeds and Laditan, 1976). Quite recently, the metabolism of 3-methyl histidine has been investigated in experimentally induced protein-energy malnutrition in rats (Nagabhushan and Rao, 1975). Urinary excretion of 3-methyl histidine increases during starvation, when muscle wasting occurs. Further study is needed to prove whether this can be a sensitive indicator to differentiate between kwashiorkor and marasmus.

It should be kept in mind, however, that protein-calorie malnutrition, either kwashiorkor or marasmus, is not a single nutrient deficiency disease except in the rarest cases. Usually there is a lack of vitamins and minerals as well as energy and protein. In children suffering from PCM, therefore, the predominating deficiency of amino acids and calories is almost invariably associated with a deficiency of other nutrients such as vitamins and trace elements which complicates the clinical and metabolic picture of PCM.

Currently available biochemical tests are of little use in the appraisal of energy intake. Anthropometric measurements such as height, weight, and skinfold thickness permit a reasonable approximation of this facet of nutritional status.

#### Experimental PCM in Rats

In 1968, Edozien claimed that all previous experimental models for kwashiorkor and marasmus using animals such as rats and weanling pigs have not been successfully reproduced. The animals showed some moderate water retention, but not the frank edema which is so characteristic of the kwashiorkor syndrome in children. He explained that the reason for failure of earlier attempts to produce experimental kwashiorkor seemed to be that the experimental diets either contained too much protein or were not given for long enough periods. Edozien produced the condition showing the features of kwashiorkor by ad libitum feeding of male rats (weighing 100-130 g) with diets containing 0.5% or 1.0% lactalbumin for two months. He produced marasmus by feeding restricted amounts of the same diets. Mortality rates among the marasmic rats were high apart from severe wasting. The final phase of kwashiorkor with edema appeared during the fourth months, with the earliest signs of "moon face" and a progressive increase in weight.

However, as early as 1920, Kohman already had experimentally produced in rats the classical "war edema" or "war dropsy" characteristic of protein malnutrition during the war years. Type of diet during those years was basically low in protein and has been the cause of development of this unrecognized disease. Feeding of low protein carrot diets

in Kohman's experiments decidedly influenced the development of edema which was not prevented by fats, vitamins, or salts added to the diet. She attributed the edema specifically to protein deficiency.

Subsequent to Edozien's (1968) study, other researchers using rats have successfully reproduced or simulated PCM in children (Whitehead and Coward, 1969; Vasantha, 1970; Sridansky and Verney, 1970; Salem et al., 1973; Anthony and Edozien, 1975; Arthur, 1976). Grimble et al. (1969) failed to produce changes in rats because the diet was not low enough in protein.

Kirsch et al. (1968) fed 100 g male rats with diets containing varying levels of mixed plant and animal protein. No external signs of abnormality were observed in rats on the 8% and 12% protein diets. The rats on 5% protein diet were stunted, had fine hair, and the signs of edema were evidenced by puffy eyes and bulging cheeks.

Salem et al. (1973) fed 7% casein diets to 100-110 g rats and produced the full picture of kwashiorkor with the characteristic edema in ten weeks. The condition which developed in half of the animals was accompanied by diarrhea and loss of hair. The edema was usually localized in the region between the forelimbs. The weight of the edematous rats was constant during a 10-day period. When the edema disappeared, the weight fell and shortly thereafter the animals died.

Philbrick and Hill (1974) fed a low protein diet of 0.5% casein supplemented with methionine. The animals lost 20% of their initial body weight during the first four weeks of the experiment, after which rehabilitation was initiated. At the fifth week, two of the four animals killed and three of the five animals rehabilitated showed edema.

Recently, Arthur (1976) demonstrated experimentally that kwashiorkor can be induced in weanling rats in 3-4 weeks when fed low calorie diets that had 8% gelatin as a sole source of protein. Whereas Edozien (1968) used very low amounts of good quality protein (lactalbumin) and Arthur (1976) a fairly high level of very low quality protein (gelatin), it is important to know how the same animals would respond to similar experimental protein-energy insults by feeding proteins of intermediate quality such as those from wheat, corn, or rice and also varying the energy levels. Such diets simulate actual dietary habits in many developing countries and impoverished communities where dietary proteins are largely derived from high intakes of staple cereal foods. In a preliminary study, Marok (1977) experimentally produced the full condition of kwashiorkor in rats fed 4% wheat gluten protein diets for 5-6 weeks.

#### Serum Total Protein and Albumin

The two tests most commonly used in assessment of protein nutrition in the past are serum total protein and albumin (Sauberlich, 1974). Children with clinical signs of protein malnutrition have low serum total protein and albumin levels (Whitehead, 1967). Albumin levels can be as low as 2 g/100 ml in children with severe kwashiorkor compared to 7-8 g/100 ml in normal children. On the other hand, children with marasmus may have normal serum total protein and albumin levels. Whitehead et al. (1971) reported that a falling serum albumin level can be a reliable indicator of developing kwashiorkor. Albumin normally constitutes more than 50% of total serum proteins and responds to PCM more readily than globulins which are relatively insensitive to nutritional alterations.

In a study of 324 preschool Lebanese children diagnosed to be in a state of suboptimal nutrition, Downs (1964) reported a drop in plasma protein levels from 8 to 7 g/100 ml in 21% of the children during the first year of life. Of the children with albumin values previously in the normal range, 11% were below 3.5 g/100 ml at the end of the year. All globulin levels rose during the 12 month period, the most dramatic increase being in the  $\gamma$ -globulin fraction. At the end of the year, the total plasma globulin levels were above 3.5 g/100 ml in over half of the children. For months, most of the children received a gradually diminishing supply of breast milk supplemented with a mixed diet which was low in calories and proteins; the condition was complicated by illnesses and infections, resulting in growth retardation.

Bengoa et al. (1959) indicated that many workers found serum protein levels in kwashiorkor to be about 4.0-4.5 g/dl. Preclinical kwashiorkor found in 6 out of 100 children studied indicated total serum protein of 4.5-5.6 g/dl compared to 6.73 g/dl in other children. Recognizing these small differences the researchers indicated that, to be of value, serum protein values must be analyzed in conjunction with other tests.

Both total protein and albumin levels in the serum generally do not fall below the normal range until clinical signs are starting to appear (Robson and Larkin, 1972; Philbrick and Hill, 1974). The changes may be restricted to the kwashiorkor type of PCM, since many cases of marasmus have serum protein levels within the normal range. However, the serum albumin level is often lowered in PCM subjects, but not until symptoms are about to appear.

Whitehead (1967) reported that the albumin/globulin ratio may fall markedly below 1.0 in kwashiorkor, compared to 1.0 or above in normal subjects. Baertl et al. (1974) found a high correlation between total protein and albumin in 41 severely malnourished infants. Also, serum albumin levels were significantly correlated ( $r = 0.947$ ) with amino acid levels, particularly of essential ones, as a group and individually. These declines in concentration were mainly the result of changes in hydration or edema. Higher correlations of tryptophan ( $r = 0.74$ ) and the branched-chain amino acids ( $r = 0.5$ ) with albumin suggested a more specific role in determining serum albumin levels. Of those determined, the valine to glycine ratio had the highest correlation ( $r = 0.85$ ) with albumin. Use of the ratios might help recognize severe protein deficiency in infants manifesting edema and yet exhibiting elevated serum protein levels. The authors elaborated that in extreme and prolonged calorie deficiency, amino acids coming from muscles and internal organs are used for albumin synthesis. The opposite situation probably exists in the early stages when weight gain and growth continue despite an inadequate protein intake; serum albumin can be metabolized to provide amino acids to internal organs, muscle, and supporting tissues. If the situation is prolonged, the protein concentration of the tissues decreases and eventually the clinical picture of kwashiorkor develops.

Graham et al. (1966) studied the effects of protein and calorie intakes on serum proteins among malnourished infants. Among the convalescent infants, serum albumin was found to be a reliable indicator of protein adequacy. However, among marasmic infants, calorie deficiency caused by starvation can be so severe that serum albumin does not give any

indication of severity of the concomitant protein deficiency; the albumin level may be high, normal, or nearly normal while calorie deprivation is obvious. Contraction of the cellular fluid due to losses of sodium and water may result in falsely elevated serum total protein and albumin levels; the values after rehydration are more reliable. Excess sodium and water retention may also cause falsely low values. There is no general agreement on the lower limit of serum albumin levels below which protein malnutrition can be confidently stated. They further pointed out that hemoglobin concentrations in hot climates may result in blood nutrient values that are elevated and misleading. Likewise, serum protein levels may be affected by environmental factors such as infections and infestations. The  $\alpha$ -globulin fraction increases and thus elevates protein levels. Serum albumin which usually represents 50% to 65% of serum total proteins might be a more reliable and sensitive index but can also appear normal in children exhibiting inadequate growth rates. In PCM, the tissues may be maintaining normal or near normal protein levels in the blood. This could happen despite inadequacy of protein intake and even in the presence of clinical signs.

Coward et al. (1971) reported values of 3.0 to 4.5 g for serum total protein and 0.8 to 1.5 g/100 ml for serum albumin in kwashiorkor children. In recovered children, total serum values varied from 6.0 to 7.5 g/100 ml and albumin from 3.0 to 4.5 g/100 ml.

In a supplementary feeding study, Habicht et al. (1973) reported values of 7.81 vs. 7.65 g/100 ml for 2-3 year old children with and without food supplements, respectively, and 4.59 vs. 4.60 g/100 ml on albumin values. None of the children were suffering from extreme protein

depletion. This finding illustrates the insensitivity of serum albumin index for the definition of moderate protein malnutrition.

Robson and Larkin (1972), in a summary of various reports, pointed out the problem of hookworm infestation affecting protein metabolism in protein-deficient children, which consequently may cause serum total protein to be extremely low. On the other hand, infections stimulate an increase in serum globulins which in turn inflate serum total protein levels. The effect of infestation and infection cannot always be assessed in nutrition surveys. For this reason, even mean values of serum protein levels in communities may not be suitable for comparative purposes.

In studies with rats, Salem et al. (1973) found a 41% fall in serum protein concentration during the first four weeks of feeding with a 1% casein protein diet. Anthony and Edozien (1975) also observed a fall in concentration of serum protein and albumin in malnourished rats fed 0.5% and 1.0% lactalbumin, and the other diets fed in restricted amounts.

Most recently, serum albumin has been recommended as a powerful diagnostic indicator of marginal and severe PCM (Reeds and Laditan, 1976; Hay et al., 1976; Golden et al., 1975). Children of height-weight measurements in the lower ranges (severely wasted) who had died in the hospitals had serum albumin levels of less than 1.5 g/dl. Serum albumin was recommended as a better indicator than any anthropometric measurement in edematous malnutrition. In the presence of edema, body weight does not estimate tissue wasting.

Though serum albumin and protein are reduced in severe protein deficiency, they are insensitive indices and their use as an index of protein status of individuals and/or populations provides objective



information for only the most severely deficient. Both are found to be insensitive of marginal or mild degrees of protein deprivation. Plasma albumin levels can relate well to protein status only after protein deprivation has been severe and albumin concentrations are abnormally low.

#### Serum/Plasma N/E Amino Acid Ratio

Blood amino acid concentrations have been suggested as potentially useful in population studies (Whitehead, 1964; Whitehead and Dean, 1964). It appears, however, that these are readily altered by more recent dietary intakes of protein and may therefore give a false impression of long-term protein status. Contradictory results are reported from various studies investigating the effects of PCM on serum or plasma amino acid levels.

Arroyave et al. (1962, 1970) and Whitehead (1964) found that total free amino acids in the plasma of children with PCM are lowered, and that the ratio of nonessential to essential amino acids is disturbed or is no longer in the usual balance. With kwashiorkor, the distortion of the ratio was mainly due to the reduction in concentration of certain essential amino acids while the nonessential or dispensable ones remained unaltered. In marasmus, the reduction in serum concentration of the essential amino acids, leucine, isoleucine, methionine, and valine, and of the nonessential amino acids, tyrosine and arginine, also was observed. In the same cases the concentration of other nonessential amino acids remained normal or were elevated. The amino acids ratios also were imbalanced as well in children who, although living on a poor diet, showed

few or none of the clinical signs except a small size for age. The imbalance suggested biochemical changes occurring during the developmental stages of PCM and can therefore be considered as one of the earliest biochemical abnormalities so far discovered.

In well-nourished young rats, Widdowson and Whitehead (1966) observed no significant changes in the amino acid ratios with increasing age. Rats fed on diets low in protein had considerably higher amino acid ratios after two, three, and four weeks. The animals receiving all their protein from "matooke" (a kind of cooking banana) and groundnuts had higher values (mean  $4.41 \pm 0.76$ ) than those obtaining their protein from casein (mean  $3.85 \pm 1.05$ ). If all the values at two, three, and four weeks are taken together on each of the low protein diets at the same ages (mean  $1.63 \pm 0.36$ ), the difference is highly significant for both low protein diets. The undernourished rats which were fed limited amounts of the control diet also showed an increase in the ratio (mean value at 2, 3, and 4 weeks =  $2.44 \pm 0.58$ ) which was smaller than for the two low protein groups.

In 1967, Whitehead recommended the serum amino acid ratio as determined from four nonessential amino acids (glycine, serine, glutamine, and taurine) and four essential amino acids (leucine, isoleucine, valine, and methionine) as a biochemical indicator of PCM. Normally, the amino acid ratio is less than 2.0. Higher ratios usually indicate primary protein malnutrition in children although a normal ratio does not necessarily mean that a child is nutritionally normal (Sauberlich, 1974).

Anasuya and Rao (1968) concluded that the reduction of serum amino nitrogen from amino acids in kwashiorkor was partly because of increased

intracellular fluid resulting in edema. In marasmus, the finding of higher concentrations of amino nitrogen is therefore expected or total amino acids may not be significantly altered.

In a study of 30 cases of marasmic and control children, Singh et al. (1973) found that the N/E amino acid ratio was significantly higher (4.15) among marasmic group of children compared to the control group (1.5-2.5). Absorbancies spectrophotometrically measuring the concentrations of the group of four essential and four nonessential amino acids in paper chromatography bands were used in computing the ratios. On the other hand, Heard et al. (1968) reported that the N/E plasma amino acid ratio was increased in kwashiorkor but not in marasmus.

In a study of 60 children, 2-3 years of age, who were given food supplements to increase protein intakes from 1.75 g/kg/day to 2.5 g/kg/day, Habicht et al. (1973) observed a significantly higher nonessential to essential amino acid ratio in the serum of the supplemented children (2.07) as compared to unsupplemented ones (1.72). This was contrary to what was expected because the ratio increases with inadequate intake and decreases with adequate protein nutrition. Because other evidence, e.g., serum protein and albumin levels, indicated that protein nutriture was adequate in the supplemented children, a high amino acid ratio may suggest that satisfactory dietary levels of different types of protein produce different amino acid ratios.

Philbrick and Hill (1974) also reported the elevation of the ratios of total serum free nonessential to essential amino acids and of free phenylalanine to tyrosine (4.2-12.5) in rats fed low protein diets containing 0.5% casein supplemented with methionine.

Salem et al. (1973) found no correlation of the ratio of nonessential to essential amino acids to the severity of protein deficiency between rats fed adequate (16%) and low (1%) casein diets for 16 weeks. Only the ratio of alanine to threonine and that of serine + glycine to threonine were always significantly higher than normal, the latter being higher in all stages of protein deficiency. There was a tendency of the ratio to increase with body weight deficit. The ratios are shown in Table 1.

Table 1. Serum N/E amino acid ratios in control rats (C) and rats given a low-protein diet (LP)<sup>a</sup>

Period no.	Ratio					
	N/E amino acids		alanine/ threonine		(serine + glycine)/ threonine	
	C	LP	C	LP	C	LP
1. 0-4 weeks	1.15	1.69	1.19	2.20	1.45	4.61
2. 5-8 weeks	1.19	3.05	0.91	1.56	1.48	3.95
3. 9-12 weeks	1.47	1.43	0.91	2.82	1.35	3.31
4. 13-16 weeks	1.27	7.71	0.92	4.01	1.36	6.03

<sup>a</sup>Salem et al. (1973).

Anthony and Edozien (1975) found the concentration of both essential and nonessential amino acids in the serum to be reduced in rats fed 0.5% and 1.0% lactalbumin diets. Valine, leucine, and isoleucine were most affected. Only histidine was markedly increased which was associated with the reported increase in urinary urocanic acid excretion in children with PCM. It was theorized that either a defect in the degradation of this acid was involved or that the turnover rate of histidine was much greater than the capacity of enzyme systems could accommodate. The N/E

amino acid ratio was higher in the marasmus than in the kwashiorkor rats. Sridansky and Verney (1970) showed that during protein deficiency, the skeletal muscles respond by decreasing the protein synthesis and increasing protein catabolism, therefore diverting the amino acids into circulation but at the same time maintaining normal or increased liver protein synthesis. Liver synthesis might be increased and the circulating amino acids are directed towards this synthesis.

It was also suggested by Anthony and Edozien (1975) that the most consistently useful of the plasma amino acid data in the evaluation of protein nutriture is the low concentration of total essential amino acids. The low serum levels, in spite of wide variations in liver amino acids, indicate that the major defect may be one of transport. Serum insulin level was consistently low and, because this hormone is known to play an important role in amino acid transport, it is possible that the low plasma concentration of essential amino acids is related to the synthesis and/or release of this hormone.

Heard et al. (1977) observed the similarity in plasma amino acid ratios in rats fed low-protein diets compared to the severely malnourished child. There were subnormal concentrations of branched-chain amino acids (valine, leucine, and isoleucine), lysine, threonine, and methionine, with maintenance levels or an elevation of the concentration of non-essential amino acids. The imbalance revealed a significantly elevated N/E amino acid ratio in the low-protein fed rats. The ratio appeared normal in the high-protein and high-protein but energy restricted animals. The study also confirmed that the amino acid pattern of chronically energy

restricted rats is qualitatively very similar to that in the plasma of rats which were acutely fasted after previously being adequately nourished.

Sauberlich (1974) recommended additional investigations to evaluate dietary factors other than calories and protein that may influence serum amino acid ratios, including vitamin and mineral deficiencies. The use of the amino acid ratio test as the sole indicator of subclinical protein malnutrition may not be warranted until further studies to validate such tests are made.

#### Urinary Urea and Creatinine

The nitrogen compounds of the urine of an individual in nitrogen equilibrium comprise three classes (White et al., 1973): 1) a large number of diverse compounds excreted daily in small but relatively constant amounts, e.g., creatinine, uric acid, etc.; 2)  $\text{NH}_3$ , the excretion of which is a function of the acid-base economy of the body and is generated in the kidney as a means of excretion of excess protein nitrogen; and 3) urea, the amount of which represents the difference between the dietary intake of nitrogen and the sum of (1) and (2). Circumstances that result in positive nitrogen balance lead to a decrease of urea excretion; in those with excessive excretion of nitrogen at the expense of body proteins the excess urinary nitrogen is excreted as urea. The rate of formation and excretion of urea is the "leveling device" by which nitrogen balance is maintained.

Beginning with Voit in 1866 (cited in Albanese and Orto, 1973) and to the first quarter of this century, urinary urea was almost universally

employed as an index of the quantity and quality of protein ingested. It was also a determinant of the effects of other foodstuffs on protein utilization (Lusk, 1928).

In 1926, Smith reported that the output of urea and ammonia varies with nitrogen intake, whereas the excretion of creatinine and uric acid remains fairly constant. In the early 1950's, it was assumed that urea was the major nitrogenous end product of protein metabolism and that it served no nutritive purpose (San Pietro and Rittenberg, 1953). The rate of excretion of urinary urea is thought to be related to the rate of total metabolism of proteins, whether of tissue or dietary origin.

Schimke (1962) found that variations in urea excretion may be largely mediated by alterations in levels of enzymes specifically associated with urea synthesis. A protein-free diet was reported to result in a 75% decrease in urea excretion compared to rats fed 15% protein diet. On the other hand, starvation was associated with a five-fold decrease in urea excretion. When the quantity or quality of dietary protein was lower than needed, or when the calories supplied with the food did not provide at least 50% or 60% of the daily caloric requirement, total catabolism outweighed anabolism. Body protein was broken down rapidly. More nitrogen was excreted with the urine than was consumed with the food, resulting in a negative nitrogen balance. Normally, 90% of the nitrogen excreted is in the form of urea. During starvation, this value decreases to levels of 53 to 60% and ammonia excretion increases. The progressive reduction of nitrogen excretion, as seen in long-term starvation, semi-starvation, or in protein-free diets, can be a sign of progressive loss of available protein fractions

in the body for urea synthesis or of body adaptation to low food intake or low protein supply.

Picou and Phillips (1972) found lower urea excretion in malnourished children and hypothesized that the reduction in protein intake led to significantly decreased rates of urea synthesis and other pathways. Changes in urea metabolism were observed in both malnourished and recovered children and may be regarded as an adaptation in protein metabolism that tends to conserve nitrogen in response to a shortage of dietary protein. When the malnourished child was compared with the well-nourished one (both on equal intakes of protein), the only difference was a relative and absolute increase in the amount of urea that is produced by the malnourished child because of energy needs.

Anthony and Edozien (1975) reported that young and severely malnourished rats appeared to have limited ability to synthesize urea. Therefore, they excreted more ammonia and other nitrogenous substances such as ethanolamine. When given an amino acid load, intermediary metabolites of the ingested amino acids were excreted. Amino acids of the urea cycle accumulated in the livers of the malnourished rats, especially of the protein deficient rats, suggesting a possible defect in the mechanism for oxidative deamination of amino acids or in the synthesis of urea by the liver. Urinary output of nitrogen fell when protein intake was reduced (Das, 1972). The activities of the urea cycle enzymes changed and paralleled the change in the pattern of nitrogen excretion which adjusted to a new level within 30 hours following a change in dietary protein levels.

A related index proposed for PCM evaluation is the urinary urea/



creatinine ratio (Arroyave and Wilson, 1961; ICNND, 1957-1972; Platt and Heard, 1958; Simmons, 1972; Luyken et al., 1970). An increase or decrease in the level of protein intake is accompanied by marked changes in the urinary urea/creatinine ratio.

In a study of 56 malnourished children, Dugdale and Edkins (1964) found that the ratio of urinary urea to creatinine in a random sample of urine reflects the amount of recent protein intake rather than nutritional status at the community level. Low ratios have been found in subjects consuming low protein diets. Similar reports from field studies were reported by Simmons (1972) wherein an increase in protein intake increases the ratio. He explained that when the glomerular filtration rate is increased in high protein diets, the excretion of urea is also increased but creatinine excretion is not affected; thus the ratio increases.

Coward et al. (1971) studied urea nitrogen/creatinine nitrogen ratios in nine children who had been treated for kwashiorkor by feeding different levels of milk protein for one week. The ratios were found to decrease from a mean value of 84 to 13 with a reduction of protein content of the diet (varying from 4.4 to 0.9 g/kg/day). Wide variabilities of values were found in untreated cases of severe malnutrition. However, values considerably overlapped as protein intakes varied among the children. Although the urea creatinine ratio reflects the amount of protein ingested, it is not established yet as to whether the measurement is precise enough to say whether a diet is adequate.

In a study of PCM rats fed varying levels of gelatin, casein, and soybeans, Arthur (1976) reported urea excretion as indicative of total

nitrogen intake and not of protein quality in the diets. The urea/creatinine ratio was observed to be a less reliable indicator than urinary creatinine excretion rates, particularly with high quality dietary protein; the ratio in relation to body weight decreased, not increased.

Another excretion product of protein metabolism that has been proposed as an index of protein nutriture is urinary creatinine (Arroyave and Wilson, 1961). Folin (1905) first observed that, unlike urea, creatinine excretion was independent of diet. He proposed the idea that protein metabolism which is associated with creatinine production be called endogenous protein metabolism, and that protein metabolism which controls urea excretion and is dependent on dietary protein be called exogenous protein.

Creatinine excretions have been reported of 60-80 mg/kg body cell mass/day for males and 47-51 mg/kg body cell mass/day for females (Randall, 1973}. Creatinine as a measure of muscle mass may, however, be questionable during serious illness when striking decrease in total creatinine excretion and plasma levels occur following a high calorie-protein diet. This can be accompanied by positive nitrogen balance, suggesting some part of creatinine production to be related to muscle catabolism.

Creatinine excretion in the urine is fairly constant. The amount excreted varies, but for each individual the daily output is almost constant. Urinary creatinine bears a direct relation to the muscle mass of the individual. It is low in obese persons and high in heavily muscled individuals of the same height. Therefore, creatinine excretion

has been used to measure total muscle mass. The assumption that creatinine excretion is a function of muscle mass is based on the fact that creatine, the only precursor of creatinine, is found principally in the muscle. In studies with rats, Borsook and Dubnoff (1947) estimated 95% of the body creatine was present in muscles. Chinn (1966) found 80% of the total body creatine in fat-free muscle and observed a high correlation ( $r = 0.93$ ) between body creatine content and urinary creatinine excretion.

In his review of early studies on creatinine, Graystone (1968) supported the high correlation between fat-free body mass and urinary creatinine levels. High correlation coefficients were obtained in normal children with no apparent sex difference when creatinine excretion in mg/day was plotted against body weight. When height was used as a baseline, no sex differences were observed in early childhood. Males and females have similar amounts of muscles per unit height, but not in late childhood when creatinine excretion is compared to either chronological age or bone age. If such relationships document normal growth in muscle mass of childhood, then they may also provide baseline information for the study of abnormal growth and abnormal growth states, such as in malnutrition.

Vestergaard and Leverett (1968) found that meat intake made little difference in creatinine excretion. Although an average coefficient of variation of 7.1% during a 10-day urine collection period was observed, there was relative constancy in the daily creatinine urinary output. On the other hand, Ritchey et al. (1973) found that creatinine excretion varied directly with dietary protein. Fomon (1974) reported that urinary creatinine excretion was 14% higher in infants on a milk formula than on

a soybean formula containing the same amount of protein. Habicht et al. (1973) reported that the urinary urea and creatinine excretion was significantly higher in preschool children given protein food supplements than among those not given any.

In a more recent study involving a group of 34 adult and child subjects, Forbes and Bruining (1976) confirmed the high correlation ( $r = 0.988$ ) between lean body mass, as determined by potassium-40 counting, and urinary creatinine excretion. The use of urinary creatinine as a reasonable index of lean body mass in humans did not contradict results from carcass analyses in animals (Van Niekerk et al., 1963).

Arroyave and Wilson (1961) and Viteri and Alvarado (1970) recommended that the amount of creatinine in a 24-hour sample of urine in relation to a child's height is a good index of the depletion of muscle mass. In malnutrition there is a cessation of growth. There is also muscle wasting associated with an excretion of creatinine which is dictated by the depletion of lean body mass during protein deprivation. Malnourished children were found to have lowered creatinine excretion compared to normal children of the same height.

The relationship of creatinine excretion to height appears useful, as does the ratio of urinary nitrogen to creatinine. Nitrogen/creatinine ratios are greater in countries with high protein intakes than those with low protein consumption (ICNND, 1967). The sulfate/creatinine ratio has also been proposed as a measure of the qualitative and quantitative aspects of dietary protein intake (NAS-NRC, 1963). This index is based on the assumptions that most of the urinary sulfate comes from the sulfur

amino acids and that content of these amino acids is closely related to protein quality.

Expressing creatinine excretion in terms of height is preferred rather than in terms of body weight because of variations in amounts of adipose tissue. Children suffering from PCM have generally lowered creatinine-height indices (Sauberlich, 1974). The index reflects the relationship of creatinine excretion by the subject (mg/day) and that of a normal child of the same height. The ratio is expressed in terms of creatinine excretion/unit of time/cm of body height.

#### Urinary Hydroxyproline

Collagen is one of the few proteins known to contain hydroxyproline. Collagen is the insoluble fiber of connective tissues, the most abundant protein in the body, and constitutes 25-33% of total protein, and therefore 6% of body weight. It is found throughout the body in connective tissues, in cartilage, ligaments, tendons, matrix of bones, the pelvis of kidneys, the ureters. It underlies the skin, serves as binding for blood vessels, and provides intercellular binding substance in parenchymatous organs, such as the liver, and muscles (White et al., 1973).

Excretion of hydroxyproline, an index of collagen formation, is thought to reflect active growth (Jasin et al., 1962; Smiley and Ziff, 1964; Jones et al., 1964; Klein and Teree, 1966). Human growth hormone administration increases urinary hydroxyproline excretion after 10-12 days (Raben, 1959). Since hydroxyproline is found almost exclusively in collagen, its urinary output may reflect bone collagen turnover and may be useful as a guide to metabolic activity of the bone.

There is increasing interest in the use of urinary hydroxyproline excretion as a measure of nutritional status. Whitehead (1965) reported that the excretion of hydroxyproline in the urine may be of help in detecting children who are growing slowly as a result of inadequacies in their diet. As in hormonal dwarfism, a general feature of PCM is a reduced growth rate (nutritional dwarfism). Since hydroxyproline is a product of collagen metabolism, low excretions in the urine reflect the decreased turnover of body collagen, specifically a depression of metabolism.

Urinary hydroxyproline in kwashiorkor children was found to be significantly lower than in normal children (Anasuya and Rao, 1966; Katz, 1970]. The authors also explained that this can be a result of reduced soluble collagen protein or a malfunctioning of its turnover caused by severe protein deficiency. Prockop (1962) found that even in growing rats, a significant fraction of urinary hydroxyproline arises from catabolism of insoluble mature collagen, in addition to that derived from the soluble collagen pool. There is more soluble collagen in the metabolically active fraction.

Widdowson and Whitehead (1966) showed that rats fed protein deficient banana diets for eight weeks and calorie-deficient restricted diets failed to grow and had lower average hydroxyproline excretion (0.8 mmoles) than the casein-fed control group (4.0 mmoles). When the undernourished animals were rehabilitated by giving plentiful amounts of a good diet they grew rapidly, and there was a corresponding rise in the excretion of hydroxyproline.

Whitehead (1969) reviewed the effect of many factors other than diet

on hydroxyproline excretion. Infections can reduce hydroxyproline excretion when there is temporary cessation or retardation of growth. Infections and infestations can actually increase hydroxyproline excretion because of increased turnover of collagen and as more tropo-collagen intermediates are formed. Whitehead and Coward (1969) found negative correlation between hydroxyproline and amino acid ratio, and positive correlation between hydroxyproline and weight for age. Also, rats fed on low protein and on low calorie diets had lower hydroxyproline excretion than those fed on control (ad libitum) diets. Hydroxyproline excretion fell more sharply in the low protein group than in the low calorie group, although growth rate was the same for both groups. The hydroxyproline excretion was found to be related to the soluble collagen concentration of the rat skin.

Anasuya and Rao (1970) studied the relationship of body collagen and urinary hydroxyproline excretion in young rats fed low protein and low calorie diets. Urinary hydroxyproline excretion ( $\mu\text{g}/24$  hours) was lowered more severely in protein deficient rats ( $10.7 \pm 1.77$ ) than in calorie deficient rats ( $33.9 \pm 6.9$ ). For the control group, hydroxyproline excretions ( $\mu\text{g}/24$  hours) were  $248 \pm 9.02$  during the initial period and  $327.2 \pm 16.74$  at the end of the experiment. There was a sharp drop in hydroxyproline excretion during the first week of the study, thus suggesting a possible use in detecting subclinical PCM. Creatinine excretion was not affected to the same degree as hydroxyproline so that the hydroxyproline/creatinine ratio was lower in the malnourished animals.

Picou et al. (1965) also observed low hydroxyproline excretion by malnourished male infants. The increase in urinary levels as recovery

proceeds suggests that low hydroxyproline excretion in man reflects a reduction in the turnover rate of collagen. It was suggested that hydroxyproline excretion can be a useful index of early growth failure before there are significant changes in body weight.

In a study using 30-day old rats, Vasantha (1970) noted that changes in soluble collagen content of the skin occurred earlier than changes in insoluble collagen content. The total collagen content of the skin is lowered in kwashiorkor which may have direct bearing on the cutaneous lesions in PCM (Vasantha et al., 1970).

The urinary content of hydroxyproline related to creatinine has been suggested as an indirect index of protein status of children (Whitehead, 1967). Based on previous studies, McLaren et al. (1970) also proposed that the urinary hydroxyproline/creatinine ratio or index be used.

Anasuya and Rao (1966) reported a high correlation between hydroxyproline and creatinine excretion both for normal ( $r = 0.7954$ ) and kwashiorkor children ( $r = 0.7353$ ). In kwashiorkor children, urinary excretion of hydroxyproline was significantly lower than in normal children. The decreased urinary excretion of hydroxyproline in kwashiorkor patients was attributed to a reduction in soluble collagen pool or its turnover caused by a severe protein deficiency.

Cabacungan et al. (1973) found that the hydroxyproline/creatinine ratios generally followed excretion values but were less closely associated with the other measures of nutritional status than the 24-hour total hydroxyproline excretion values. A decrease from 75.3 to 56.3 mg/24 hours hydroxyproline excretion was observed among girls receiving low protein diets (24 g daily); excretion remained constant among those



on normal dietary proteins (46 g daily). They suggested that the ratio may be valuable as an index in the early stages of subclinical protein malnutrition among young children rather than among older ones.

In a recent study involving 247 four- to five-year old black children in two counties in Mississippi, where undernutrition was thought to be prevalent, Futrell et al. (1975) tested the relationship of hydroxyproline excretion and other indices of nutritional status. A computer program was used to calculate regression coefficients for the subjects. The hydroxyproline index (mmoles hydroxyproline/liter divided by mmoles of creatinine/kg body weight) and hydroxyproline/creatinine ratio (mmoles hydroxyproline divided by mmoles of creatinine) were used as dependent variables, with hemoglobin, hematocrit, height, weight, head circumference, and dietary intake of various nutrients as independent variables. Results showed that hydroxyproline index was the better indicator compared to the hydroxyproline/creatinine ratio. The partial regression coefficients for weight and height were the only significant variables. Only 23 to 34% of the variation in the hydroxyproline index was explained by the other variables. These data seem to agree with the results of Anasuya and Rao (1966) who felt that the variation of the index simply reflects differences in body size. Futrell and coworkers felt that perhaps the children in the population studied were not sufficiently deficient in protein and energy for the index to be a valid indicator. It may thus appear to have no advantage over height and weight as an indicator of nutritional status, since the index is primarily affected by the size of the child.

## EXPERIMENTAL

Four experiments were conducted in this study. The number of rats and the treatments in each experiment are shown in Table 2.

Table 2. Experimental design

						<u>Total no. of rats</u>
<u>Experiment 1</u>						
Protein from wheat gluten	0.4%	1%	2%	10%	25%	
No. of rats	8	8	8	8	8	40
<u>Experiment 2</u>						
Protein from wheat gluten, lactalbumin	2% -	4% -	8% -	16% -	-- 16%	
No. of rats: 5% cellulose	8	8	8	8	8	80
55% cellulose	8	8	8	8	8	
<u>Experiment 3</u>						
Protein from wheat gluten, lactalbumin	2% -	4% -	- 8%	- -	- -	
No. of rats: ad lib.	8	8	8	-	-	48
50% ad lib.	8	8	8	-	-	
<u>Experiment 4</u>						
Protein from wheat gluten, lactalbumin, gelatin	2% - -	4% - -	- 0.5% -	- - 8%	- 8% -	
No. of rats	8	8	8	8	8	<u>40</u>
Total number of rats in four experiments						208

Experiment 1:  
Wheat Gluten Protein for  
Production of Experimental PCM

Objective

The purpose of this experiment was to investigate the effect of varying levels of wheat gluten (WG) on the growth, survival, and production of experimental kwashiorkor in weanling rats. In an earlier study, Marok (1977) was able to produce the full condition of kwashiorkor in rats by feeding 4% WG protein for 6-7 weeks. WG protein is of intermediate biological value, and closely simulates the quality of protein in actual dietary habits in many developing countries where dietary proteins are largely derived from high intakes of staple cereal foods. Comparisons were made of the changes in selected biochemical indicators (serum total proteins, albumin, N/E amino acid ratio, urinary urea, creatinine, and hydroxyproline) and the clinical signs of deficiency.

Animals

Forty male weanling rats of Sprague-Dawley strain were obtained from the stock colony of the Food and Nutrition Department of Iowa State University.<sup>1</sup> The rats were selected from litters of five after weaning at 21 days of age. The rats weighed from 51-65 g with a mean of 56 g. The littermates of five were allotted at random with one to each of the five diets. Eight litters were used to give eight rats per treatment in a randomized block design. All rats were housed individually in suspended wide-meshed stainless steel cages and placed in a constant room

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<sup>1</sup>Original breeding stock from Simonsen Laboratories, White Bear Lake, Minnesota.

temperature (approximately  $76 \pm 2^\circ\text{F}$  and 42-45% relative humidity). The rats received the laboratory stock diet and distilled water for 12 hours after weaning before they were fed the experimental diets. Initial weights were equalized in each treatment. Rats were weighed and tail lengths measured at the beginning and each week to the end of the experiment. The stages of development of the clinical signs of malnutrition were observed.

### Diets

Five experimental rations were formulated to contain WG at 0.4, 1, 2, 10, and 25% protein levels (Table 3). Cornstarch and corn oil were used as main sources of energy. The composition of vitamin and mineral mixtures added to the diets is shown in Appendix A-1 and A-2, respectively.

Table 3. Composition of diets (%), Experiment 1

Ingredients	Protein (%)				
	0.4	1	2	10	25
Wheat gluten <sup>a</sup>	0.5	1.3	2.6	13.1	32.9
Cornstarch <sup>b</sup>	79.5	78.7	77.4	66.9	47.1
Corn oil <sup>c</sup>	8.0	8.0	8.0	8.0	8.0
Nonnutritive fiber <sup>d</sup>	5.0	5.0	5.0	5.0	5.0
Vitamin mix <sup>e</sup>					
Fat soluble	1.0	1.0	1.0	1.0	1.0
Water soluble	1.0	1.0	1.0	1.0	1.0
William Briggs mineral salt mix <sup>a</sup>	5.0	5.0	5.0	5.0	5.0
Total	100.0	100.0	100.0	100.0	100.0

<sup>a</sup>General Biochemicals, Chagrin Falls, Ohio.

<sup>b</sup>Clinton Corn Products, Clinton, Iowa.

<sup>c</sup>Mazola, Best Foods Division, Corn Products Company, New York, New York.

<sup>d</sup>Teklad Test Diet, Madison, Wisconsin.

<sup>e</sup>See Appendix, Tables A-1 and A-2.

The rats were fed ad libitum for 4-5 weeks, or until the clinical signs of kwashiorkor were observed in any one rat within a littermate group. Food jars were changed every two days. A clean jar was weighed together with 25-35 g of diet. Two days later, the jar was weighed again; any food spilled was collected, separated from the feces, and added to the food that was left. Consumption was calculated as the difference of the two weights. Water bottles were filled as needed and changed weekly.

#### Sample collection

The last food intake was weighed in the morning of the last day of the experimental period. The rats were then placed in individual metabolism cages and urine was collected for 24 hours. Water was supplied but food was withheld during the urine collection. At the end of the collection period, the rats were anesthetized with sodium pentobarbital<sup>1</sup> (0.25 mg) per 100 g body weight). After weighing, each rat was incised lengthwise and crosswise in the abdomen and thoracic regions to expose the heart and liver. Blood was drawn while the heart was still beating. Centrifuge tubes (15-ml) containing the blood were placed in ice. After 2-3 hours, the blood was centrifuged at a speed of 7,500 rpm for 20 minutes. The serum was separated, placed in vials, and frozen until time of analyses. The liver was excised and nonliver tissue was removed. The liver was blotted with damp paper towels and weighed.

The urine was diluted with the washings from the metabolism pans and funnels. Total diluted urine volume was recorded. The diluted urine was

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<sup>1</sup>Nembutal, Abbott Laboratories, North Chicago, Illinois.

adjusted to pH 1-3 with HCl and tested with litmus paper. Samples were subsequently frozen until time of analyses.

Experiment 2:  
Effects of Protein and Energy Levels  
Restricted by Dilution with Cellulose

Objective

Experimental kwashiorkor had been produced in Experiment 1 by feeding rats WG protein at the 2% level for 4-5 weeks. This experiment was designed to determine the effects on the production of PCM in rats of different levels of WG protein with energy content diluted with cellulose. Clinical signs were compared to those in Experiment 1. The effects on the biochemical indices also were compared. Lactalbumin protein at 16% level was fed to the control group. Since kwashiorkor is usually associated with a low protein-high carbohydrate diet, but marasmus with a wide variety of diets consumed in restricted amounts, this experiment was designed to determine whether marasmus and kwashiorkor can be developed separately by restricting energy intake through fiber dilution in the diet.

Animals

Eighty male weanling rats of the same strain and source as in Experiment 1 were selected from litters of five rats or more. Rats weighed 47-60 g with a mean of 53 g. The 16 litters of five rats each were randomly assigned to diets containing two levels of energy. One rat from each litter was randomly allotted to each of five protein treatments at each energy level. Eight rats from eight different litters comprised

each treatment group. All rats were fed, cared for, and handled in the same manner as in Experiment 1. Changing of feed, weighing, and tail measurements were done every three days. The experimental period lasted from 4-5 weeks. Observations were made on the clinical symptoms of malnutrition in the animals.

### Diets

Wheat gluten at 2, 4, 8, and 16% protein levels and lactalbumin at 16% protein level were used as sources of protein in this study. Lactalbumin served as control protein. In the formulation of the diets, nonnutritive fiber was included at 5% level to provide a high energy diet (3.65 mcal/kg) and at 55% level to provide a low energy diet (1.84 mcal/kg). Composition of diets is given in Table 4. Food was weighed and changed every three days.

### Sample collection

Urine, blood, and liver samples were collected and handled in the same manner as in Experiment 1. However, since plasma and serum samples were both needed for chemical analyses, an anticoagulant, sodium citrate, was added to the blood for plasma collection. To every 2 ml of blood, 0.05 ml of sodium citrate (3.8% w/v) was used. For serum samples, 1 ml of the blood with sodium citrate was separated and 10  $\mu$ l calcium chloride solution (10% w/v) was added to coagulate the fibrinogen in the plasma. The peritoneal fluid in the abdominal cavity of the rats was aspirated with a syringe and the total volume recorded.

Table 4. Composition of diets (%), Experiment 2

Ingredient	Protein (%)				Lactalbumin
	Wheat gluten				
	2	4	8	16	
					16
Wheat gluten <sup>a</sup>	2.6	5.3	10.5	21.0	--
Lactalbumin <sup>b</sup>	--	--	--	--	20.4
Cornstarch <sup>c</sup>					
High energy level	77.4	74.7	69.5	59.0	59.6
Low energy level	32.4	29.7	24.5	14.0	14.6
Corn oil <sup>d</sup>	8.0	8.0	8.0	8.0	8.0
Nonnutritive fiber <sup>e</sup>					
High energy level	5.0	5.0	5.0	5.0	5.0
Low energy level	55.0	55.0	55.0	55.0	55.0
Vitamin mix <sup>f</sup>					
Fat soluble	1.0	1.0	1.0	1.0	1.0
Water soluble	1.0	1.0	1.0	1.0	1.0
William Briggs salt mix <sup>a</sup>	5.0	5.0	5.0	5.0	5.0
Total	100.0	100.0	100.0	100.0	100.0

<sup>a</sup>General Biochemicals, Chagrin Falls, Ohio.

<sup>b</sup>Nutritional Biochemicals Corporation, Cleveland, Ohio.

<sup>c</sup>Clinton Corn Products, Clinton, Iowa.

<sup>d</sup>Mazola, Best Foods Division, Corn Products Company, New York, New York.

<sup>e</sup>Teklad Test Diet, Madison, Wisconsin.

<sup>f</sup>See Appendix, Tables A-1 and A-2.

### Experiment 3: Effects of Protein and Energy Levels Varied by Restriction of Food Intake

#### Objective

Because kwashiorkor but not marasmus had been produced in Experiment 2, this experiment was designed to develop kwashiorkor and marasmus separately by feeding rats lactalbumin and two different levels of WG



protein. Rats were fed for 3 weeks instead of 4-5 weeks. Any PCM developed would be in the preclinical stage. Energy intakes were varied by restriction of food intake. Symptomatology and biochemical indices were compared with those of Experiments 1 and 2.

### Animals

Forty-eight male weanling rats of the same age, strain, and source as in Experiments 1 and 2 were fed the stock diet for two days to bring their weights to at least 50 g. Weights of the rats at the start of the experiment ranged from 50-67 g with a mean of 57 g. Twenty-four pairs of sibling rats were each randomly allotted to three protein treatments. One rat from each pair was fed ad libitum and the other, 50% ad libitum. For each of the six ration treatments, eight rats from eight different litters were used. The animals were fed, cared for, and handled in the same manner as in Experiments 1 and 2.

### Diets

Three experimental diets were formulated to contain 2 and 4% protein from WG and 8% from lactalbumin (control diet). The composition of these diets is shown in Table 5. For the experiment, however, six dietary regimens were used. They were:

1. 2% WG protein (ad libitum feeding)
2. 2% WG protein (50% ad libitum feeding)
3. 4% WG protein (ad libitum feeding)
4. 4% WG protein (50% ad libitum feeding)
5. 8% LA protein (ad libitum feeding)
6. 8% LA protein (50% ad libitum feeding)

Feeding was done every day and lasted for 21 days. Half of the food consumed by the rats on ad libitum feeding was weighed and given to its paired rat in the restricted (50% ad libitum) diet regimen.

Table 5. Composition of diets (%), Experiment 3

Ingredient	Protein (%)		
	2	4	8
Wheat gluten <sup>a</sup>	2.6	5.3	--
Lactalbumin <sup>b</sup>	--	--	10.2
Cornstarch <sup>c</sup>	77.4	74.7	69.8
Corn oil <sup>d</sup>	8.0	8.0	8.0
Nonnutritive fiber <sup>e</sup>	5.0	5.0	5.0
Vitamin mix <sup>f</sup>			
Fat soluble	1.0	1.0	1.0
Water soluble	1.0	1.0	1.0
William Briggs mineral salt mix <sup>a</sup>	5.0	5.0	5.0
Total	100.0	100.0	100.0

<sup>a</sup>General Biochemicals, Chagrin Falls, Ohio.

<sup>b</sup>Nutritional Biochemicals Corporation, Cleveland, Ohio.

<sup>c</sup>Clinton Corn Products, Clinton, Iowa.

<sup>d</sup>Mazola, Best Foods Division, Corn Products Company, New York, New York.

<sup>e</sup>Teklad Test Diet, Madison, Wisconsin.

<sup>f</sup>See Appendix Tables A-1 and A-2.

#### Sample collection

Urine and blood samples were collected and handled in the same manner as in Experiments 1 and 2. However, the rats were fasted for 12 hours before urine collection during the next 12 hours. Aside from serum, fasting blood samples also were collected in heparinized capillary tubes which were centrifuged for 20 minutes for hematocrit readings.<sup>1</sup> Plasma in the capillary tubes were separated and frozen for plasma amino acid analyses.

<sup>1</sup>Microhematocrit MB centrifuge and microcapillary reader, Damon/IEC Division, Needham Hts., Massachusetts.

Experiment 4:  
Comparison of Biochemical Indicators of PCM  
in Rats Fed Different Protein Diets

Objective

Since experimental PCM was successfully simulated by feeding 2% WG protein diets in these experiments, and by 4% wheat gluten diet in the experiments by Marok (1977), it was deemed important to compare the effects of those diets with other types of protein on the biochemical indices chosen for this study. The effects of 2% WG protein was thus compared with those of a very low amount of good quality protein, 0.5% lactalbumin (Edozien, 1968), and a fairly high level of very low quality protein, 8% gelatin protein (Arthur, 1976). Feeding these two proteins in previous studies resulted in the production of similar symptoms of full kwashiorkor in rats.

Animals

Forty male weanling rats of the same strain and from the same source as in Experiments 1, 2, and 3 were randomly assigned to five diet treatments. Because of time limitations, this experiment was conducted simultaneously with Experiment 3. Rats in three of the diet treatments in Experiment 3 were used in comparisons with two other treatments in Experiment 4. Handling, feeding, and care of the animals were done in the same manner as in Experiment 3.

Diets

The five diet regimens used for this experiment were 2 and 4% protein from wheat gluten, 0.5 and 8% from lactalbumin, and 8% from gelatin.

The rats fed 8% lactalbumin protein diets served as the control group.

The composition of the different diets is given in Table 6.

Table 6. Composition of diets (%), Experiment 4

Ingredient	Protein (%)				
	Lactalbumin		Gelatin	Wheat gluten	
	8	0.5	8	2	4
Lactalbumin <sup>a</sup>	10.2	0.6	--	--	--
Gelatin <sup>b</sup>	--	--	8.9	--	--
Wheat gluten <sup>b</sup>	--	--	--	2.6	5.3
Cornstarch <sup>c</sup>	69.8	79.4	71.1	77.4	74.7
Corn oil <sup>d</sup>	8.0	8.0	8.0	8.0	8.0
Nonnutritive fiber <sup>e</sup>	5.0	5.0	5.0	5.0	5.0
Vitamin mix <sup>f</sup>					
Fat soluble	1.0	1.0	1.0	1.0	1.0
Water soluble	1.0	1.0	1.0	1.0	1.0
William-Briggs mineral salt mix <sup>b</sup>	5.0	5.0	5.0	5.0	5.0
Total	100.0	100.0	100.0	100.0	100.0

<sup>a</sup>Nutritional Biochemicals Corporation, Cleveland, Ohio.

<sup>b</sup>General Biochemicals, Chagrin Falls, Ohio.

<sup>c</sup>Clinton Corn Products, Clinton, Iowa.

<sup>d</sup>Mazola, Best Foods Division. Corn Products Company, New York, New York.

<sup>e</sup>Teklad Test Diet, Madison, Wisconsin.

<sup>f</sup>See Appendix, Tables A-1 and A-2.

#### Sample collection

Urine and blood samples were collected in the same manner as in Experiment 3. Rats were fasted for 12 hours before urine collection during the next 12 hours to eliminate any effects of gelatin on urinary hydroxyproline excretion (Prockop and Sjoerdsma, 1961).

### Chemical Analyses

The amount of serum and urine required made many analytical procedures impossible to use in these experiments. Consequently, the microtechniques for the automated analysis of serum total protein and albumin, urinary urea, creatinine, and hydroxyproline for nutrition surveys in developing countries as recommended by Coward et al. (1971) have been used for this study. However, some minor modifications were made. The autoanalyzer<sup>1</sup> apparatus in the Swine Nutrition Research Laboratory at Iowa State University was used. For the amino acid ratios, the paper chromatography method of Whitehead (1964) was employed.

#### Serum total protein and albumin

Total protein was estimated on the basis of the color reaction with biuret. The composition of the biuret is described in the Technicon method sheet N-14b.<sup>2</sup> The method, as adapted to the autoanalyzer, was based on the technique by Failing et al. (1960).

The biuret reaction depends upon the formation of a purple colored complex of copper in an alkaline solution with two or more carbamyl groups which are joined directly together or through a single atom of nitrogen or carbon. In the autoanalyzer, the serum sample was automatically diluted with an air-segmented stream of biuret reagent. After passing through delay coils to allow time for completion of the reaction, the absorbancies at 550 nm were measured by a colorimeter using a flow

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<sup>1</sup>Technicon AutoAnalyzer, Technicon Corporation, Tarrytown, New York.

<sup>2</sup>Technicon AutoAnalyzer Methodology, Technicon Instrument Corporation, Chauncey, New York, 1965.

cuvette with a 15 mm light path. The readings were automatically recorded. The sampling time was 18 seconds, thus only 30  $\mu$ l of serum was used. The sampler was set for 50 determinations per hour. For the standard curve, bovine serum albumin was used at concentrations of 2, 5, and 7 g/dl. Correction for turbid and hemolyzed sera was made by running the serum samples using the blank reagent in place of the biuret reagent. The reagent baseline was readjusted. The g/dl protein equivalents of the blank for standard curve were determined and subtracted from the biuret values.

Total albumin was estimated on the basis of the color reaction with 2,4 (hydroxyazobenzene) benzoic acid (HABA). The working solution for HABA is described in Technicon method sheet N-15c.<sup>1</sup> The procedure for automated analysis is based on the quantitative binding of the anionic HABA dye specifically to the serum albumin. It was founded on the work of Ness et al. (1965). In the analyzer, the serum sample was automatically diluted and reacted with the HABA dye. After passing through delay coils to complete the reaction, the absorbancies were measured by the colorimeter at 505 nm using a flow cuvette with 15 mm light path. Readings were automatically recorded. The sampling was also 18 seconds, thus only 30  $\mu$ l serum was used. The sampler was set for 50 samples per hour. For the standards, bovine serum albumin was used at concentrations of 2, 5, and 7 g/dl. For blank corrections, serum samples were run using blank solutions in place of the HABA reagent. The resultant values were subtracted from the HABA values.

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<sup>1</sup>Technicon AutoAnalyzer Methodology, Technicon Corporation, Ardsley, New York, 1967.

The same autoanalyzer manifold was used for both albumin and total protein determination. When changing from one procedure to another, the manifold was washed thoroughly with distilled water.

#### Urinary urea nitrogen

The autoanalyzer urea nitrogen method is a slightly modified version of the procedure described by Marsh et al. (1965). Urea nitrogen is measured as a result of its direct reaction with diacetyl monoxime (2,3, -Butanedione 2-oxime) in the presence of thiocarbazide under acid reactions. Thiosemicarbazide intensifies the color of the reaction product and enables the determination to be run without need of concentrated acid reagents. The colored reaction product was measured at 520 nm in a flow cuvette with a 15 mm light path. The sampler was set for 50 samples per hour. The reagents are described in Technicon method sheet N-1c.<sup>1</sup> For the standards, concentrations of 2.5, 5, 10, 15, 20, 30, and 40 mg urea nitrogen/dl were used. All urine samples were further diluted to bring the concentrations into the range of the standards. Urea nitrogen values were multiplied by a factor of 2.14 for conversion to mg urea.

#### Urinary creatinine

The method employed for creatinine analysis is a modification of the procedure of Folin and Wu method (Hawk et al., 1947). The adapted method for automation is described in Technicon method sheet N-11b.<sup>1</sup> The sample, diluted with an air-segmented stream of 0.9% sodium

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<sup>1</sup>Technicon AutoAnalyzer Methodology, Technicon Instruments Corporation, Chauncey, New York, 1965.

chloride, was combined with an air-segmented water stream after passing through a dialyzer. The stream was mixed with a combination of saturated picric acid and 0.5 N sodium hydroxide. Upon mixing, the streams were sent through a time delay coil. The absorbancies of the resulting reaction solutions were measured in a colorimeter at 505 nm using a 15 mm tubular flow cell. The sampler was set for 50 determinations per hour. For the standards, concentrations of 1, 3, 5, 7, 10, 15, and 20 mg creatinine/dl were used. Concentrations of the diluted urine samples were adjusted to bring concentration within the range of the standards.

#### Urinary hydroxyproline

The automated analysis method for urinary hydroxyproline by Hosley et al. (1970) was employed. Slight modifications were made. The method is based on work by Grant (1964). Urine samples were prepared for the autoanalyzer using the initial stages of Prockop and Udenfriend's (1960) manual method. Hydroxyproline in urine is 97% peptide bound and is liberated to the free form by acid hydrolysis, achieved by adding concentrated hydrochloric acid and autoclaving at 15 lbs pressure for three hours. The acidified mixture was decolorized with charcoal resin, placed in Sorvall plastic tubes, and then centrifuged for 10 minutes at 10,000 rpm. Two ml of the clear supernatant was titrated to approximately pH 6.0 with two drops of methyl red indicator and 0.4N sodium hydroxide in a 10-ml volumetric flask, and brought to volume with distilled water, achieving a dilution factor of 40. The sampler was set for 30 determinations per hour with alternating water cups to give



a double wash. Hydroxyproline standards at concentrations of 0.25, 0.5, 1.0, and 2.0 µg/ml preceded each run. The autoanalyzer was run initially with reagents and water blank, until a steady baseline was achieved. A 40-ft time delay coil in a water bath at room temperature prevented the Erlich's reagent from precipitating, and facilitated peak reading. Before acid hydrolysis, diluted urine samples were concentrated by boiling in a water bath in order to bring concentration within the range of the standards. The free hydroxyproline is first oxidized and decarboxylated to pyrrole with an oxidant, Chloramine T (sodium p-Toluenesulfon chloramide), and heating. The pyrrole reacts with Erlich's reagent (p-dimethyl aminobenzaldehyde), forming a red colored complex. The absorbancies were measured at 557 nm and automatically recorded.

#### Serum/plasma N/E amino acid ratio

The method of Whitehead (1964) for rapid determination of plasma amino acids in subclinical kwashiorkor was used. Approximately 150 µl of plasma or serum were pipetted out of storage vials or were blown out of heparinized capillary tubes which were previously centrifuged to separate the plasma and to make hematocrit readings. The plasma or serum was added to 4 ml of 90% ethanol in a test tube to deproteinize the sample. After vigorous mixing with a Vortex<sup>1</sup> mixer, the mixture was allowed to stand for 10 minutes and then centrifuged at a speed of 6,000 rpm for 15 minutes. The clear supernatant was aspirated off into a flat-bottomed Beckman vial (25-ml) and evaporated to dryness

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<sup>1</sup> Vortex Jr. mixer, Scientific Industries Inc., Springfield, Massachusetts.

with nitrogen gas over a water bath at a temperature of 60-65°C. Drying time was approximately 15-20 minutes, depending on the amount of flow of nitrogen gas.

The residue was redissolved in 200  $\mu$ l of 10% isopropanol, taking care that all residues were scraped off from the sides and the bottom of the vial. The amino acids were then determined by a one-dimensional ascending paper chromatography using Whatman paper No. 1. The sample was applied to the paper in amounts of approximately 25  $\mu$ l in streaks 2 cm long. An exact quantitative transfer was not done since the method estimates the ratio of the groups of amino acids and not the absolute concentrations of amino acids. The eight standard amino acids each dissolved in 10% isopropanol at .025M concentrations were mixed and also streaked in each paper containing four samples. They served as guides in determining the spots.

The chromatography papers were placed in equilibrated tanks with butanol-acetic acid-water mixture (12:3:5, by volume). The chromatogram was run in the solvent for 8.5-9.0 hours or until the solvent front has risen to approximately 21-23 cm. After drying for 30-45 minutes or until the smell of acetic acid was removed, the papers were dipped briskly through a pan of ninhydrin solution (2 g/l acetone), drained, and air-dried for five minutes. To develop the color, the papers were placed for exactly five minutes in an oven at 110°C. Upon cooling at room temperature, the color of the spots changed from red-purple to blue-purple. Using the spots of the standard amino acid mixture as a guide, the two separate spots for the essential amino acids, leucine and isoleucine, and for methionine and valine, and one spot for the

nonessential amino acids, glycine, serine, taurine, and glutamine together, were ringed with a pencil. The papers were then dipped in copper nitrate solution (1 ml saturated copper nitrate solution/1000 ml ethanol) and subsequently air-dried. The colors of the spots turned from purple to salmon-red. The spots were cut out and placed as small strips in test tubes. The two spots for the essential amino acids (leucine-isoleucine and methionine-valine) were placed in one tube and the other spot for the nonessential amino acids in another tube. The color from the papers was eluted by adding 4 ml methanol to the tubes and allowing to stand for one hour with occasional shaking. The absorbancies were measured at 509 nm in a Gilford spectrophotometer<sup>1</sup> using methanol as the blank. The ratio was computed as absorbancy of the nonessential amino acids divided by that of the essential amino acids as follows:

$$\text{Ratio} = \frac{\text{A, 509 nm for gly + ser + gln + tau color}}{\text{A, 509 nm for leu + ile + met + val color}}$$

### Statistical Analyses

#### Experiment 1

Eight litters were used. The rats within each litter were randomly assigned to the five protein treatments. Litters were considered as blocks. Analysis of variance (ANOVA) was used to test differences among protein treatments. The least significant difference (LSD) was used to judge differences between any two groups.

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<sup>1</sup>Gilford Spectrophotometer 240, Gilford Instrument Laboratories, Oberlin, Ohio.

### Experiment 2

Energy levels were assigned to litters which served as the whole plots of a split-plot design. Within each litter, the five protein levels were assigned at random to individual rats. In the ANOVA, the differences between energy levels were tested using the variation among litters treated alike, the whole-plot error. Differences among protein levels and the interaction of protein and energy were tested using the subplot error. Comparisons of any two treatment means by LSD determined significance.

### Experiment 3

Levels of protein were assigned to litters which served as the whole plot in a split-plot design. Within each litter, the two methods of feeding were assigned at random to individual rats. The differences between protein levels were tested using the variation among litters treated alike, the whole-plot error. Differences between methods of feeding and the interaction of protein and method of feeding were tested using the subplot error. To judge differences between any two groups, the LSD was used.

### Experiment 4

Forty rats were allotted to five protein treatments in a completely randomized design. In the ANOVA, the differences among protein treatments were tested using the pooled error from the five groups. The LSD was used to test differences between any two groups.

On the average, eight rats were used for each treatment in all four experiments. All animals were fed in individual cages; therefore, each

animal was a replication in itself. All tests were considered significant at the  $P < .05$  or  $P < .01$  (Snedecor and Cochran, 1972).

Statistical design and analyses were done in consultation with Dr. David Cox, Department of Statistics, and data were handled by the Iowa State Computation Center, Iowa State University.

## RESULTS AND DISCUSSION

Experiment 1:  
Wheat Gluten Protein for  
Production of Experimental PCM

To investigate the effect of varying levels of WG proteins on growth, survival, and production of experimental PCM, 40 weanling rats were allotted to each of five protein diets with eight rats in each group. Comparisons of biochemical indicators and clinical symptoms were made.

General appearance and clinical symptoms

Apart from severe emaciation and stunting of growth as the feeding period progressed, rats fed 2% WG protein diets started showing signs of edema during the third week of feeding. At first, the edema appeared as puffiness of the face and/or the body. Some rats developed "moon" faces. On the fourth and fifth week, 7 of 8 rats in the group had edema which developed into pendular sacs of subcutaneous fluid between the forelimbs. In one of the rather severe cases of edema, the pendular sac was large enough to touch the floor when the rat was standing. In some, the abdomens were bloated with ascites fluid. The edema varied in severity and often disappeared and reappeared rather suddenly during a few days following its onset. As edema developed, weight of some rats increased by as much as 20%. One rat fed 2% protein diet lost hair in the upper area of the outside of the thigh and developed skin lesions before the full condition of edema and pendular sac developed. Animals fed 0.4 and 1% protein showed more severe emaciation and growth retardation but with moderate rather than severe signs of edema and in fewer animals per group

than did those fed 2% protein. Rats fed 10% WG protein appeared stunted in growth with no signs of edema, while those fed 25% appeared normal.

It appeared that the symptoms in rats fed 0.4 and 1% WG protein diets should be described as marasmic or marasmic-kwashiorkor condition rather than kwashiorkor per se. The clinical signs of gross or severe edema in these groups were not seen in as many rats as in the group fed 2% protein. Instead, the appearance of emaciation with or without some edema was more common. Two rats died after fast, prior to necropsy. The clinical symptoms of experimental kwashiorkor in rats fed 2% protein were similar to those developed by Kohman (1920) in rats fed low protein carrot diets and by Edozien (1968) in rats fed 0.5 and 1% lactalbumin diets. Arthur (1976) produced the condition in rats by feeding 8% gelatin protein for 3-4 weeks and Marok (1977) by feeding WG at 4% protein levels for 6-7 weeks. Heard et al. (1958) reported that pigs fed low protein diets developed a syndrome resembling marasmus in children; feeding of extra calories in form of carbohydrates for ameliorating the condition resulted in a form of protein malnutrition resembling kwashiorkor.

### Growth

Results on the effects of varying levels of WG protein on total weight gains and tail length gains are presented in Table 7. The weekly weight gains and tail length gains are shown in Figure 1.

The highest average gain in weight at the end of four weeks was attained by rats fed 25% protein. Rats fed 0.4, 1, and 2% protein lost as much as 11 to 16 g by the end of the feeding period. Rats fed 10%

Table 7. Summary of results on body measurements in rats fed five levels of wheat gluten, Experiment 1

Dietary protein (%)	Body weights (g)			Liver (g)	Tail length (cm)	
	Initial	Total gains	Final		Initial	Final
0.4	56.6 <sup>a</sup>	-16.5	38.4	1.43 (6)	8.4	9.5
1	57.6	-14.2	42.1	1.73 (6)	8.3	9.6
2	57.8	-11.2	44.9	1.92 (6)	8.3	9.8
10	56.2	-0.2	53.4	1.82 (6)	8.3	10.0
25	54.6	46.9	110.1	3.21 (6)	8.2	13.9
<hr/>						
F-test	n.s. <sup>b</sup>	**	**	**	n.s.	**
LSD .01	3.17	6.79	11.31	0.60	0.39	0.46
.05	2.35	5.03	8.38	0.44	0.30	0.34
Overall S.E.	2.29	4.91	8.18	0.37	0.29	0.54

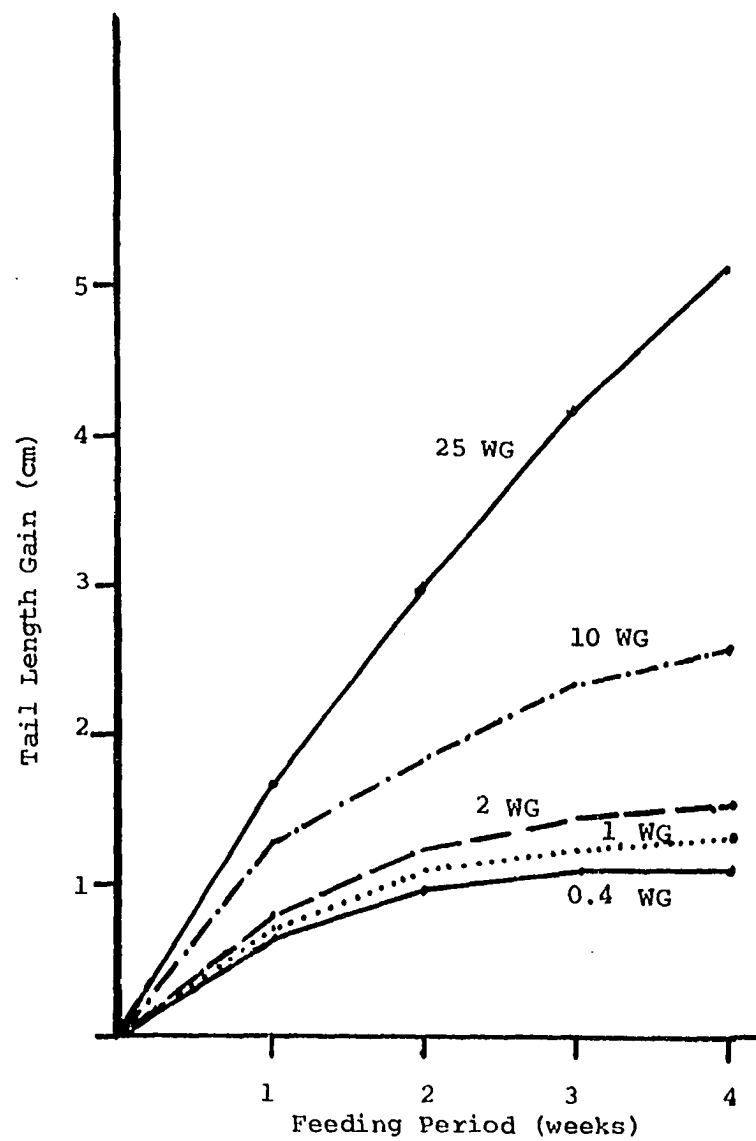
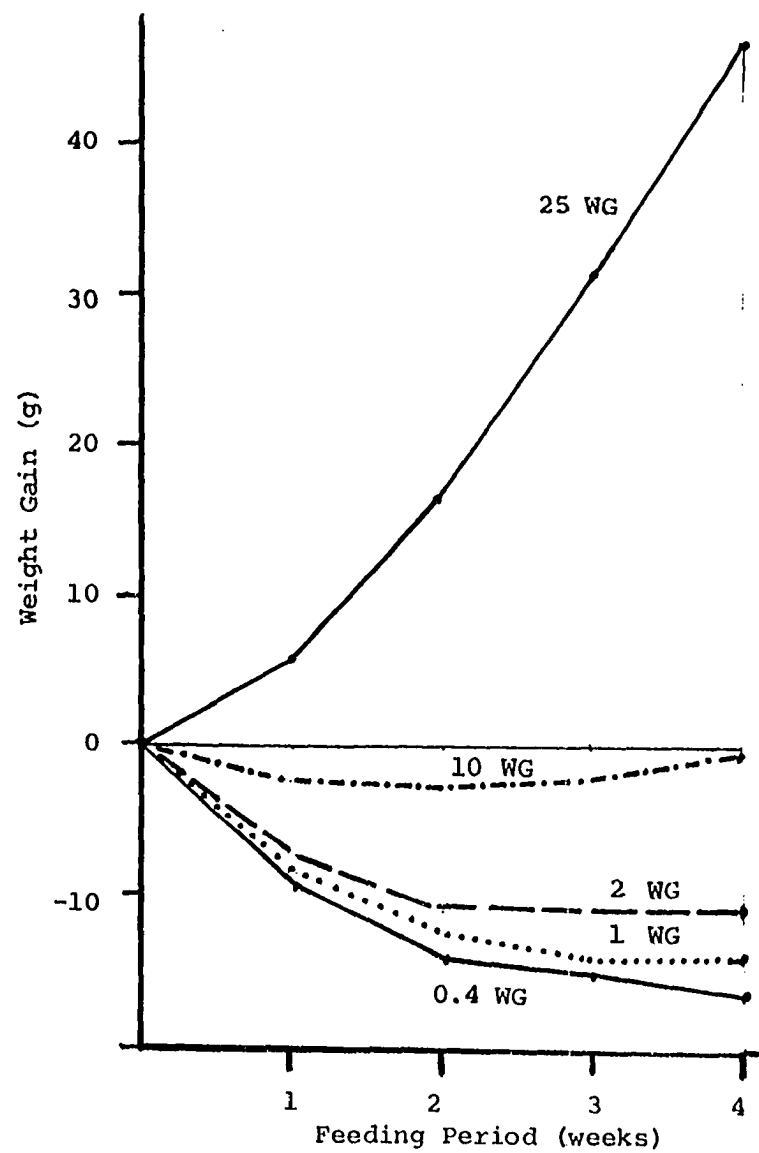
<sup>a</sup>n = 8 unless indicated in parentheses ( ).

<sup>b</sup>n.s. = not significant.

\*\*Significant at .01 level.



Figure 1. Average gain in weight and tail length of rats fed five levels of wheat gluten protein for four weeks, Experiment 1



protein diets lost some weight during the first three weeks but regained their starting weights during the fourth week. Growth patterns among rats fed 0.4, 1 and 2% levels of protein were almost the same, a progressive trend to weight loss compared to weight gain in those fed 25% protein. During the fourth and fifth weeks, there were slight increases in weight gains in rats fed 1 and 2% protein diets; these rats had lost weight during the first three weeks. The slight increase in weight could result from the developing edema, or an adaptation to the low protein and high energy levels allowing small increments in weight due to development of some body fat. Mayer (1958) observed that weight of rats fed low protein diets was disproportionately high in fat, suggesting that rats partly compensate for the inadequate supply of protein by overeating energy.

Likewise, average final body weight of rats fed 25% WG protein diet was more than double the weights of rats fed 0.4, 1, 2, and 10% protein. No significant differences in body weights were found among rats fed 0.4, 1, and 2% proteins; those fed 2 and 10% protein diets were different. Rats lost weight during the 24-hour fast period prior to necropsy. The differences between final body weights at the end of the experiment and the weights prior to necropsy were partly due to immediate food intakes prior to the fast.

For rats fed 0.4, 1, and 2% protein diets, tail lengths increased substantially during the first three weeks although there was no gain in body weight (Figure 1). The tails grew by as much as 1.2-1.5 cm until the third week, after which no further growth occurred. Furthermore, tails of rats fed 10 and 25% protein grew by as much as 2.6-5.1 cm, respectively, by the fourth week (Table 7). The use of tail length

as an index of growth in rats in this experiment was assumed to be analogous to increases in height among children during the early years of rapid growth.

Liver weights of rats fed 25% protein diets were significantly higher ( $P < .01$ ) than those receiving lower protein diets. Liver weights of rats fed the other protein diets were practically the same, except when comparing liver weights of rats fed 0.4 and 2% protein. Anthony and Edozien (1975) reported that weights of individual livers of rats reflected the weight loss produced by chronic protein and energy deprivations. The liver weights, in proportion to body weight, were essentially the same for all rats of the same age irrespective of dietary treatment. However, in the malnourished rats, the livers were smaller in proportion to the body weight than those in the 100-g control animals.

#### Food intake

The average total food, protein, and energy intakes of rats fed the different WG protein diets is summarized in Table 8. Rats fed 0.4, 1, and 2% protein diets consumed, on the average, only 48% of the total food consumed by those fed 25% protein diets. The increase in food intake, as protein levels of the diet increased, generally resulted in increments in both total protein and energy intakes. However, since the diets were isocaloric, the increase in protein intake of rats fed 25% protein compared to the other groups was almost 50 times higher, while that for energy intake was only double. This indicates that the increase in energy intakes, particularly at the higher levels of dietary protein, was not parallel to the increase in protein intakes. Rats which consumed the

Table 8. Mean total food, protein, and energy intakes of rats fed five levels of wheat gluten, Experiment 1

Dietary protein (%)	Total food intake (g)	Total protein intake <sup>a</sup> (g)	Total energy intake <sup>a</sup> (kcal)
0.4	89.8 <sup>b</sup>	0.36	327
1	87.2	0.87	318
2	88.4	1.76	323
10	121.1	12.44	451
25	181.9	45.47	687
<hr/>			
F-test	**	**	**
LSD .01	16.76	4.11	65.72
.05	12.42	3.05	48.72
Overall S.E.	12.13	2.98	47.57

<sup>a</sup>Computed values.<sup>b</sup><sub>n</sub> = 8.

lowest total amounts of protein (0.36 g) had slightly higher total energy intakes due to a slightly higher total food intake than those fed 1 and 2% protein diets. Musten et al. (1974) explained that rats regulate their protein intakes as well as total calorie intakes. Regulation of energy intakes seems to be stronger than regulation of protein intakes. In their experiments, when diets were low in protein, rats were unable to increase protein intake by increasing intake of total diet, and hence calories.

This appeared to have been due to an energy regulating mechanism which maintained energy balance. When concentration of protein in the diet is above levels preferred, rats eat high amounts of protein in order to consume adequate energy.

#### Serum total protein and albumin

Examination of results in Table 9 show that, on the average, serum total protein was lowest (3.2-3.3 g/dl) among rats fed the three lowest WG protein diets, 0.4, 1, and 2%. Levels of serum total protein increased with increasing dietary protein, reaching 5.3 g/dl in rats fed 25% WG protein.

Table 9. Serum total protein and albumin, and albumin/globulin (A/G) ratio of rats fed five levels of wheat gluten, Experiment 1

Dietary protein (%)	Serum protein (g/dl)	Serum albumin (g/dl)	Serum A/G ratio <sup>a</sup>
0.4	3.27 (7) <sup>b</sup>	1.80 (7)	1.74 (7)
1	3.24 (7)	1.62 (7)	1.03 (7)
2	3.17	1.35	0.87
10	3.83	2.54	2.18
25	5.26	3.85	3.41
<hr/>			
F-test	**	**	**
LSD .01	0.73	0.65	1.44
.05	0.54	0.48	1.06
Overall S.E.	0.52	0.47	1.04

<sup>a</sup>A/G ratio = albumin ÷ globulin with G = protein-albumin.

<sup>b</sup>n = 8 unless indicated in parentheses ( ).

Likewise, serum albumin levels were lowest (1.4-1.8 g/dl) with PCM rats. The values increased to 2.4 and 3.8 g/dl albumin in rats fed 10 and 25% WG protein, respectively. These results indicate a fall in serum total protein and albumin concentration as a result of PCM. Edozien (1968) reported values of 3.00 and 4.00 g/dl serum total protein and 1.55-2.40 g/dl for serum albumin for PCM rats. For control rats the values were 6.66 and 3.96 g/dl for serum total protein and albumin, respectively.

Albumin/globulin (A/G) ratios among rats fed the three lowest protein diets (1.74, 1.03, 0.87) were significantly lower ( $P < .01$ ) than those fed 25% protein (3.41). However, the ratios for rats fed 10 and 0.4% protein were not significantly different. Edozien (1968) also found A/G ratios of 1.07-1.50 for PCM rats. The ratio of 1.60 for control rats fed 16% LA in his experiment was lower than the values for rats fed 10 and 25% WG protein in the present experiment. This difference could be attributed to variations in the quality of proteins used, in the ages of the animals, or in the method by which the globulin values were derived. Serum total protein is essentially comprised of albumin and globulin fractions (White et al., 1973). Since albumin is approximately 50-65% of the serum total protein, the difference between total protein and the albumin fraction can be assumed to be the globulin fraction. No separate chemical determinations were made for serum globulins in this experiment. Edozien (1968) determined total albumin and globulin directly by electrophoresis on cellulose acetate. The globulin fraction often separated poorly from the albumin and sometimes was included with the albumin values. Among humans, Whitehead (1967) found the A/G ratio to fall markedly below 1.0 in kwashiorkor compared to 1.0 or above in normal subjects. Although

serum total protein and albumin levels were distinctly lowered in kwashiorkor type of PCM, many cases of marasmus have serum protein levels within the normal range. In the present experiment, it appears that rats fed 0.4 and 1% WG protein diets had developed into marasmic or marasmic-kwashiorkor condition. Serum albumin levels were higher (1.80, 1.62) in these rats compared to rats fed 2% protein (1.35 g/dl). In marginal malnutrition, e.g., in rats fed 10% WG protein, the A/G ratio was practically the same as in rats fed 0.4% WG protein.

#### Serum N/E amino acid ratio

Data in Table 10 reveal that there were no significant differences in the N/E amino acid ratios among rats fed the various WG protein diets. However, the ratios seem to increase with development of PCM. Rats fed 0.4, 1, and 2% protein had higher N/E amino acid ratios than those fed 10 and 25% protein. Whitehead (1964) reported that ratios of 2.0-3.0 are considered within the normal range for children and for rats. The ratio is raised in children with distinct kwashiorkor but not in distinctly marasmic children. In normal children, the ratio is less than 2.0, while in kwashiorkor, ratios above 3.0 are generally observed. The increase in the N/E ratio is generally associated with decreased essential amino acid concentrations and elevated or normal nonessential amino acid levels.

In this experiment, N/E amino acid ratios for rats fed 10 and 25% protein were almost the same, indicating perhaps the test is not sensitive to differentiate levels of protein intakes above those which generate PCM. Heard et al. (1977) reported subnormal concentrations of branched-chain essential amino acids with normal or elevated concentrations of



Table 10. Nonessential/essential (N/E) amino acid ratio in serum of rats fed five levels of wheat gluten, Experiment 1

Dietary protein (%)	Serum N/E amino acid ratio <sup>a</sup>
0.4	2.20 (7) <sup>b</sup>
1	2.15 (7)
2	2.01 (7)
10	1.79 (7)
25	1.82 (8)
<hr/>	
F-test	n.s.
LSD .01	0.65
.05	0.48
Overall S.E.	0.43

$$^a \text{ N/E amino acid ratio} = \frac{\text{A, 509 nm for gly + ser + gln + tau color}}{\text{A, 509 nm for leu + ile + met + val color}}$$

<sup>b</sup>Number of rats per group is in parentheses.

nonessential amino acids. However, they found that chronically mal-nourished and energy restricted rats had qualitatively very similar plasma amino acid patterns to rats which were severely fasted after previously being well-nourished. On the other hand, Anthony and Edozien (1975) found the concentrations of both essential and nonessential amino acids in the serum of rats to be reduced without any significant effect on the ratio in rats fed 0.5 and 1.0% LA diets. Also, histidine was markedly increased. Hence, they suggested that the use of changes in total amino acid concentration in evaluation of protein nutriture might be more useful.

### Urinary urea and creatinine

The results shown in Table 11 reveal that urea excretions increased from 1.10 to 89.65 mg/day as the level of protein in the diet increased from 0.4 to 25%. With the increase in total food intakes, the total protein intakes increased proportionately. The 81-fold increase in urinary urea excretion for rats fed 25% protein compared to those fed 0.4% protein paralleled the 126-fold increase in total protein intakes (Figure 2). Because the diets were isocaloric, the increase in food intake as the protein level in the diet increased from <1 to 25% caused greater increases in protein consumption than in energy consumption. With food intakes increasing from 90 g for rats fed 0.4% protein to 180 g for those fed 25% protein, energy intakes only doubled. The high net losses in nitrogen as urea in proportion to the protein intake, and the observed loss in body weight in rats fed 0.4, 1, and 2% protein diets, reflect degradation of tissue proteins. On the other hand, the amount of nitrogen losses in the form of urea during increased protein intake among those rats fed 25% protein allowed steady increases in body size and weight, indicating tissue protein synthesis. These comparisons can be substantiated by computing the urea excretion per gram total protein intake for each group of rats. As total protein intakes increased, the amount of urea excreted per gram of protein intake became less. The amount of urea excretions approximately reflects the quantity of protein intake. The level of urea excretion could be increased when tissue catabolism is taking place, as in protein malnutrition during stage of growth. Schimke (1962) reported a decrease in urea excretion during starvation. The low urea excretion in malnourished children must be a

Table 11. Urinary urea, creatinine, urea/creatinine (U/C) ratio, and creatinine/tail length (C/TL) index in rats fed five levels of wheat gluten, Experiment 1

Dietary protein (%)	Urinary urea (mg/day)	Urinary creatinine (mg/day)	U/C ratio <sup>a</sup>	C/TL index <sup>b</sup>
0.4	1.10 <sup>c</sup>	0.34	3.04	3.57
1	2.46	0.36	6.71	3.67
2	6.29	0.44	15.20	4.53
10	23.17	0.97	24.20	8.59
25	89.65	2.34	39.00	16.69
<hr/>				
F-test	**	**	**	**
LSD .01	25.50	0.40	11.56	2.75
.05	18.89	0.30	8.57	2.04
Overall S.E.	18.45	0.29	8.37	1.99

<sup>a</sup> Calculated as:  $\frac{\text{urea (mg/day)}}{\text{creatinine (mg/day)}}$  .

<sup>b</sup> Calculated as:  $\frac{\text{creatinine (mg/day)}}{\text{final tail length (cm)}} \times 100$ .

<sup>c</sup><sub>n</sub> = 8.

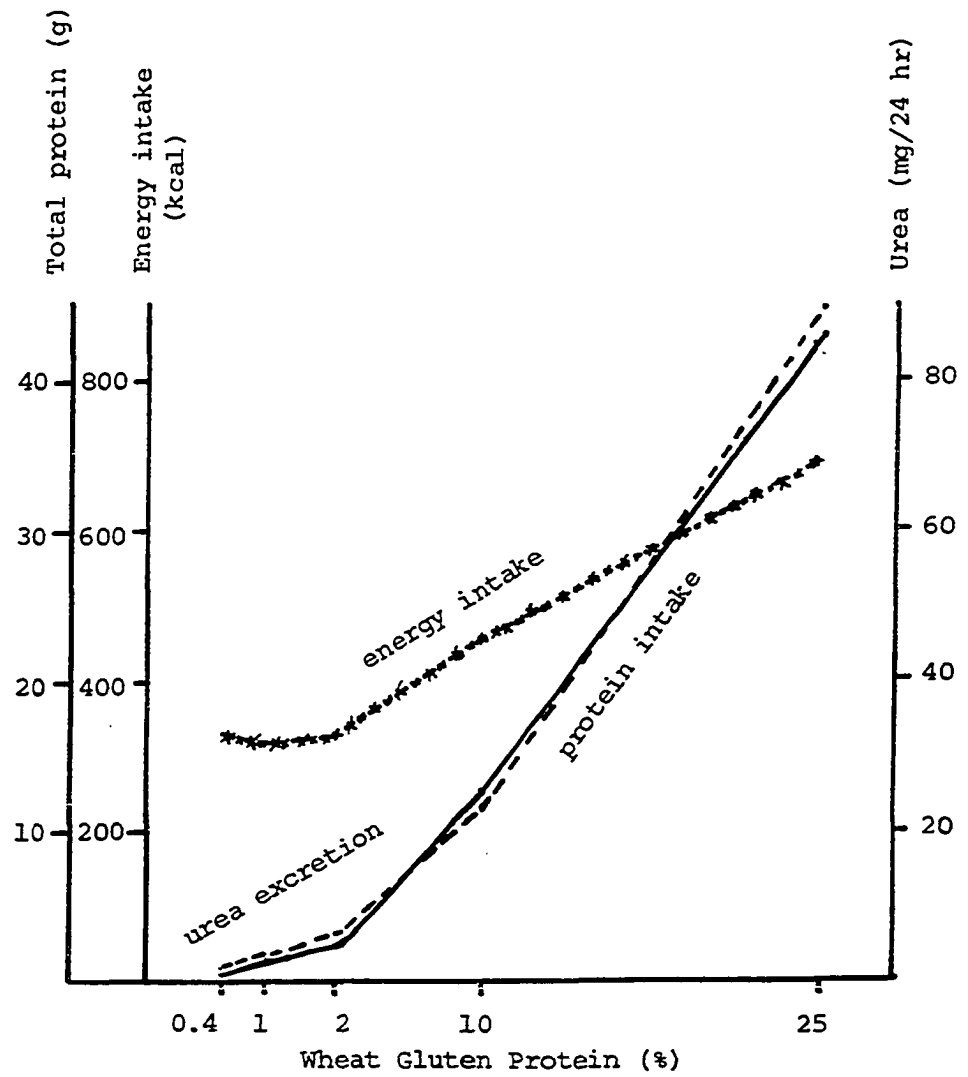


Figure 2. Relationship of urea excretion to total protein and energy intake in rats fed five levels of wheat gluten protein for 4-5 weeks, Experiment 1

result of the reduction in protein intakes leading to significantly decreased rates of urea synthesis (Picou and Phillips, 1972). With increased levels of dietary protein and energy, excretion also increases tremendously indicating perhaps an increase in the rate of turnover of body proteins and metabolism of dietary protein.

Table 11 also shows the comparisons in creatinine excretions as affected by WG protein levels in the diet. Creatinine excretion increased significantly as the level or amount of wheat gluten fed increased. Rats fed 0.4, 1, and 2% WG protein had significantly lower ( $P < .01$ ) creatinine excretion than those fed 10 and 25% protein; the values were practically the same for rats fed each of these three very low protein diets. Creatinine levels in a 24-hour collection is generally believed to reflect total lean body mass (Graystone, 1968). Forbes and Bruining (1976) confirmed the high correlation between lean body mass, as determined by potassium counting, and urinary creatinine excretion. In this experiment, the low levels of creatinine excretion for rats fed very low levels (0.4, 1, and 2%) of WG protein therefore reflected smaller lean body mass, compared to those fed 10 and 25% protein. Rats fed 25% protein diets were more than two times heavier and, therefore, excreted more than twice the amount of creatinine of those fed 10% WG protein diets (2.34 mg/day compared to 0.97 mg/day). Rats with PCM have smaller body sizes, smaller lean body mass, and therefore excreted very low amounts of creatinine.

When urea/creatinine (U/C) ratios were computed, results showed significant increases in the ratios as WG protein levels in the diet increased. The lowest ratio was observed in rats fed 0.4% protein. Rats fed 2% protein had significantly higher ( $P < .01$ ) ratios than those

fed 0.4%, but not 1% protein, and were significantly lower than those fed 10 and 25% protein. The magnitude of the differences in the ratios between rats fed 10 and 25% protein (24.20 vs. 39.00) was lower than when urea and creatinine excretions were compared separately; the differences were, however, still highly significant. Simmons (1972) reported an increase in U/C ratio with increase in protein intake. Dugdale and Edkins (1964) also found low U/C ratios in children consuming low protein diets. In previous tabulated results, it will be recalled that the increase in amount of protein intake was associated with increase in urea loss. The resulting increase or loss in weight also reflected the amount of total protein and food intake. If weight changes are primarily a result in changes in lean body mass and not in water or fat content of the body, creatinine excretion can be directly related to weight changes resulting from increases in lean body mass. The relation of protein intake to lean body mass during growth perhaps can be reflected in the ratio of urea/creatinine excretion, since urea depends on total nitrogen or protein intake, and creatinine reflects total fat-free body mass. The U/C ratio perhaps can be a useful indicator of dietary protein levels in nutrition surveys where average dietary protein quality is known.

Data on the relationship between tail length and creatinine excretion in rats fed different WG protein diets are also summarized in Table 11. Rats fed 25% protein diets had the longest tails and also had the highest mean creatinine/tail length (C/TL) index among the five groups of rats. Rats fed 0.4, 1, and 2% protein had significantly lower indices than those fed 10% protein but were not significantly different from each

other. The increase in tail length seems to parallel the increase in creatinine excretion as protein levels in the diet and total protein intakes increased. Both creatinine excretion and tail length seem to be more related to the development of lean body mass and growth than is weight alone, especially in severe PCM when weight increments can be a result of edema. Expressing creatinine excretion in terms of height (or tail length in rats) is more informative than its relationship to body weight because of variations in amounts of adipose tissues and edema. For the same reason, expressing creatinine excretion in relation to tail length seems more useful than in relation to body weight. The creatinine/height index has been suggested in evaluating protein nutriture (Sauberlich, 1974). Urinary creatinine excretion is principally determined by lean body mass, whereas height indicates growth in stature. Children with stunting of growth from PCM usually have lowered creatinine/height indices.

#### Urinary hydroxyproline

Hydroxyproline (OHPr) excretions are summarized in Table 12. The excretions of rats fed 25% WG protein diets were significantly higher than among rats fed 0.4, 1, 2, and 10% protein. Rats fed 0.4, 1, and 2% protein had significantly lower OHPr excretions than those fed 10% protein. These rats had the lowest weights, were stunted in growth, and appeared very malnourished. The excretion of OHPr in the urine may be helpful as an indicator of slow growth consequential to inadequacies in the diet (Whitehead, 1965). Rats fed low protein diets excreted low amounts of OHPr which seem to also parallel changes in creatinine

excretions. Both OHPr, a product of collagen metabolism, and creatinine, a product of creatine metabolism in the muscle, are excreted in reduced amounts in the malnourished and protein-depleted rats.

Table 12. Urinary hydroxyproline (OHPr), hydroxyproline/creatinine (OHPr/C) ratio, and OHPr index in rats fed five levels of wheat gluten, Experiment 1

Dietary protein (%)	Urinary OHPr (µg/day)	OHPr/C ratio <sup>a</sup>	OHPr index <sup>b</sup>
0.4	27 (6) <sup>c</sup>	9.92 (6)	0.36 (6)
1	31	9.24	0.38
2	51	12.56	0.56
10	94	9.94	0.53
25	403	17.59	1.90
<hr/>			
F-test	**	**	**
LSD .01	56.44	3.76	0.19
.05	41.75	2.78	0.14
Overall S.E.	40.62	2.70	0.14

<sup>a</sup> Calculated as:  $\frac{\text{hydroxyproline (µg/day)}}{\text{creatinine (mg/day)}} \times 100.$

<sup>b</sup> Calculated as:  $\frac{\text{hydroxyproline (µg/day)} \times \text{body weight (kg)}}{\text{creatinine (mg/day)}} \times 100.$

<sup>c</sup>n = 8 unless indicated in parentheses ( ).



When the hydroxyproline/creatinine (OHP<sub>r</sub>/C) ratio was computed and related to dietary protein concentration, the changes in the ratio as a result of variation in protein level were not quantitatively proportional to each of these biochemical parameters considered separately (Table 12).

The incorporation of body weight for adjustment in the computation of the OHP<sub>r</sub> index from the OHP<sub>r</sub>/C ratio allows for adjustments in the changes in creatinine excretion with changes in body weight. The data in Table 12 show an increased sensitivity of the OHP<sub>r</sub> index when adjusted for weight compared to the OHP<sub>r</sub>/C ratio. The OHP<sub>r</sub> index rose with smaller changes in protein levels of the diet and as total protein intake increased. The index was significantly lower ( $P < .01$ ) in the malnourished and protein-depleted animals fed 0.4, 1, 2, and 10% protein diets than it was in the better-nourished rats fed 25% protein. In normal growing children, between one and ten years of age, the ratio of the excretion of OHP<sub>r</sub> to creatinine per kg of body weight remains constant. Whitehead (1965) reported in his studies that the values for malnourished rats were much lower than those for well-nourished ones belonging to the same age.

#### Experiment 2: Effects of Protein and Energy Levels Restricted by Dilution with Cellulose

This experiment was designed to determine whether kwashiorkor and marasmus can be developed separately by restricting energy intake through fiber dilution in the diet and to determine accompanying PCM indices. Eighty weanling male rats were assigned to ten diets containing two levels of energy, high (HE, 3.65 mcal/kg) and low (LE, 1.84 mcal/kg). There were five protein treatments at each energy level: 2, 4, 8, and 16% protein

from wheat gluten, and 16% from lactalbumin which served as the positive control.

#### General appearance and clinical symptoms

The experiment failed to develop the conditions of kwashiorkor and marasmus separately in the rats. The clinical signs of kwashiorkor in rats fed diets containing 2% WG protein in Experiment 1 were also observed with rats fed 2% WG protein diets at both HE and LE levels during the 4-5 week feeding period in Experiment 2. Rats fed high fiber LE diets consumed more food and compensated for the calorie deficit in the diet. Therefore, the total energy intakes were not restricted in proportion to dietary composition. Early in the experiment the rats lost weight, became emaciated, and growth was greatly retarded. Symptoms of edema developed in 3-4 weeks, appearing initially on the face and/or body. These signs appeared in varying degrees and had different times of onset and duration. The edematous condition was more extreme and appeared in all the rats fed 2% WG protein LE diets, whereas those receiving HE regimens at the same protein levels had less extreme edema and not all developed the symptom. In some rats, the pendulous sac of fluid between the forelimbs developed into a larger sac which enveloped the forelimbs, the entire thoracic area, and abdomen. These rats could barely walk. On the average, rats gained from 8-10 g during edema development. One rat gained 71% body weight within the five days following the onset of edema. On the other hand, PCM rats fed the HE diet increased in weights by only 10-15%.

This clinical picture of experimental kwashiorkor in rats fed 2% WG

protein LE diet was similar to those developed by Kohman (1920) in rats fed low protein wet carrot diet, and by Arthur (1976) in rats fed 8% gelatin protein diet at LE levels for 3-4 weeks. The rats fed LE gelatin diet produced more severe edema than those fed the same diet at HE level. Edozien (1968) fed 0.5 and 1% LA protein to 100-130 g rats and it took four months to develop the clinical picture of kwashiorkor in the animals. In this experiment, the weanling rats used weighed only 53-54 g on the average. Diarrhea and loss of hair as reported by Salem et al. (1973) were not observed. Two of the PCM animals were found dead at the end of the 24-hour fast prior to necropsy.

The question is raised why the rats fed 2% WG protein diets containing low levels of energy developed edema more severely and more readily than those fed 2% protein diets but with high energy levels. Kwashiorkor is generally associated with low protein-high carbohydrate diets. Heard et al. (1958) confirmed this observation with pigs fed on a low protein diet which developed the condition resembling kwashiorkor if additional carbohydrate was added to the diet. The dilution of cornstarch in the diet with cellulose decreased energy density. Apparently, the inclusion of 55% fiber in the diet did not result in substantial lowering of energy intakes of the animals because they were fed ad libitum. The presence of high amounts of fiber in the diet was probably a contributory factor in the aggravation of the edematous condition. Fiber in the digestive tract imbibes and retains much water. Rats fed the high fiber diets were observed to drink much more water than those fed low fiber HE diets. As the stool expanded, the greater bulk may have caused more rapid transit and expulsion of the fiber residues. It seems that nutrient absorption

was slowed down. With increased water intake, fluid retention in the tissues also increased and aggravated the edema. Kohman (1920) found that water intake of rats fed on a low protein dried carrot diet decidedly influenced the development of edema. Edema developed more frequently, more severely, and sooner in the animals fed a wet diet.

### Growth

During the first 30 days of feeding, growth patterns of rats fed the various diets at HE and LE levels are shown in terms of mean weight gains in Figure 3 and in terms of mean tail length gains in Figure 4. The results show, as expected, that protein quality and quantity and level of energy in the diet influence growth response of the animals.

The effects of protein quality and quantity on growth were different at the two levels of energy used in the experiment. On the average, rats on LE diets grew better than those on HE diets. This can be attributed to higher food intakes of rats freely fed LE diets compared to those fed HE diets containing similar amounts of protein. Rats fed 16% LA protein diets had almost linear growth response and grew much better than rats fed the different WG diets. Rats fed 2% WG protein diets lost weight steadily with greater losses among those on LE diets. In the latter group, progressive increase in weights was observed during the last week of the feeding experiment, after weight loss earlier during the period. The increase in weight during this period can be attributed to increases in body fluid during the development of edema. Animals fed 4% WG protein diets at HE levels had smaller weight losses; those fed 8% protein diet almost maintained initial weight. Their counterparts fed the LE diets

Figure 3. Average gain in weight of rats fed lactalbumin (LA) and wheat gluten (WG) protein at high and low energy levels for 30 days, Experiment 2

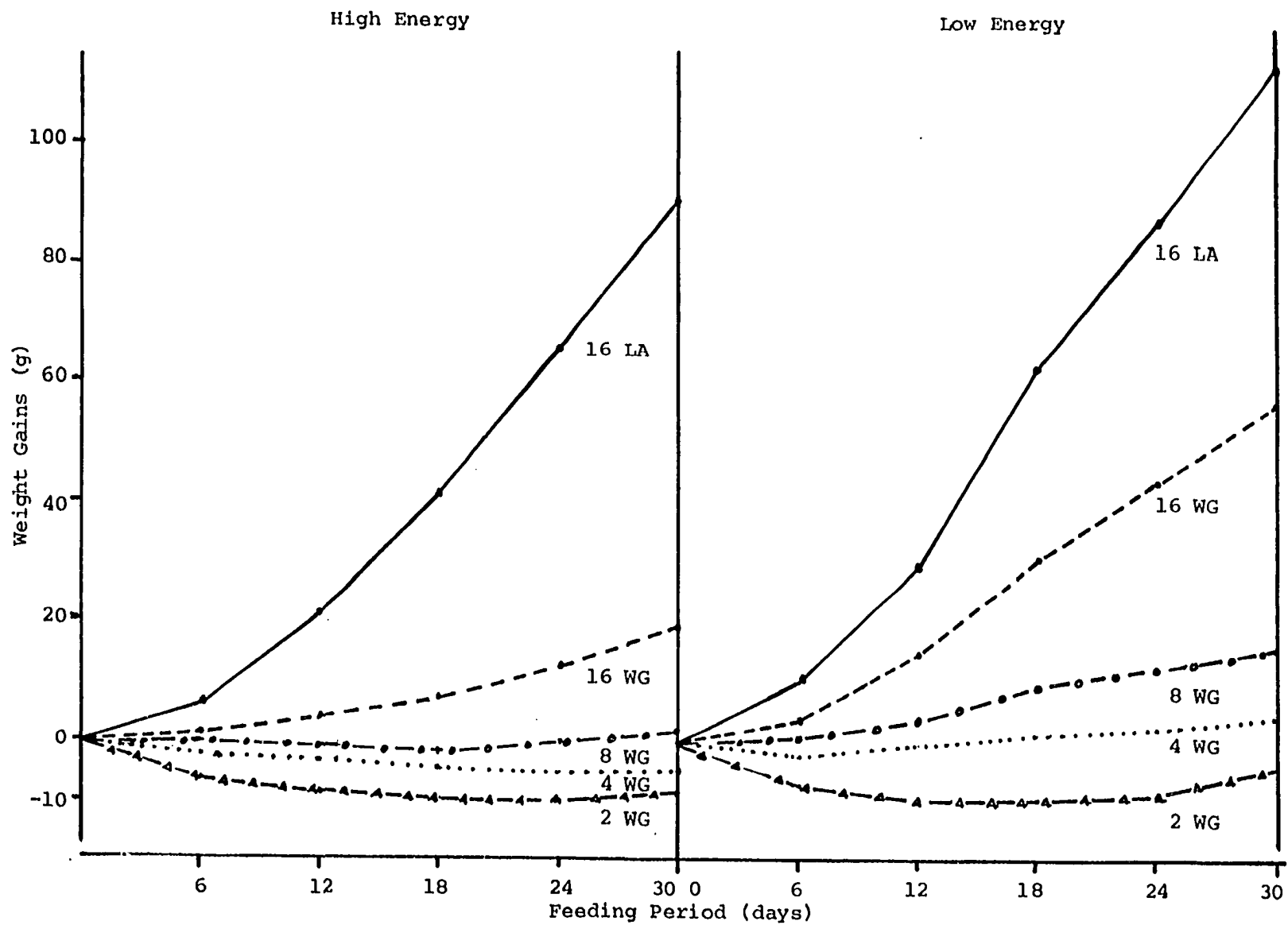
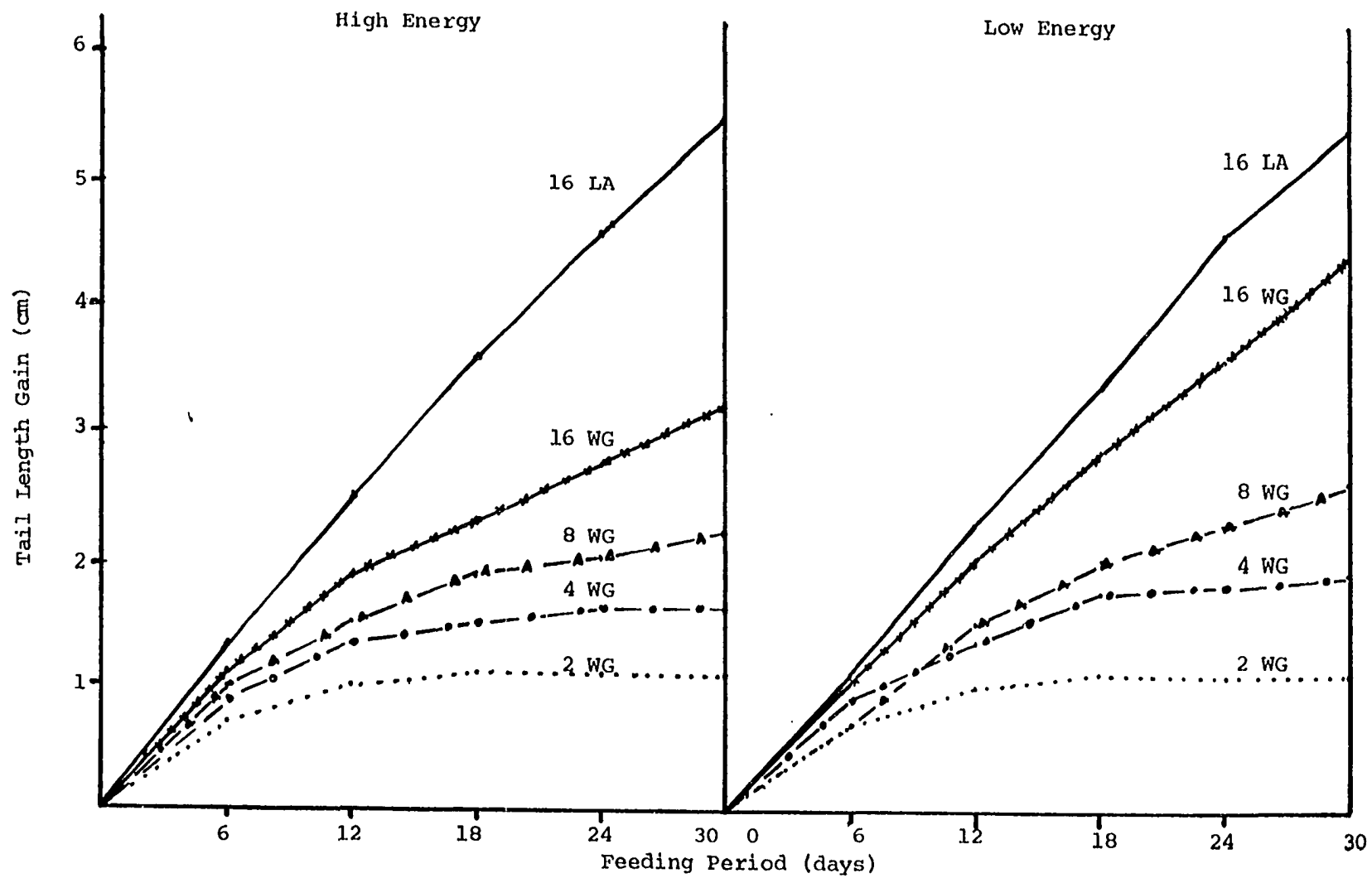


Figure 4. Average gain in tail length of rats fed lactalbumin (LA) and wheat gluten (WG) protein at high and low energy levels for 30 days, Experiment 2





grew about twice as much. The growth patterns of rats fed 16% WG protein at LE level showed steadier and better growth than their counterparts fed the HE diet. Rats fed 16% WG protein LE diets also grew better than all those fed the other WG diets, but not as well as those fed the control LA diets.

Initially, the rats had an average weight of 53-54 g with average tail length of 7.6-7.7 cm (Table 13). By the 30th day of the feeding period, rats fed 16% LA protein diet had significantly higher ( $P < .01$ ) weight gains than those fed WG diets. The differences in weight gains among rats fed varying levels of WG protein were also significant ( $P < .01$ ), except between those fed 2 and 4% WG protein at HE levels. Analysis of variance showed interaction between energy and protein concentrations, indicating that changes in weight as an effect of protein levels are not the same for the two levels of energy used. Rats fed LE diets had significantly higher total weight gains than those fed HE levels. The rats were fed ad libitum and, therefore, those fed the LE diets ate more to meet energy needs.

As in Experiment 1, tails of rats fed 2% WG protein diets grew in length notwithstanding a failure to gain in body weight during the first three weeks (Figure 4). Tails grew by as much as 1.0-1.1 cm up to the third week after which no further growth occurred. On the other hand, tails of rats fed LA protein grew much faster than those receiving WG protein diets. Tails of rats fed LA protein at HE and LE levels attained almost the same length by the 30th day of the feeding period. On the average, tails of rats fed higher levels of WG protein LE diets grew faster than those receiving lower amounts of protein at HE levels.

Table 13. Mean weight gains and tail lengths of rats fed lactalbumin (LA) and wheat gluten (WG) at high and low energy levels, Experiment 2

Dietary protein (%)	Energy:	Weight (g)				Tail length (cm)			
		Initial		Total gains		Initial		Final	
		HE	LE	HE	LE	HE	LE	HE	LE
2 WG		53.0 <sup>a</sup>	54.9	-8.5	-4.2	7.6	7.5	8.6	8.5
4 WG		53.1	54.4	-3.6	4.1	7.5	7.6	9.1	9.5
8 WG		53.1	54.6	2.1	15.6	7.6	7.8	9.8	10.4
16 WG		52.8	54.1	19.5	57.5	7.6	7.8	10.8	12.5
16 LA		52.5	53.3	90.7	113.8	7.5	7.6	13.1	13.5
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F-test:									
Energy		n.s.		**		n.s.		**	
Protein		n.s.		**		n.s.		**	
En x Prot		n.s.		**		n.s.		**	
LSD .01 Energy		4.16		8.71		0.63		0.65	
.05		3.00		6.28		0.45		0.47	
.01 Protein		2.32		9.26		0.24		0.48	
.05		1.75		6.96		0.18		0.36	
Overall S.E.		2.47		9.82		0.26		0.51	

<sup>a</sup><sub>n</sub> = 8.

The occurrence of significant protein x energy interaction in the analysis of variance reveals that the total growth in tail lengths as affected by level of protein is not the same for the two energy levels. Except for rats fed 2% WG protein diets, final tail lengths of rats fed LE diets were higher than those fed the same diet at HE levels. Rats fed LE diets ate more, consumed more protein, and consequently had

greater tail growth than those receiving HE diets. The differences in final tail lengths between rats fed HE and LE diets were much less than differences in final body weights (Figures 3 and 4). Weights seem to readily fluctuate with compositional body changes while tail lengths, like height in humans, increase steadily for a time even under severe dietary restrictions.

#### Food intake

Data in Table 14 reveal that the energy density of the diet greatly affects the amount of food intake. Rats fed LE diets ate about twice as much food as those on high energy diets. The amount of food consumed increased with increasing levels and quality of protein. Rats fed the 16% WG protein LE diet ate 114% more food than their counterparts on HE diets. Rats fed the other four WG protein LE diets ate only 56-83% more food than their counterparts fed the HE regimens. Food intake of rats significantly improved with increasing WG protein levels, except for those fed 8% WG protein HE diets, compared to those fed 4% protein diets with the same energy level.

As a consequence of increased food intakes, rats fed LE diets ate more protein than those fed HE diets with equal protein concentrations. The increased food consumption brought total energy intakes of rats fed LE diets to almost the same levels as their counterparts fed HE diets. Exceptions were rats fed the 2% WG protein LE diet that had significantly lower ( $P < .01$ ) energy intakes than their counterparts fed HE diets (Table 14). These results were similar to the findings of Arthur (1976). Rats

Table 14. Mean total food, protein, and energy intakes of rats fed lactalbumin (LA) and wheat gluten (WG) at high and low energy levels, Experiment 2

Dietary protein (%)	Total food intake (g)		Total protein intake <sup>a</sup> (g)		Total energy intake <sup>a</sup> (kcal)	
	Energy: HE	LE	HE	LE	HE	LE
2 WG	114.5 <sup>a</sup>	178.5	2.30	3.57	419	328
4 WG	140.4	247.0	5.61	9.88	513	456
8 WG	146.7	267.7	11.73	21.42	533	499
16 WG	175.5	376.2	28.08	60.19	650	711
16 LA	308.4	544.8	49.15	87.17	1138	1033

F-test:

Energy	**	**	n.s.
Protein	**	**	**
En x Prot	**	**	**

LSD .01 Energy	27.98	3.28	84.30
.05	20.16	2.36	61.97
.01 Protein	22.23	3.37	60.74
.05	16.70	2.53	46.52
Overall S.E.	23.58	3.58	65.69

<sup>a</sup>Computed values.

<sup>b</sup><sub>n</sub> = 8.

freely fed low calorie diets generally ate more food and compensated for the low energy concentration despite the bulk of the LE diet. However, this was not always completely true for all kinds and at all

concentrations of protein. In experiments on food regulation in weanling rats, Musten et al. (1974) observed that when diets with protein varying in quantity and quality and with varying caloric density were offered simultaneously to rats, the protein intake in relation to total energy was constant as a result of appropriate selection. In their experiments, when energy of a WG diet was diluted by replacing cornstarch with cellulose, the rats selected to maintain a constant intake of protein. Petersen and Baumgardt (1971) reported that rats adjust their food intake when the energy density of the diet is altered. The total influences of energy and protein on food intake are not fully understood.

Osborne and Mendel (1918) observed that rats ate only small amounts of protein-free or low protein diets if given a choice between 0, 6, and 18% casein diets. The rats were unable to increase their protein intake by increasing intake of total diet, and hence, calories. Likewise, in the present experiments, rats ate in proportion to energy needs rather than protein needs. When diets were diluted with cellulose to lower the energy level, rats ate more to approximately maintain energy intake. This could be due to the effect of an energy intake regulating mechanism which tends to maintain energy balance. Similarly, when concentrations of protein in the diet are much above the level needed, rats ate excess amounts of protein in order to consume adequate energy.

#### Liver weight, body weight, and peritoneal fluid

Data in Table 15 show that, on the average, rats fed LE diets had significantly higher ( $P < .01$ ) final body weights and liver weights and more fluid in their peritoneal cavities at the time of necropsy than

Table 15. Body weight, liver weight, and peritoneal fluid of rats fed lactalbumin (LA) and wheat gluten (WG) at high and low energy levels, Experiment 2

Dietary protein (%)	Energy:	Final body weight (g)		Liver weight (g)		Peritoneal fluid (ml)	
		HE	LE	HE	LE	HE	LE
2 WG		43.3 <sup>a</sup>	49.8	1.72	1.50	0.29	2.52
4 WG		47.1	52.3	1.89	1.81	0.39	0.62
8 WG		53.3	61.7	1.86	1.86	0.33	0.33
16 WG		69.7	101.8	2.22	3.40	0.18	0.27
16 LA		140.4	159.1	4.17	5.32	0.38	0.82
<hr/>							
F-test:							
Energy		**		*		*	
Protein		**		**		**	
En x prot		**		**		**	
LSD .01 Energy		9.38		0.47		0.61	
.05		6.76		0.34		0.44	
.01 Protein		10.36		0.45		0.92	
.05		7.78		0.34		0.62	
Overall S.E.		10.99		0.48		0.97	

<sup>a</sup><sub>n</sub> = 8.

\* Significant at .05 level.

those fed HE diets. Exceptions are rats fed 2 and 8% WG diets where no significant differences in body weights of the rats were observed. Likewise, the livers of rats fed 2, 4, and 8% WG protein diets were practically the same except for rats fed 2% protein LE diet that had significantly lower ( $P < .01$ ) liver weight than all other rats. The latter

group of rats also had greater amounts of fluid in the peritoneal cavity and developed the more severe symptoms of edema. In one grossly edematous rat weighing 82 g, approximately 9.2 ml of fluid was drawn from the peritoneal cavity. When edematous rats were autopsied, the fluid in the pendulous sac which bulged between the forelimbs was not found to be watery and free-flowing; rather the fluid was intimately immeshed into the tissue and appeared like a wet gelatinous mass between the skin and the breast muscle.

Rats fed the LA protein (control) diet had the highest body and liver weights, and with relatively small amounts of peritoneal fluid. If the average amounts of peritoneal fluid per kg of body weight in the different groups of rats are calculated and compared, the greater amount of fluid retention can be found in rats fed the LE diets. The water retention in the peritoneal compartment of the body perhaps can be partly attributed to the increased water intakes resulting from high fiber content and bulk in the diet. Waterlow and Alleyne (1971), however, stated that from a strict point of view there is no justification in putting emphasis on edema, since children with marasmus as well as those with kwashiorkor have increased amounts of body water.

Liver weights of rats fed 16% LA and 16% WG protein were significantly different ( $P < .01$ ) from each other and significantly higher ( $P < .01$ ) than those fed lower levels of protein; the weights of their livers were lower, however, when expressed in terms of per unit body weight. Hansen-Smith (1977) reported that differences in organ weights arise because of changing body composition of the animals as they mature; heart, kidney, and liver become a smaller proportion of body weight, whereas muscle mass

progressively increases. With chronic dietary restriction during the period of maturation, organ proportions more nearly resemble the well-nourished age controls than the well-nourished weight controls which are younger. Different types of dietary restrictions as well as age and stage of maturation of experimental animals may affect organ weights. On the contrary, McNaulty and Dickerson (1974) found that, in rats, weights of the majority of organs relative to body weights were maintained both during undernutrition and rehabilitation. Weights of livers remained unchanged during undernutrition despite changes in the amount of DNA and protein.

#### Serum total protein and albumin

Results presented in Table 16 show that serum total protein is influenced by protein quantity and quality and energy levels of the diet. At most protein levels, rats fed the WG and LA protein HE diets had higher serum total protein concentrations than their counterparts fed LE diets. These serum total protein levels increased significantly as the levels of dietary proteins increased from 2 to 16% and as the quality of protein was improved by a change from wheat gluten to lactalbumin. However, the levels of increments in serum total protein as dietary protein levels changed were not the same for the rats in the two energy groups. Rats fed LA diets had the highest serum protein values at both energy levels of the diet. Serum total protein concentrations in rats fed HE and LE diets at 8 and 16% protein levels were almost the same. However, serum total protein of rats fed 2 and 4% WG proteins and 16% LA protein at HE levels was significantly higher ( $P < .01$ ) than their counterparts on LE diets.



Table 16. Serum total protein and albumin, albumin/globulin (A/G) ratios in rats fed lactalbumin (LA) and wheat gluten (WG) at high and low energy levels, Experiment 2

Dietary protein (%)	Serum protein (g/dl)		Serum albumin (g/dl)		Serum A/G ratio <sup>a</sup>		
	Energy:	HE	LE	HE	LE	HE	LE
2 WG		3.50	2.58 (7) <sup>b</sup>	1.81	1.29 (7)	1.19	1.19 (7)
4 WG		3.72 (7)	3.44	2.29 (7)	2.15	1.57 (7)	1.81
8 WG		4.21	4.20	2.88	3.11	2.37	3.53
16 WG		4.52	4.63	3.32	3.24	3.10	2.60
16 LA		5.39	5.04	4.29	3.48	4.91	2.43
<hr/>							
F-test:							
Energy		*		n.s.		n.s.	
Protein		**		**		**	
En x prot		**		**		**	
LSD .01 Energy		0.35		0.46		1.29	
.05		0.25		0.33		0.93	
.01 Protein		0.32		0.24		1.01	
.05		0.24		0.18		0.76	
Overall S.E.		0.34		0.26		1.07	

$$^a \text{A/G ratio} = \frac{\text{albumin (g/dl)}}{\text{globulin (g/dl)}} \text{ where G = protein-albumin.}$$

<sup>b</sup><sub>n</sub> = 8 unless indicated in parentheses ( ).

Likewise, serum albumin levels were somewhat lower among rats fed LE diets (except with those fed 8% WG protein) than their counterparts fed HE diets. These differences, however, were not statistically significant. Serum albumin levels significantly ( $P < .01$ ) increased as quantity and

quality of protein in the diet improved, although the responses were not the same for the different protein treatments at the two energy levels. The highest albumin concentration of 4.29 g/dl was observed to be among rats fed 16% LA protein at HE level; the lowest of 1.33 g/dl was among rats fed 2% WG protein LE diet. Both groups of rats developed clinical signs of edema but more severely among those on low energy-high fiber diets.

Serum albumin/globulin (A/G) ratios were not significantly different between rats fed HE and LE diets (Table 16). There were marked differences in A/G ratios between rats fed the different protein diets, although the magnitude and direction of the change in the ratios were not systematically the same as the quantity and quality of protein in the diets changed. A/G ratios were lowest (1.19) among rats fed 2% WG protein and were not statistically different from those fed 4% WG (1.57-1.81). Among rats fed HE diets, the A/G ratio steadily increased as protein levels and quality improved. Among rats fed LE diets, the ratio increased as protein levels changed from 2 to 8% WG, and decreased with 16% WG and LA proteins.

These results are congruent with those in Experiment 1 and in studies of Edozien (1968) who reported ratios of 1.07-1.50 for the PCM rats. Whitehead (1967) also found the A/G ratio to fall below 1.0 in human kwashiorkor. Normal subjects have ratios above 1.0. Both serum total protein and albumin levels are lowered in kwashiorkor rats; the fall in the serum concentrations become more dramatic as edema becomes more severe. Whitehead (1967), Golden et al. (1975), and Hay et al. (1976) reported that a falling serum albumin level can be a reliable indicator

of developing kwashiorkor. On the other hand, because of increased globulin fractions during infections and infestations among population groups, serum total protein can be increased and appear to be within normal limits. No signs of serious infections were observed in the rats in this experiment. The proportional differences observed in total serum protein concentrations were smaller than those in serum albumin concentrations. Philbrick and Hill (1974) found that the fall in serum albumin coincided with the onset of edema in kwashiorkor rats. Serum albumin concentrations of the rats remained the same until the end of the third week of the experiments in spite of steady loss in weight. Whitehead et al. (1971) reported Ugandan children with kwashiorkor all had lower serum albumin concentrations. This was not true for children with marginal PCM. Similarly, serum albumin levels were maintained for a long period of time before dropping in children consuming adequate amounts of poor quality proteins such as corn (Schendel and Hansen, 1962). The drop in serum albumin associated with the development of edema has been found to be a consequence of considerable decreases in extravascular albumin pools of the body while intravascular pool was maintained (Kudlicka and Kudlickova, 1973; Yuile et al., 1959). Albumin in the extravascular compartments shifts to the intravascular pools. The shift in albumin seems to occur after the ability of the body to synthesize albumin is reduced. Lowered rates of synthesis as well as rate of catabolism of the albumin are apparently adaptations that occur following reduced intakes of protein (Picou and Waterlow, 1962; James and Hay, 1968; Waterlow, 1975).

It should be recalled that the total energy intakes of rats fed LE diets were almost at equal levels to those fed HE diets (Table 14). Hence, it is not correct to consider rats fed LE diets to have low energy intakes compared to those fed HE diets. In fact, protein intakes of rats on LE diets were about twice as high as those fed HE diets. There was no real restriction of energy intake; instead, there was an increased intake of fiber which was added at 55% level in place of cornstarch to produce the LE diets. A decidedly increasing trend was observed in serum total protein and albumin as protein quality and quantity increased. The possibility exists that all the extra protein consumed by rats fed the LE diets was catabolized for energy; the results support this possibility.

#### Serum/plasma N/E amino acid ratios

Table 17 shows the comparison in the N/E amino acid ratio in serum and plasma of rats fed various protein diets at the two different energy levels. Examination of the data reveals that, in general, there are small differences in the N/E amino acid ratio between serum and plasma (except for rats fed 4 and 8% WG protein diets at HE levels). In both plasma and serum, the ratios were significantly higher ( $P < .01$ ) in rats fed LE diets than their counterparts receiving HE diets. Since rats fed LE diets had almost doubled protein intakes and similar energy consumption as those fed HE diets, these results reflect the effects of increased fiber in the diet, and not those of protein alone. Analysis of variance shows that there were significant differences ( $P < .01$ ) in the ratios among rats fed different levels of protein. There was no one-directional trend in response with increasing levels of protein in the diet. Rats with

kwashiorkor and fed 2% WG protein LE diets had the lowest N/E amino acid ratios which were significantly lower ( $P < .01$ ) than their counterparts fed HE diets.

Table 17. Nonessential/essential (N/E) amino acid ratios in serum and plasma of rats fed lactalbumin (LA) and wheat gluten (WG) at high and low energy levels, Experiment 2

Dietary protein (%)	N/E amino acid ratio				
	Energy:	Serum		Plasma	
		HE	LE	HE	LE
2 WG		1.96 (7) <sup>b</sup>	1.40 (6)	2.02 (6)	1.25 (7)
4 WG		2.93 (7)	2.26	3.35	2.05
8 WG		2.19 (7)	2.12 (7)	2.99	1.90
16 WG		2.92	1.92	3.03	1.83
16 LA		2.75 (7)	1.77	2.84	1.66
-----					
F-test:					
Energy		**		**	
Protein		**		**	
En x prot		**		**	
LSD .01 Energy		0.37		0.55	
.05		0.27		0.40	
.01 Protein		0.47		0.48	
.05		0.35		0.36	
Overall S.E.		0.48		0.49	

<sup>a</sup>  $\frac{A, 509 \text{ nm for gly} + \text{ser} + \text{gln} + \text{tau color}}{A, 509 \text{ nm for leu} + \text{ile} + \text{met} + \text{val color}}$ .

<sup>b</sup><sub>n</sub> = 8 unless indicated in parentheses ( ).

The declining trend in the N/E amino acid ratios as protein levels increased from 4 to 16% and changed from WG to LA protein was consistent in both serum and plasma. Among rats fed HE diets, the highest ratios were found in rats fed 4% WG protein HE diets; the case was the same for their counterparts fed LE diets. Plasma N/E amino acid ratios for rats fed 8 and 16% WG and 16% LA at HE levels were practically the same; this trend was observed for their counterparts fed LE diets. In the case of serum N/E amino acid ratios, more variability was noted in rats fed higher protein diets at high energy levels.

Whitehead (1964) and Arroyave et al. (1970) reported imbalances in amino acids not only in severe cases of kwashiorkor but also in children who were living on poor diets without showing clinical signs of PCM except a small size for age. Widdowson and Whitehead (1966) found the N/E amino acid ratio to be considerably higher in rats fed diets low in protein (4.41) than those fed the control casein diet (2.50). Normally, the ratio is less than 2.00 (Whitehead, 1967). Singh et al. (1973) found the N/E amino acid ratio also to be higher (4.15) in marasmic children compared to the control healthy group (1.5-2.5). In the present experiment, rats fed the LE diets had significantly higher total protein intakes (Table 14) than those fed HE diets. The results on the N/E amino acid determinations seem to follow the reports of other researchers on the increase in the ratios with decreasing levels of dietary protein. However, if the dietary protein intake alone determined the plasma ratio of N/E amino acids, then the values for rats fed 8% WG protein HE diet should be similar to those fed 4% WG protein LE diet because of the nearly identical protein consumption in these treatments. The N/E

amino acid ratios (Table 17) for rats fed these diets support this concept.

The imbalance in plasma amino acids, significantly higher N/E amino acid ratios, is apparent among rats fed 4% WG protein HE diets. At 4-5 weeks, these rats are presumed to be in preclinical stages of PCM. Marok (1977) was able to produce the full condition of kwashiorkor in rats by feeding 4% WG protein for 6-7 weeks. This finding suggests that this imbalance of amino acids perhaps can be considered as one of the earliest biochemical abnormalities which occur in the early stages of PCM. Widdowson and Whitehead (1966) also observed rats fed on diets low in protein to have higher N/E amino acid ratios as early as two weeks. In this experiment, the low N/E amino acid ratios of 1.25-1.40 in rats fed 2% WG protein diets, that had developed clinical signs of kwashiorkor, are not congruent with results reported by many workers previously cited. However, Sauberlich (1974) expressed the opinion that although higher ratios occur in primary protein malnutrition, normal ratios do not necessarily mean that a child is nutritionally normal. It is possible that the concentrations of both essential and nonessential amino acids during the preclinical and clinical stages of the disease have declined considerably, and therefore the ratios appear to be normal. Salem et al. (1973) found no correlation of the N/E amino acid ratios to the severity of protein deficiency in rats fed adequate and low casein diets. Anthony and Edozien (1975) found the concentrations of both essential and nonessential amino acids to be reduced in rats fed 0.5 and 1% LA diets. They concluded that the low serum amino acid levels, in spite of wide variations in liver amino acids, may indicate that the

major defect is one of amino acid transport. Another source of variability in the amino acid ratios, as reported by various workers, could be in the specific amino acids used in computing the ratio.

#### Urinary urea and creatinine

As in Experiment 1, the increase in urea excretions paralleled the increase in total protein intakes and was independent of total energy consumption (Table 18). The amount of urea excreted in a day seemed to reflect the protein level of the diet or, perhaps more accurately, the amount of recent protein intake. Rats fed LE diets ate greater amounts of protein than those fed HE diets, and this was reflected in the increased urea excretion. Examination of urinary urea values for rats in the individual protein treatments indicates that rats fed 2% WG protein HE diets excreted the lowest amount of urea; their protein intakes also were the lowest among all the groups. Their counterparts fed the LE diets ate more food and, therefore, had higher protein intakes which were also reflected in urea excretions. The differences in both urea and protein intakes in these two groups of rats were, however, not statistically significant. Also, urea excretions seem not to be related to the severity of the kwashiorkor condition. When the quality of dietary protein was improved by replacing wheat gluten with lactalbumin, the level of urea excretion per gram of protein consumed was lower in rats fed 16% lactalbumin protein compared to those fed 16% wheat gluten protein diets. This was not surprising because rats fed the better quality protein could be expected to be in more positive nitrogen balance. The difference in urea excretion per unit consumption of



Table 18. Urinary urea, creatinine, urea/creatinine (U/C) ratio, and creatinine/tail length (C/TL) index in rats fed lactalbumin (LA) and wheat gluten (WG) at high and low energy levels, Experiment 2

Dietary protein (%)	Urea (mg/day)		Creatinine (mg/day)		U/C ratio		C/TL index <sup>a</sup>	
	Energy: HE	LE	HE	LE	HE	LE	HE	LE
2 WG	10.87 <sup>b</sup>	20.96	0.47	0.26	23.93	80.74	5.42	2.96
4 WG	13.80	37.32	0.60	0.81	24.19	46.54	6.54	8.48
8 WG	23.14	78.65	0.81	1.15	29.19	76.96	9.52	10.98
16 WG	54.59	131.71	1.30	2.60	42.83	49.86	12.09	20.99
16 LA	50.53	172.35	3.57	4.27	14.66	40.04	27.16	31.58
-----								
F-test:								
Energy	**		**		**		*	
Protein	**		**		**		**	
En x prot	**		**		**		**	
LSD .01 Energy	27.70		0.45		18.78		3.04	
.05	20.00		0.32		13.53		2.19	
.01 Protein	28.27		0.45		16.46		3.41	
.05	21.24		0.33		12.37		2.56	
Overall S.E.	29.99		0.47		17.46		3.62	

<sup>a</sup> Calculated as:  $\frac{\text{creatinine (mg/day)}}{\text{final tail length (cm)}} \times 100.$

<sup>b</sup><sub>n</sub> = 8.

protein is more noticeable in rats fed HE diets. These results show that protein quality also influences urea excretion, especially when growth is occurring. Brown and Cline (1974) observed urea excretion in the pig to be a reliable indicator of protein quality in the diet. Supplementation of a corn diet with L-lysine or tryptophan resulted in a significant decrease in total urinary urea excretion as compared with pigs fed the same diet without amino acid supplementation. In PCM, urea excretions decrease as a result of adaptive enzyme changes (Waterlow, 1975). Rats fed low protein diets show a reduction in urea cycle enzymes. Briggs and Freeland (1977) observed maximum rates of urea synthesis in isolated rat hepatocytes to be increased with increases in the protein content of the diet. Das (1972) reported that when rats are changed from a high to a low protein diet, urinary nitrogen output reached a new lower level in 30 hours. The pattern of nitrogen excretion paralleled the change in urea cycle enzyme activity. However, it is still unclear as to how the enzyme system recognizes that there is greater or lesser amount of nitrogen to be excreted. In normal growing animals, the increase in dietary protein leads to greater nitrogen losses as urea because of increases in the rates of turnover of body proteins and metabolism of dietary protein.

Table 18 shows that the excretion of creatinine increases significantly with increases in the level of protein in the diet. Rats fed LE diets had higher total food and protein intakes, grew better, and excreted more creatinine than those fed similar HE diets. An exception is the PCM edematous rats fed 2% WG protein LE diet. These rats ate more and had higher protein intakes but had greater weight losses and more

severe edema than their counterparts given HE diets. A marked difference in creatinine excretion can be seen between rats fed HE and LE diets containing 16% WG protein. The difference in body weights between these two groups of rats was greater than any other two groups fed the same protein but different energy levels. Rats fed higher levels and better quality protein gained more weight and had higher creatinine excretion rates. These results seem to agree with reports in the literature that creatinine is more related to lean body mass than to protein intakes per se. In normal growing individuals, total body weight is correlated to lean body mass. Creatinine excretions have been found to be associated with endogenous protein metabolism, more specifically with body creatine present in the muscles (Chinn, 1966). Folin (1905) concluded that creatinine is independent of the diet. As a measure of muscle mass, and therefore growth and development in young children, it has been recommended as a useful index in the appraisal of PCM (Viteri and Alvarado, 1970). Malnourished children have been found to excrete low levels of creatinine which is indicative of poor muscle development.

The U/C ratios were significantly higher ( $P < .01$ ) for rats fed LE diets and with higher protein intakes than for those receiving HE regimens (Table 18). These results are compatible with the findings of Simmons (1972) and Dugdale and Edkins (1964) that the U/C ratio increases with increasing protein intake and, therefore, reflects the amount of recent protein intake rather than the nutritional status of population groups. Since urea relates well to total nitrogen intake and creatinine reflects body weight, it follows that the U/C ratio would increase with increasing levels of protein consumption. However, this was not always the case in

this experiment. Urea excretions in PCM rats increased, perhaps due to nitrogen losses caused by excessive muscle wasting during weight loss. As a consequence, creatinine excretions decreased and thus the ratios were higher. The findings of this study indicate that the U/C ratio may not be a useful indicator in PCM since the values decreased instead of increased with high protein intakes, particularly the better quality proteins. The U/C ratio also remained the same or increased with low protein intakes.

The use of creatinine as a measure of growth becomes more meaningful when expressed in relation to stature (creatinine/height index). For this study, the C/TL index was chosen as an analogous measurement. An increasing trend in the C/TL index of rats as protein levels increased can be seen in Table 18. Rats fed LE diets had significantly higher ( $P < .05$ ) C/TL indices than those fed high energy diets, except for PCM rats fed 2% WG proteins. These rats had the lowest C/TL index, 2.96. Whereas creatinine reflects body weight and lean body mass, tail lengths relate to stature. As a child grows, height steadily increases whereas weight can fluctuate readily as food intake is increased or decreased. In PCM there is weight loss or failure to gain weight, but the child may still continue to increase in height to some extent, although the rate may be decreased. Thus, the creatinine/height index is decreased. During adequate intakes of protein, the rate of increase in body weight exceeds the rate of increase in stature and, therefore, the index increases. Such was the case in PCM rats when tail lengths were used in place of heights. Rats fed low protein diets gained less weight but their tails continued to grow. Therefore, this index was lower in rats

fed low protein diets. Those fed high levels of WG protein and the better quality protein (LA) gained weight at a much faster rate, causing the index to be much higher. The use of height (in case of humans) or of body/tail length (in case of rats) as the denominator in determining creatinine indices is a more sensitive measure than using body weight alone. Results of this experiment indicate that creatinine/height index in humans or C/TL index in rats can be a useful tool in assessing protein nutriture.

#### Urinary hydroxyproline

Data presented in Table 19 show that urinary hydroxyproline (OHPr) excretions are significantly lowered ( $P < .01$ ) as protein levels and quality in the diet decreased. Except for rats fed 2% WG protein diets, OHPr excretions were significantly higher ( $P < .01$ ) among rats fed LE diets than those receiving HE rations. As expected, these rats fed the LE diets generally grew better because of higher total food and protein intakes. As body weights of rats fed 16% LA protein HE diet doubled, OHPr excretions more than doubled in amount. As protein levels increased from 2 to 4% and from 4 to 8% in WG protein diets fed at the LE levels, OHPr excretions also approximately doubled. However, severely edematous rats fed 2% WG protein LE diets had slightly lower levels of OHPr excreted over the 24-hour period than their counterparts receiving HE diet, although food and protein intakes were higher in the former than in the latter. The excretion of OHPr is a measure of collagen metabolism. Therefore, it is an indirect measure of growth. As in Experiment 1, OHPr excretions seem to parallel those of creatinine excretions.

Table 19. Urinary hydroxyproline (OHP<sub>r</sub>), hydroxyproline/creatinine (OHP<sub>r</sub>/C) ratio, and OHP<sub>r</sub> index in rats fed lactalbumin (LA) and wheat gluten (WG) at high and low energy levels, Experiment 2

Dietary protein (%)	OHP <sub>r</sub> (μg/day)		OHP <sub>r</sub> /C ratio <sup>a</sup>		OHP <sub>r</sub> index <sup>b</sup>	
	Energy: HE	LE	HE	LE	HE	LE
2 WG	37 <sup>c</sup>	34	8.42	12.46	0.36	0.58
4 WG	56	78	9.75	9.93	0.46	0.52
8 WG	84	145	10.37	12.84	0.56	0.79
16 WG	186	386	14.75	14.80	1.02	1.52
16 LA	687	718	20.78	16.88	2.86	2.68
<hr/>						
F-test:						
Energy	**		n.s.		*	
Protein	**		**		**	
En x prot	*		**		**	
LSD .01 Energy	84.40		2.32		0.23	
.05	60.41		1.67		0.16	
.01 Protein	63.41		2.46		0.24	
.05	43.53		1.85		0.18	
Overall S.E.	89.53		2.61		0.25	

<sup>a</sup> Calculated as:  $\frac{\text{hydroxyproline } (\mu\text{g/day})}{\text{creatinine } (\text{mg/day})} \times 100.$

<sup>b</sup> Calculated as:  $\frac{\text{hydroxyproline } (\mu\text{g/day}) \times \text{body weight (kg)}}{\text{creatinine (mg/day)}}$

<sup>c</sup><sub>n</sub> = 8.

When OHP<sub>r</sub>/C ratios were computed, the ratio did not show any marked differences between energy treatments (Table 19). Analysis of variance indicated that differences among protein treatments, within energy levels, were highly significant. The effects of protein levels in the diet on OHP<sub>r</sub> excretions were not the same for the two energy levels used compared to rats fed 2% WG protein HE diets; those fed 2% WG protein LE diets had higher ratios because differences in creatinine excretion were much greater than in OHP<sub>r</sub> excretions.

In determining the OHP<sub>r</sub> index (Table 19), the body weights of rats were incorporated in the computation for OHP<sub>r</sub>/C ratio. This method increased the sensitivity of the measurement. Multiplying the value by 100 brought the results to comparable values for humans. It seems that the index is influenced by the amount and kind of protein in the diet. As levels of protein increased from 2 to 16%, the index increased also. The same trend is true when the protein quality improved from wheat gluten to lactalbumin. The OHP<sub>r</sub> index for rats fed 2% WG diets was high because body weights were higher as a result of edema. At the same time, creatinine excretion was lower because of failure to grow or maintain enough lean body mass. A higher body weight factor and a low creatinine denominator in the formula will result in a higher value for the OHP<sub>r</sub> index. The analysis of variance showed that the effects of protein were not the same for the two energy levels. As levels of protein consumption increased, the OHP<sub>r</sub> index also increased, but not at the higher dietary protein levels; it increased with the better quality protein (LA). In these groups, the OHP<sub>r</sub> index was lower for those rats fed the LE diets although they had higher total protein consumption than those fed the HE

diets. This finding suggests that the OHP<sub>r</sub> index can be useful at low and marginal protein nutriture but not at high protein intake levels.

Experiment 3:  
Effects of Protein and Energy Levels  
Varied by Restriction of Food Intake

Because kwashiorkor but not marasmus was produced in Experiment 2, this experiment was designed to develop kwashiorkor and marasmus separately by feeding rats with lactalbumin and two levels of wheat gluten protein and varying the energy intake in all three protein treatments by restriction of food intake. A total of 40 rats was assigned to the six diet treatments. Rats were pair-fed for 50% restriction; the ad libitum group was allowed to eat freely, and those on the 50% restriction were given half the average amount of the food the next day. The feeding period was 21 days instead of the usual 4-5 weeks. Preclinical signs of PCM were observed and biochemical measurements were compared. Hematocrit measurements were included as an added biochemical indicator.

General appearance, clinical signs, and survival

Since rats were fed for only three weeks, the features of gross kwashiorkor with obvious edema were not produced. By the end of the third week, 4 of 8 rats fed 2% WG protein diet ad libitum showed signs of developing pendulous sacs of edema between the forelimbs. The puffy face and somewhat bloated body were observed in most of these rats. Stunting of growth was obvious. Rats appeared apathetic and lethargic. On the other hand, rats fed 2 and 4% WG protein diets in 50% restricted amounts were severely emaciated, appearing practically "skin and bones".



Growth retardation was even more severe. Bloating of abdomens was observed in more rats in the restricted than the ad libitum-fed groups. During necropsy, the bloating of abdomen of these rats was found to be due to gas and not fluid. By the end of the second week, rats fed 50% restricted amount of 2% WG protein diets had died. Only 3 of 8 rats fed 2% WG protein in restricted amounts survived long enough for sample collection and necropsy. All rats receiving the 4% WG protein diets in 50% restricted amounts survived to the 21st day of the feeding period, but they were severely emaciated. They appeared comparatively aggressive and difficult to handle, especially during the time of feeding and weighing. The body proportions of rats fed on both restricted and ad libitum low protein dietary regimens were different from those observed in rats fed 8% LA protein. There were no external signs of abnormality in the latter which were much larger than those fed the same diet at restricted levels.

The clinical signs of kwashiorkor, although not severe, were seen in rats fed 2% WG protein diets ad libitum for 21 days. Marasmus developed in rats fed 2 and 4% WG protein diets in 50% restricted amounts. Kirsch et al. (1968), Anthony and Edozien (1975), and Philbrick and Hill (1974) successfully produced kwashiorkor in rats by ad libitum feeding of low levels of protein, and marasmus, by restricted feeding of the same diets. Similar clinical signs were observed by these workers but with much more exaggerated features of the disease. The animals were initially older and heavier, and they were fed the experimental diets in less restricted amounts for longer periods of time. Diarrhea and loss of hair were observed in only one or two rats in these studies. There were several

cases of bleeding and blackening of tip of tails in both kwashiorkor and marasmic rats.

### Growth

The growth responses of rats fed the various diets ad libitum and in restricted amounts are presented in Figures 5 and 6 and Table 20. Initial mean weights were 57-58 g. At the end of the feeding period, rats fed ad libitum had higher weight gains or lower weight losses than those fed the same diets in restricted quantities. Tails of ad libitum-fed rats were longer than those fed restricted quantities of the same diets. The differences in weight gains and final tail lengths were significant ( $P < .05$ ). Rats fed restricted amounts of 4% WG protein diets lost practically the same weight as those fed 2% WG protein diets in restricted amounts. This was not true for ad libitum feeding of the same diets. Rats fed freely the same diets lost 17% of their original weights. On the other hand, rats fed 8% LA protein diets ad libitum had the highest weight gains while those fed the same diets in restricted amounts gained less than one-fourth as much. Tails of rats fed 2% WG protein diets in restricted amounts were shorter than those fed 4% WG protein. Rats fed 8% LA protein diets had significantly longer ( $P < .01$ ) tails than those fed WG protein diets.

Rats fed 2 and 4% WG protein diets ad libitum lost weight steadily during the first two weeks and maintained their weight thereafter (Figure 5). Weights of rats fed 8% LA protein diets ad libitum increased continuously. In comparison, progressive weight losses in rats fed 2 and 4% WG protein diets in restricted quantities were even greater than those

Figure 5. Average gain in weight of rats fed lactalbumin (LA) and wheat gluten (WG) protein diets ad libitum or 50% ad libitum for 21 days, Experiment 3

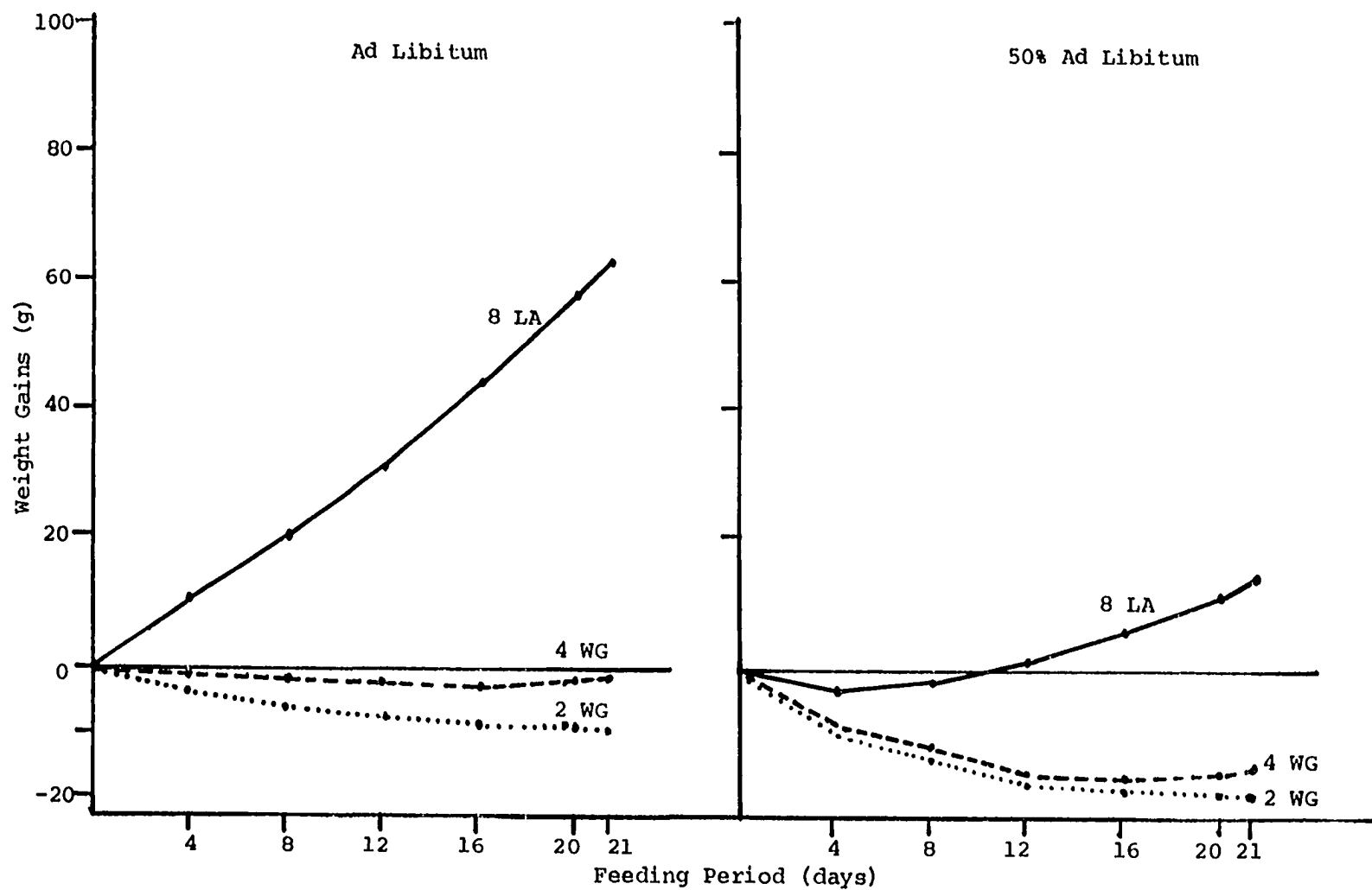


Figure 6. Average gain in tail length of rats fed lactalbumin (LA) and wheat gluten (WG) protein diets ad libitum or 50% ad libitum for 21 days, Experiment 3

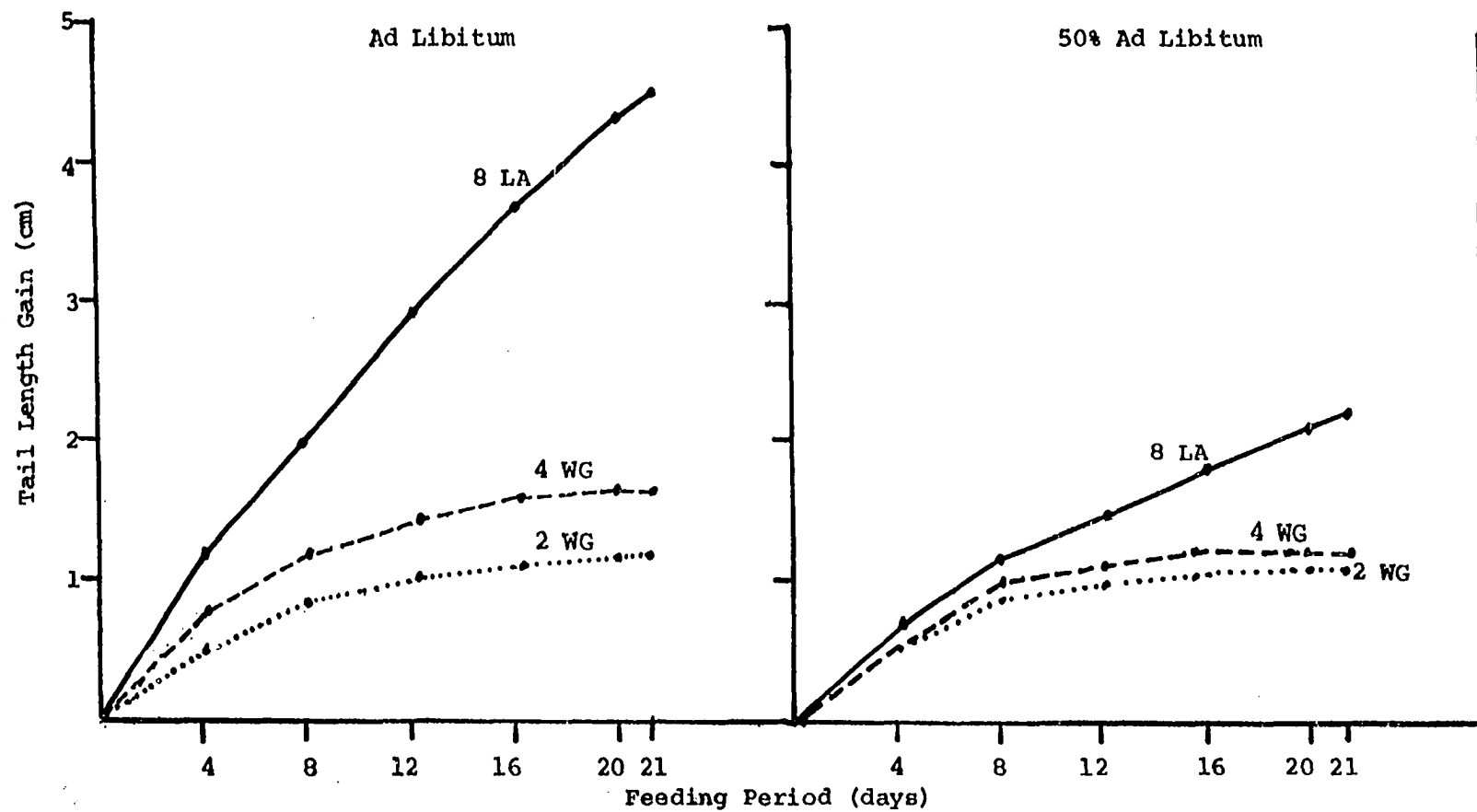


Table 20. Average total weight gains, body weights, and tail lengths of rats fed lactalbumin (LA) and wheat gluten (WG) diets ad libitum or 50% ad libitum for 21 days, Experiment 3

Dietary protein (%)	Feeding:	Weight (g)						Final tail length (cm)	
		Total gains		Body (final)		Body (before necropsy)			
		Ad lib	50% Ad lib	Ad lib	50% Ad lib	Ad lib	50% Ad lib	Ad lib	50% Ad lib
2 WG		-10.1 <sup>a</sup>	-17.0 (3)	47.0	37.8 (3)	45.1	37.2 (3)	8.7	8.2 (3)
4 WG		-2.0	-14.0	55.2	40.6	52.0	40.0	9.3	8.8
8 LA		63.2	14.8	121.2	73.0	110.8	66.5	12.4	10.6
-----									
F-test:									
Protein		**		**		**		**	
Feeding		**		**		**		**	
Prot x feeding		**		**		**		**	
LSD .01 Protein		11.66		9.90		9.06		0.90	
.05		4.37		7.27		6.66		0.66	
.01 Feeding		8.57		4.47		3.68		0.32	
.05		3.17		3.29		2.69		0.24	
Overall S.E.		4.96		5.47		4.22		0.37	

<sup>a</sup>n = 8 unless indicated in parentheses ( ).

fed the same diets ad libitum; on the 21st day of the feeding period, they had lost almost 40% of their original weights. Rats fed restricted 8% LA protein lost 3-4 g during the first four days and subsequently gained weight.

Tails of rats fed restricted diets also grew steadily despite failure to gain weight. The patterns of tail growth (Figure 6) of rats fed 2 and 4% WG protein diets in restricted amounts were almost the same, but were much lower than those fed the same diets ad libitum. Rats fed ad libitum ate twice as much protein as the restricted rats. Tails of rats fed 8% LA protein diets ad libitum grew steadily and linearly, and twice as fast as those on restricted feeding. Tail growth of marasmic rats occurred during the first two weeks, after which the same lengths were maintained.

Degree of losses in weight during fast and urine collection at end of the feeding period and prior to necropsy seem to be related to physical condition of the rats at the beginning of that period, particularly to state of dehydration. Rats fed ad libitum had greater weight losses during fast than those fed restricted diets (Table 20). Marasmic rats fed restricted diets, particularly 2 and 4% WG protein, were more emaciated and had much less body water to lose than kwashiorkor rats fed the same diets ad libitum. Food still present undigested in the gastrointestinal tract at start of fasting may have partly accounted for greater weight losses in ad libitum-fed rats than in those fed restricted diets.

#### Food intake

Considering the exact meaning of 50% ad libitum, rats on restricted diets did not eat exactly 50% of total amounts eaten by ad libitum-fed



rats because of rounding-off errors (Table 21). Each day, the food to be given to the restricted rats was calculated and weighed on the basis of that eaten by the ad libitum-fed rats. However, the actual restrictions which occurred were large enough to generate in the rat the desired effect of producing marasmus separately from kwashiorkor. Rats fed restricted 2 and 4% WG protein diets received only 184-197 kcal and 1.01-2.15 g protein in 21 days. These amounts were only 56-62% of the total calorie and protein intakes, respectively, of rats fed the same diets ad libitum. Heard et al. (1977) reported that rats freely fed low protein (4%) diets consumed an average of 55% as much food as the high protein (12%) diet groups fed ad libitum and, therefore, only 18% as much protein. Growth was minimal, being only 7% of that achieved by rats fed high dietary protein.

#### Serum total protein and albumin

Rats fed on ad libitum and 50% ad libitum diets had practically the same serum total protein concentration (Table 22). Although it appears that rats fed 2 and 4% WG protein ad libitum had somewhat higher serum protein levels than those fed restricted amounts, the differences were not significant. In both groups of rats, serum protein levels were raised as the quantity and quality of protein increased. Doubling the amount of WG protein from 2 to 4% in the diet resulted in a much lesser degree of improvement in serum protein levels than did raising protein levels from 4% WG to 8% LA.

The serum albumin levels of rats fed diets in restricted amounts were significantly higher ( $P < .01$ ) than those fed ad libitum except that

Table 21. Average total food, protein, and energy intakes of rats fed lactalbumin (LA) and wheat gluten (WG) diets ad libitum or 50% ad libitum for 21 days, Experiment 3

Dietary protein (%)	Total food intake (g)		Total protein intake <sup>a</sup> (g)		Total energy intake <sup>a</sup> (kcal)	
	Feeding:	50%	50%	50%	50%	50%
	Ad lib	Ad lib	Ad lib	Ad lib	Ad lib	Ad lib
2 WG	81.7 <sup>b</sup>	50.4 (3)	1.64	1.01 (3)	298	184 (3)
4 WG	95.5	53.8	3.82	2.15	350	197
8 LA	191.5	100.6	15.32	8.05	703	369
-----						
F-test:						
Protein		**		**		**
Feeding		**		**		**
Prot x feeding		**		**		**
LSD .01 Protein		21.58		1.61		79.15
.05		15.85		1.19		58.14
.01 Feeding		6.31		0.47		23.16
.05		4.58		0.34		16.81
Overall S.E.		7.16		0.53		26.30

<sup>a</sup> Computed values.

<sup>b</sup> n = 8 unless indicated in parentheses ( ).

Table 22. Serum total protein and albumin, albumin/globulin (A/G) ratios in rats fed lactalbumin (LA) and wheat gluten (WG) ad libitum or 50% ad libitum for 21 days, Experiment 3

Dietary protein (%)	Serum protein (g/dl)		Serum albumin (g/dl)		Serum A/G ratio <sup>a</sup>	
	Feeding:	50%	Ad lib	50%	Ad lib	50%
	Ad lib	Ad lib	Ad lib	Ad lib	Ad lib	Ad lib
2 WG	3.78 <sup>b</sup>	3.56 (3)	2.17	2.65 (3)	1.45	3.20 (3)
4 WG	4.37	4.30 (7)	2.52	3.10 (7)	1.48	3.44 (7)
8 LA	5.71	6.13	4.14	4.34	3.34	3.06
<hr/>						
P-test:						
Protein		**		**		n.s.
Feeding		n.s.		**		**
Prot x feeding		n.s.		n.s.		n.s.
LSD .01 Protein		0.64		0.57		1.86
.05		0.47		0.42		1.37
.01 Feeding		0.55		0.50		1.42
.05		0.39		0.36		1.02
Overall S.E.		0.60		0.56		1.56

<sup>a</sup>  
A/G ratio =  $\frac{\text{albumin (g/dl)}}{\text{globulin (g/dl)}}$  where G = protein-albumin.

<sup>b</sup><sub>n</sub> = 8 unless indicated in parentheses ( ).

rats fed 8% LA protein ad libitum had practically the same serum albumin levels as those fed in restricted amounts. The serum albumin levels almost doubled when the level of dietary protein in the diet was increased from 2 to 4% WG protein to 8% LA protein. Among rats fed WG protein diets, serum albumin levels did not improve significantly by raising protein levels from 2 to 4%. Results reflect the effects of protein level and quality and the energy content of the diet on serum albumin levels.

The A/G ratios for rats fed restricted amounts of 2 and 4% WG protein diets were significantly higher ( $P < .01$ ) than their counterparts fed the same diets ad libitum (Table 22). Feeding 8% LA protein diets ad libitum did not raise A/G ratios significantly above those fed restricted diets.

Marasmic rats (fed 2 and 4% WG protein in restricted quantities) have higher serum albumin levels than kwashiorkor rats (fed 2 and 4% WG protein ad libitum); the albumin values of all these rats were still lower, however, than those of rats fed 8% LA protein diets ad libitum or in restricted amounts. Also, A/G ratios for marasmic rats were higher than those for kwashiorkor rats, but were similar to those of more normally growing rats fed lactalbumin protein diets. These results agree with reports by other researchers that a falling albumin consistently occurs in developing kwashiorkor but not in marasmus. Albumin levels are generally lower in kwashiorkor than in marasmic children, and could be a useful tool in differentiating between the two forms of PCM (Whitehead, 1964, 1967). Serum total protein level, although reduced in concentration both in kwashiorkor and marasmus, is not a sufficient test to distinguish between kwashiorkor and marasmus. In addition to the serum

albumin concentration, an index that could be useful is the A/G ratio. Significant differences in A/G ratios were found between kwashiorkor and marasmic rats, between kwashiorkor and normal rats, but not between marasmic and normal rats. Philbrick and Hill (1974) reported that changes in serum albumin may be seen only in kwashiorkor type of PCM since many cases of marasmus have serum levels within the normal ranges. In their study, marasmic rats had significantly lowered serum albumin levels which were still higher than those in kwashiorkor rats. However, the fall in serum albumin levels in PCM may only appear when symptoms are about to appear. Such was the case in the PCM rats fed 2 and 4% WG protein in this study. The decline in serum albumin concentrations in kwashiorkor as compared to marasmus could be mainly a result of changes in hydration or edema in the rat, or the edema may partly result from the decreased serum albumin concentration. The A/G ratios may be more meaningful for assessment of PCM during the preclinical stage of kwashiorkor separately from marasmus in infants who may at the same time be exhibiting elevated serum protein levels. Serum albumin levels can be a reliable indicator of protein inadequacy or adequacy but not necessarily of protein-calorie deficiency. It is suggested that the determination of the globulin fraction in the blood should be directly determined to further validate the above findings.

#### Plasma N/E amino acid ratio

Unexpectedly, no significant differences were found in the N/E amino acid ratios of rats fed the various protein diets ad libitum and in restricted amounts (Table 23). However, there is a possible trend

Table 23. Plasma nonessential/essential (N/E) amino acid ratios and hematocrit readings in rats fed lactalbumin (LA) and wheat gluten (WG) ad libitum or 50% ad libitum for 21 days, Experiment 3

Dietary protein (%)	Plasma N/E ratio <sup>a</sup>		Hematocrit	
	Feeding:	50%	Ad lib	50%
	Ad lib	Ad lib		Ad lib
2 WG	2.76 <sup>b</sup>	2.71 (3)	33.5	35.3 (3)
4 WG	3.68	2.67	33.4	37.9 (7)
8 LA	2.95	2.57	40.9	45.5
<hr/>				
F-test:				
Protein		n.s.		**
Feeding		n.s.		n.s.
Prot x feeding		n.s.		n.s.
LSD .01 Protein		0.93		4.17
.05		0.68		3.06
.01 Feeding		0.78		3.38
.05		0.57		2.44
Overall S.E.		0.89		3.70

<sup>a</sup> Calculated as:  $\frac{A, 509 \text{ nm for gly} + \text{ser} + \text{gln} + \text{tau color}}{A, 509 \text{ nm for leu} + \text{ile} + \text{met} + \text{val color}}$ .

<sup>b</sup><sub>n</sub> = 8 unless indicated in parentheses ( ).

in the results toward higher N/E amino acid ratios in rats fed ad libitum than those fed the same diets in restricted amounts. Also, rats fed 4% WG protein ad libitum had significantly higher ( $P < .01$ ) ratios than any of those fed the restricted diets, or fed either 2% WG or 8% LA protein diets ad libitum. The N/E ratios have been claimed to increase during the development of the disease (Widdowson and Whitehead, 1966). It is probable that rats fed 4% WG protein diets ad libitum are in the early developmental stage of kwashiorkor, while those freely fed 2% WG protein diets are already in a more malnourished state. Similarities in the N/E amino acid ratios among rats fed 2% WG or 8% LA protein ad libitum and all those fed restricted diets possibly reflects decreased concentrations of both essential and nonessential amino acids in the kwashiorkor and marasmic rats, without any similar changes in the normal rats fed LA protein diets. When both concentrations of nonessential and essential amino acids decrease in the same proportions, the ratio remains the same. Anthony and Edozien (1975) reported a decrease in concentration of both nonessential and essential amino acids in kwashiorkor rats. Other researchers have reported the ratio to increase with kwashiorkor or during low intakes of protein, and to decrease with adequate protein nutrition (Whitehead, 1964; Philbrick and Hill, 1974; Arroyave et al., 1970; Heard et al., 1977). Singh et al. (1973) found the ratios to be raised in children with marasmus compared with the controls. On the contrary, no correlations of N/E amino acid ratio to the severity of the protein deficiency have been reported by Salem et al. (1973). These workers also used the same analytical method as Whitehead (1964) in determining the N/E amino acid ratio. Several of the researchers mentioned above have

also proposed the use of different amino acids to compute the N/E ratio, such as alanine to threonine, phenylalanine to tyrosine, and/or concentration of individual amino acids such as histidine. Others have simply recommended the comparisons in the rise and fall of absolute amounts of some amino acids in the plasma without computing a ratio. Based on the present study and those of others reported in the literature, the usefulness of the N/E amino acid ratio in nutritional assessment needs further validation.

#### Hematocrit

Hematocrit readings among rats fed ad libitum and 50% ad libitum protein diets are shown in Table 23. Analysis of variance shows that hematocrit readings were significantly higher ( $P < .01$ ) in rats fed restricted amount of diets than those fed ad libitum. Also, as protein levels increased, hematocrit values improved significantly ( $P < .01$ ). However, there were no significant differences in hematocrit values between rats fed 2 and 4% WG protein and between rats fed ad libitum and those fed restricted amounts of 2 and 4% WG protein diets. Control rats receiving 8% LA protein diet had the highest hematocrit.

Results indicate that hematocrit is lowered in PCM. Comparing the two types of PCM, kwashiorkor rats have lower hematocrit readings than marasmic rats. Similarly, Edozien and Switzer (1977) found hemoglobin, hematocrit, and mean corpuscular hemoglobin concentrations (MCHC) to be sensitive to the levels of protein intake. However, in their experiments, rats fed on restricted diets had lower hematocrit than those fed the same diets ad libitum; these results are contradictory to the findings in this



study. Keys et al. (1954) expounded that the relationship of blood hemoglobin, hematocrit, and MCHC in PCM is complex. The hematological picture represents a state of balance between alterations in plasma volume, in water content, and adjustments in red cell mass. In kwashiorkor, anemia is a constant feature and increases in plasma volume usually occur. Because of protein or energy deficiency, hemoglobin and erythropoiesis may be inhibited. These changes alter hemoglobin concentration and hematocrit while changes in the amount of intercellular water can affect MCHC.

#### Urinary urea and creatinine

Urea excretion values shown in Table 24 represent total amounts of urea for a 12-hour urine collection following a 12-hour fast. No significant differences in urea excretion were found between rats fed the same diets ad libitum and in restricted amounts, except for rats fed 2% WG protein ad libitum which had significantly higher ( $P < .01$ ) mean urea excretions than their pairs fed the same diet in restricted quantities. Analysis of variance indicates that urea excretion was significantly influenced by protein levels in the diet. Except for rats fed 4% WG protein, urea excretions increased as protein levels in the diet were raised. However, the differences were significant ( $P < .01$ ) only between rats fed WG and LA protein diets but not between those fed two levels of WG protein. Unexpectedly, rats fed 4% WG protein diet ad libitum, and ate more food and thus had higher protein intakes, had urea excretions which appeared lower than those fed the same diets on restricted levels. The differences were, however, not significant. The reason is

Table 24. Urinary urea, creatinine, urea/creatinine (U/C) ratios, and creatinine/tail length (C/TL) index in rats fed lactalbumin (LA) and wheat gluten (WG) ad libitum or 50% ad libitum for 21 days, Experiment 3

Dietary protein (%)	Urea (mg/12 hr)		Creatinine (mg/12 hr)		U/C ratio		C/TL index <sup>a</sup>	
	Feeding:	Ad lib	50% Ad lib	Ad lib	50% Ad lib	Ad lib	50% Ad lib	Ad lib
2 WG		10.29 <sup>b</sup>	5.65 (3)	0.34	0.21 (3)	31.40	30.67 (3)	3.89
4 WG		7.42	9.70 (7)	0.47	0.27 (7)	15.71	39.54 (7)	5.06
8 LA		16.93	14.43	1.34	0.83	12.40	17.44	10.76
-----								
F-test:								
Protein		**		**		*		**
Feeding		n.s.		**		n.s.		**
Prot x feeding		n.s.		**		*		**
LSD .01 Protein		5.68		0.20		18.14		1.58
.05		4.17		0.15		13.32		1.16
.01 Feeding		6.87		0.14		12.52		1.20
.05		4.37		0.10		9.06		0.86
Overall S.E.		7.55		0.15		13.77		1.31

<sup>a</sup>  
Calculated as:  $\frac{\text{creatinine (mg/12 hr)}}{\text{final tail length (cm)}} \times 100$ .

<sup>b</sup><sub>n</sub> = 8 unless indicated in parentheses ( ).

not apparent for this lack of definite trend in urea excretions as an effect of variation in energy level by restriction of food intake. There was a high variability in urea excretion among individual rats (overall S.E. of 7.75). Rats fed LA protein diets grew better and consumed twice or more protein than rats fed WG protein diets. However, the total urea excretions in rats fed lactalbumin were only double or less than double the amount excreted by rats fed wheat gluten. The low excretion rate of urea nitrogen per unit protein intake in rats fed 8% LA protein compared to those fed WG protein probably indicates more nitrogen utilization for tissue synthesis during growth and, therefore, less nitrogen converted to urea. Picou and Taylor-Roberts (1969) indicated that reduction in protein intake leads to significantly decreased rates of urea synthesis and excretion with a resulting conservation of nitrogen. In this study, total urea nitrogen excretion did not seem to reflect small changes in protein intake at low levels of dietary protein. In severe protein deficiency, there is a definite decrease in total nitrogen excretion and urea excretion due to reduced protein turnover rates (Stephen and Waterlow, 1968).

Urinary creatinine values (Table 24) reflect the influence of dietary protein and calorie levels in the diet on the growth of rats fed ad libitum and 50% ad libitum. Rats fed LA and WG protein diets ad libitum had significantly higher ( $P < .01$ ) urinary creatinine excretions than those fed restricted amounts of the same diets. Likewise, as the level of protein in the diet was increased, the amount of creatinine excreted increased. Rats fed ad libitum had higher protein intakes and higher body weights than those fed 50% ad libitum. With greater lean body mass,

creatinine excretions thus would be expected to be higher. Kwashiorkor rats fed 2 and 4% wheat gluten ad libitum excreted higher amounts of urea than marasmic rats. No significant differences in creatinine excretions were found to exist between marasmic and kwashiorkor rats fed 2 and 4% WG protein diets. The above results are consistent with reports in the literature that creatinine excretions are correlated with growth expressed as increases in body weight and stature but, more specifically and directly, relates to the amount of muscles or lean body mass. PCM causes decreased lean body mass which lowers creatinine excretion. Therefore, urinary creatinine can be a useful indicator of poor growth as a result of PCM.

Tabulated results in Table 24 indicate significant differences in U/C ratios in rats as levels and quality of dietary protein changes. On the average, no significant differences in U/C ratios were found between rats fed ad libitum and those fed restricted amounts of food. The amount of change in the ratios as protein levels increase is not the same at the two levels of calorie consumption. Control rats were observed to have significantly lower ( $P < .01$ ) U/C ratios than kwashiorkor and marasmic rats, except for those fed 4% WG protein ad libitum which exhibited the highest U/C ratio. Marasmic rats fed restricted amounts of WG protein diets (except for 2% WG) had higher ratios than kwashiorkor rats fed the same diets ad libitum. The marasmic rats had lower weight losses and higher total protein intakes than the kwashiorkor rats. These results are contradictory to reports in the literature that the U/C ratios in humans increase with increasing levels of protein in the diet. Like urea excretion rates, it is likely that the U/C ratio is a less reliable

indicator than urinary creatinine or creatinine/tail length index (creatinine/height index in humans) for assessing protein-calorie nutriture. If excretion rates are not consistently and proportionately increased with increased protein intakes to a greater extent than are creatinine excretion rates, the U/C ratio will not show any definite increasing trend.

As shown in Table 24, there is a close relationship between creatinine excretion and tail length. As protein levels increase, the creatinine/tail length index (C/TL) increases also. Rats fed ad libitum have significantly higher ( $P < .01$ ) C/TL indices (3.85-10.76) than those fed restricted amounts of the same diets (2.58-7.84). The differences in C/TL index between marasmic rats fed 2 and 4% WG protein diets in restricted amounts are, however, not statistically significant. The increases in the index as protein levels increase are of the same general magnitude at high and low energy intakes. Marasmic rats that are not getting enough calories and protein to meet needs for growth and energy are smaller in stature, have similar bodies, and less developed muscles; therefore, they also had lower C/TL indices than kwashiorkor rats which were fed diets adequate in calories but not in protein. These results support the observations of other workers that kwashiorkor children generally suffer less severe growth retardation than marasmic infants, especially if the kwashiorkor is the result of a long-term protein deprivation. Marasmus often results in a more dramatic growth failure and muscle wasting, and even in earlier death, than does kwashiorkor.

Urinary hydroxyproline

Rats fed the various protein diets ad libitum had significantly higher ( $P < .01$ ) excretions of OHPr than those fed restricted amounts of the same diets (Table 25). The differences are significant at all three levels of protein. As the protein level increased, urinary OHPr excretion increased. The differences in OHPr excretion between rats fed 2 and 4% WG protein diets are, however, not statistically significant. In rats fed ad libitum and in restricted amounts, the OHPr excretion with LA protein diets was more than five times higher than with the WG protein diets. When growth in rats fed adequate protein from lactalbumin was retarded by food restriction, OHPr excretion was significantly decreased ( $P < .01$ ). As an index of growth, urinary OHPr excretion rates are useful in assessment of PCM. Significant differences were found between kwashiorkor and marasmic rats fed the same low levels of protein but with different energy intakes. Kwashiorkor rats have higher OHPr excretions than marasmic rats. On the other hand, more normally growing rats, such as those fed 8% LA protein, had significantly higher OHPr excretions.

Urinary OHPr excretions seem to parallel those of urinary creatinine levels (Tables 24 and 25). As creatinine excretions rise and fall with increases or decreases in energy and protein in the diet, OHPr excretions follow the same trend. However, when the derived OHPr/C ratios were calculated, statistical tests failed to show significant differences between rats fed ad libitum and those receiving diets in restricted amounts. The test was able to differentiate only between protein levels when the difference in level and quality of dietary protein was large,

as in the case of rats fed 8% LA versus those fed 2 and 4% WG protein diets. The test lost its sensitivity as far as differentiating between types of PCM.

Table 25. Urinary hydroxyproline (OHPr), hydroxyproline/creatinine (OHPr/C) ratio, and OHPr index in rats fed lactalbumin (LA) and wheat gluten (WG) ad libitum or 50% ad libitum for 21 days, Experiment 3

Dietary protein (%)	OHPr ( $\mu\text{g}/12 \text{ hr}$ )		OHPr/C ratio		OHPr index <sup>a</sup>	
	50%		50%		50%	
	Feeding: Ad lib	Ad lib	Ad lib	Ad lib	Ad lib	Ad lib
2 WG	46 <sup>a</sup>	25 (3)	14.02	11.77 (3)	0.63	0.41 (3)
4 WG	50	29 (7)	10.68	12.47 (7)	0.55	0.47 (7)
8 LA	277	166	20.61	19.57	2.30	1.31
<hr/>						
F-test:						
Protein		**		**		**
Feeding		**		n.s.		**
Prot x feeding		**		n.s.		**
LSD .01 Protein		56.04		3.51		0.34
.05		41.16		2.58		0.25
.01 Feeding		29.25		2.76		0.18
.05		21.16		2.00		0.13
Overall S.E.		32.16		3.04		0.20

<sup>a</sup> Calculated as:  $\frac{\text{hydroxyproline } (\mu\text{g}/12 \text{ hr}) \times \text{body weight (kg)}}{\text{creatinine (mg}/12 \text{ hr})} \times 100.$

<sup>b</sup>  $n = 8$  unless indicated in parentheses ( ).

The sensitivity of the test increased when the OHP<sub>r</sub>/C ratio was converted to OHP<sub>r</sub> index including a body weight factor in the formula (Table 25). Significant differences in OHP<sub>r</sub> index were found between marasmic rats fed 2 and 4% WG protein diets. A marked significant difference ( $P < .01$ ) in OHP<sub>r</sub> index can be seen between ad libitum-fed rats and those receiving restricted amounts of 8% LA protein diet. If weight is more variable than height or stature, introducing the height factor (in case of tail lengths) might be even more advantageous in increasing the sensitivity and accuracy of the OHP<sub>r</sub> index as a test. As it is, the index has been shown to be a useful biochemical indicator in the assessment of different types of PCM in human surveys. There is no doubt that healthy, normally growing children would have higher OHP<sub>r</sub> indices than malnourished children. The problem lies in determining the values at which to set the demarcation lines between normality, marasmus, kwashiorkor, and marasmic-kwashiorkor. Research emphasis should be on identifying biochemical characteristics which can distinguish between the lower end of the normal and the beginning of the malnourished states. The recommendations of many other workers for OHP<sub>r</sub> index as a biochemical tool in PCM assessment is further justified based on results of this experiment.

#### Experiment 4: Comparisons of Biochemical Indicators of PCM in Rats Fed Different Protein Diets

In the previous three experiments, kwashiorkor was successfully produced in rats by ad libitum feeding of 2% wheat gluten diets, whereas in the experiments by Marok (1977) 4% wheat gluten diet was used to



produce the condition. This experiment was designed to compare the effects of these kwashiorkor-producing wheat gluten protein diets with 0.5% lactalbumin protein diet used by Edozien (1968) and 8% gelatin (GL) protein diet used by Arthur (1976) to produce experimental PCM in rats. Forty rats were randomly allotted to five protein diet treatments using LA, GL, and WG as protein sources. The experiment was conducted simultaneously with Experiment 3, and the same rats fed 8% LA, 2% WG, and 4% WG protein ad libitum were used for comparisons in both experiments. Additionally, this experiment included 0.5% LA and 8% GL protein diets fed ad libitum.

#### Growth

The growth patterns in Figure 7 reflect the influence of quality and quantity of dietary protein on growth. Rats fed 0.5% LA, 8% GL, and 2% WG protein diets had very similar growth patterns. They steadily lost weight for two weeks, after which weight was maintained. Rats fed 4% WG protein lost less than 5% of their initial weight during the first two weeks, and were almost back to original weight by the end of the third week. Total weight losses by rats fed 0.5% LA, 8% GL, and 2% WG protein diets were almost the same and were significantly greater than those fed 4% WG protein (Table 26). As expected, the positive control rats fed 8% LA protein had the highest total weight gain and the higher tail growth rate. Tails of rats fed 8% LA protein grew fastest. Despite weight losses, tails of rats fed 0.5% LA, 8% GL, and 2% WG protein diets grew by as much as 1.0-1.3 cm during the first two weeks, after which no further growth occurred. Tails of rats fed 4% WG protein grew faster

Figure 7. Average gain in weight and tail length of rats fed lactalbumin (LA), gelatin (GL), and wheat gluten (WG) protein diets for 21 days, Experiment 4

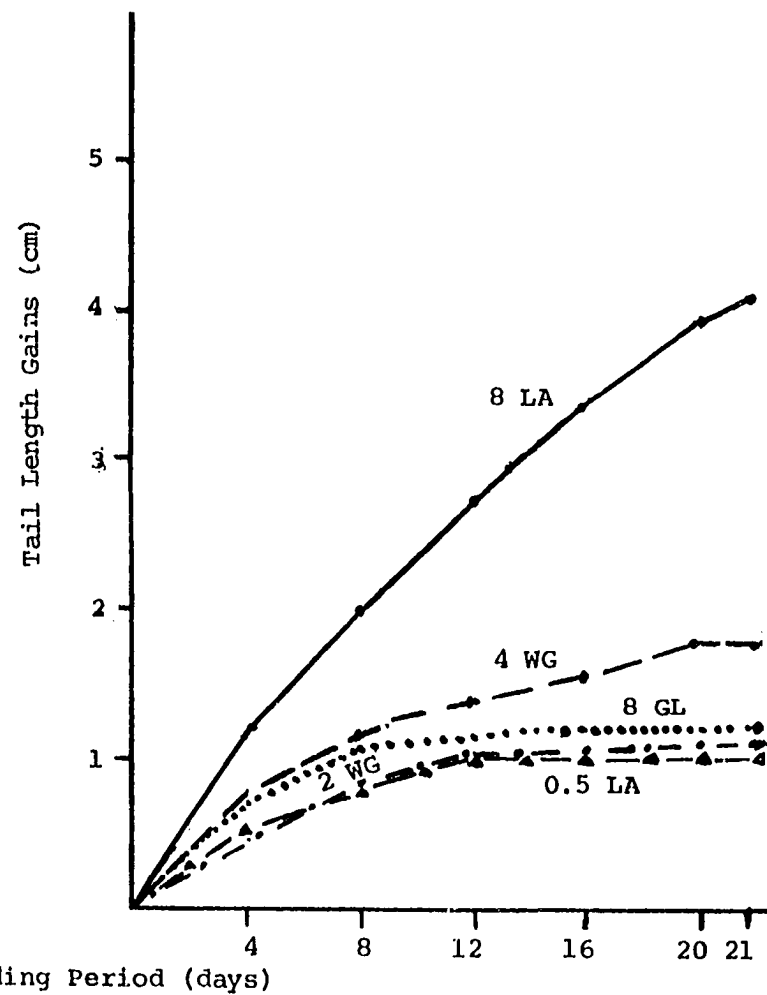
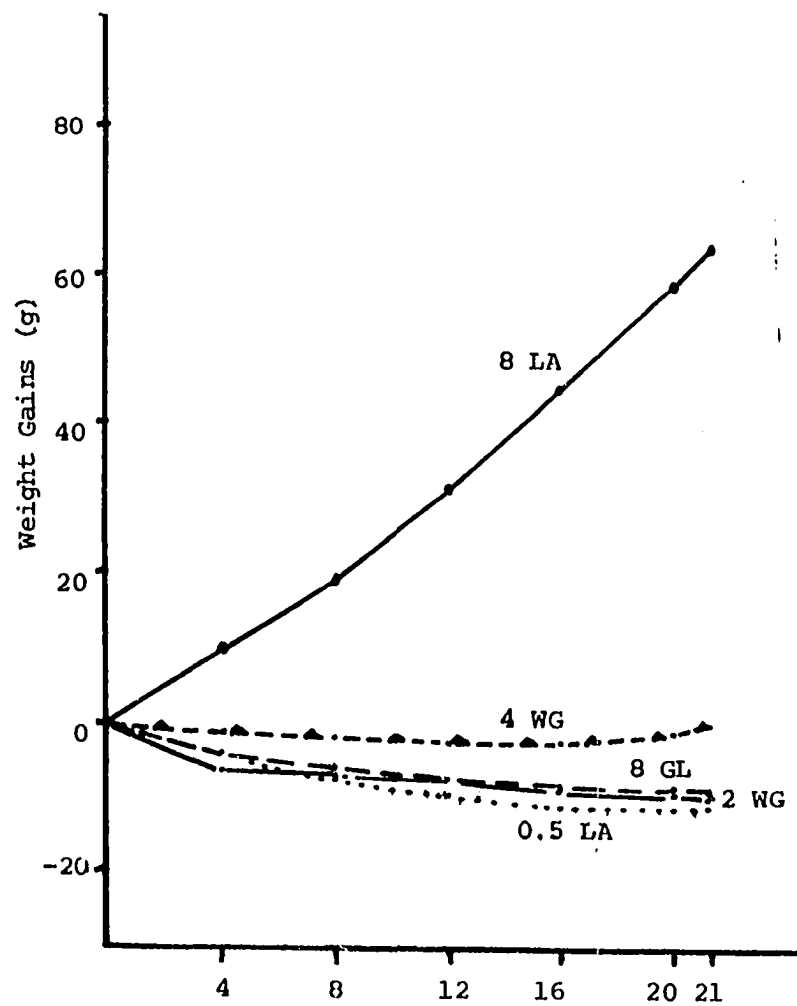


Table 26. Summary of results on mean body measurements in rats fed lactalbumin (LA), gelatin (GL), and wheat gluten (WG) for 21 days, Experiment 4

Dietary protein (%)	Weight (g)			Tail length (cm)	
	Initial	Total gains	Before necropsy	Initial	Final
8 LA	58.0 <sup>a</sup>	63.2	110.9	8.0	12.4
0.5 LA	56.9	-11.8	43.6	7.6	8.7
8 GL	57.2	-9.2	45.0	7.4	8.7
2 WG	57.1	-10.1	45.1	7.6	8.7
4 WG	57.4	-2.0	52.0	7.7	9.3
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F-test	n.s.	**	**	n.s.	**
LSD .01	6.78	9.79	9.08	0.92	1.10
.05	5.05	7.30	6.77	0.69	0.82
Overall S.E.	4.98	7.19	6.66	0.68	0.81

<sup>a</sup><sub>n</sub> = 8.

than those fed 0.5% LA, 8% GL, and 2% WG proteins, but much slower than tails of rats fed 8% LA diet. Data on the final lengths show that at 21 days, rats fed 0.5% LA, 8% GL, and 2% WG protein for the production of kwashiorkor models had the same tail lengths but were significantly shorter ( $P < .01$ ) than rats receiving 4% WG and 8% LA proteins. The results reflect that quality and quantity of protein affect growth. Gelatin is the poorest quality of all the proteins used and, therefore, even a dietary level of 8% did not support growth any better than 2% WG or 0.5% LA protein. Gelatin is lacking in tryptophan, whereas WG is limiting in lysine. Lactalbumin contains all essential amino acids in

proportions appropriate for good nutrition. Most of the amino acid nitrogen of gelatin was excreted as nitrogenous products of metabolism. Wheat gluten is intermediate in quality and, therefore, an intermediate quantity (2% protein) produced effects similar to those of 8% gelatin and 0.5% lactalbumin proteins.

#### Food intake

Data in Table 27 show that food intakes of rats fed 0.5% LA, 8% GL, and 2% WG protein diets were not significantly different. Since the diets were isocaloric, the total energy intakes of these rats also were practically the same. Total protein intakes were higher for rats fed 8% GL protein than for those fed 2% WG protein which were higher than for those fed 0.5% LA protein diets. As expected, rats fed 4% WG protein ate more and had higher total energy intakes than those fed 0.5% LA and 8% GL proteins. These rats had higher protein intakes than those fed the other PCM diets. Total food, protein, and energy intakes were highest in rats fed 8% LA diet. These results reconfirm that quality and quantity of protein affect total food intake. At very low dietary protein levels, rats ate only enough to maintain energy balance and not enough to meet protein needs. When level of protein was increased from 2 to 4% WG, food intake also was increased. When good quality protein such as LA is fed at higher levels in the diet, food consumption increases and growth ensues. The regulatory mechanisms which dictate increase or decrease in food intake seem to be complex, and involve an interplay of many factors impinging on the appetite centers (Scharrer et al., 1970). Total food consumption over a longer period of time is closely related to body size.

Table 27. Mean total food, protein, and energy intakes of rats fed lactalbumin (LA), gelatin (GL), and wheat gluten (WG) for 21 days, Experiment 4

Dietary protein (%)	Total food intake (g)	Total protein intake <sup>a</sup> (g)	Total energy intake <sup>a</sup> (kcal)
8 LA	191.5 <sup>b</sup>	15.36	705
0.5 LA	69.9	0.35	254
8 GL	69.8	5.60	257
2 WG	81.7	1.64	298
4 WG	95.5	3.82	350
F-test	**	**	**
LSD .01	22.76	1.56	83.32
.05	16.96	1.17	62.10
Overall S.E.	16.71	1.15	61.18

<sup>a</sup>Computed values.

<sup>b</sup><sub>n</sub> = 8.

#### Serum total protein and albumin

A comparison of serum total protein and albumin levels among kwashiorkor models in Table 28 shows that rats fed 0.5% LA, 8% GL, and 2% WG proteins have similarly lowered serum proteins, serum albumin, and A/G ratios during the preclinical stages of the disease. Rats fed 4% WG protein diets had higher serum protein and albumin levels but similar A/G ratios to other kwashiorkor models. It seems that the A/G ratio is a more sensitive tool than serum protein and albumin in differentiating not only between marasmus and kwashiorkor but also in differentiating severe and marginal cases of kwashiorkor from normal cases.

Table 28. Serum protein and albumin, albumin/globulin (A/G) ratio of rats fed lactalbumin (LA), gelatin (GL), and wheat gluten (WG) for 21 days, Experiment 4

Dietary protein (%)	Serum protein (g/dl)	Serum albumin (g/dl)	Serum A/G ratio <sup>a</sup>
8 LA	5.71 <sup>b</sup>	4.14	3.34
0.5 LA	3.78	2.22	1.53
8 GL	3.63	2.15	1.54
2 WG	3.78	2.17	1.45
4 WG	4.37	2.52	1.49
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F-test	**	**	**
LSD .01	0.48	0.61	1.28
.05	0.36	0.45	0.95
Overall S.E.	0.36	0.44	0.94

<sup>a</sup> A/G ratio = albumin ÷ globulin where G = protein-albumin.

<sup>b</sup><sub>n</sub> = 8.

Rats fed 4% WG protein diets are in a less advanced stage of kwashiorkor than those fed 2% WG protein; the low serum albumin and protein levels are higher than in the other kwashiorkor rats but the A/G ratio is the same. On the other hand, normal rats fed the 8% LA protein diet had the highest serum protein, albumin, and A/G ratios in the experiment. Arthur (1976) reported lowered serum albumin levels with kwashiorkor rats fed 8% GL protein diets. Edozien (1968) and Anthony and Edozien (1975) observed similar decreases in concentration of serum protein and albumin in rats fed 0.5 and 1% LA protein, and other diets fed in restricted

amounts. Baertl et al. (1974) stated that during inadequate protein intakes, serum albumin can be metabolized to provide amino acids to body organs, muscle, and other tissues. If the situation is prolonged, the protein concentration in the tissues decreases and eventually the clinical picture of kwashiorkor develops. Graham et al. (1966) noted that among marasmic infants, calorie deficiency caused by starvation can be so severe that albumin levels do not show any indication of the severity of a protein deficiency; albumin level may be high, normal, or nearly normal but calorie deprivation is obvious. They explained that contraction of the cellular fluid due to losses of sodium and water in marasmus may result in falsely elevated serum total protein and albumin levels. The question that remains to be answered is what would be the lower limits in serum albumin levels below which one can say that protein malnutrition exists.

#### Plasma N/E amino acid ratio

Analysis of variance reveals significant differences ( $P < .01$ ) in plasma N/E amino acid ratios among rats fed the five different protein diets (Table 29). The LSD test, however, indicates that the differences in the ratios among rats fed 0.5% LA, 8% GL, and 2% WG protein diets were not statistically significant. The average N/E amino acid ratio for rats fed 8% GL protein was not quite significantly higher ( $P < .05$ ) than those fed 0.5% LA protein. Only N/E ratios in rats fed 4% WG and 0.5% LA protein diets were found to be significantly different ( $P < .01$ ) from each other. For kwashiorkor rats fed 2% WG protein, the average N/E amino acid ratio of 2.76 obtained in this experiment of three weeks was



Table 29. Plasma nonessential/essential (N/E) amino acid ratios and hematocrit readings in rats fed lactalbumin (LA), gelatin (GL), and wheat gluten (WG) for 21 days, Experiment 4

Dietary protein (%)	Plasma N/E amino acid ratio <sup>a</sup>	Hematocrit (%)
8 LA	2.95 <sup>b</sup>	41.0
0.5 LA	2.40	32.7
8 GL	3.14	38.6
2 WG	2.76	33.5 (7)
4 WG	3.68	33.4
F-test	*	**
LSD .01	1.03	5.00
.05	0.77	3.73
Overall S.E.	0.76	3.67

<sup>a</sup> Calculated as: 
$$\frac{A, 509 \text{ nm for gly} + \text{ser} + \text{gln} + \text{tau color}}{A, 509 \text{ nm for leu} + \text{ile} + \text{met} + \text{val color}}$$

<sup>b</sup><sub>n</sub> = 8 unless indicated in parentheses ( ).

higher than those observed in rats fed the same diets for four weeks (1.96-2.02). On the other hand, ratios obtained in rats fed 4% WG protein HE diets in Experiment 2 (2.93-3.35) were closer to those obtained in rats fed the same diets for three weeks in Experiments 3 or 4 (3.68). These comparisons do not confirm the findings of Widdowson and Whitehead (1966) that amino acid ratios begin to increase during the early stages of development of PCM. On the other hand, the data agree with observations of Salem et al. (1973) on erratic changes in N/E

amino acid ratios in low protein-fed rats during a 12-week period. Those ratios were not correlated with the degree of protein deficiency; only the ratio of alanine to threonine was found to be always significantly higher in the low protein-fed rats than in normal rats. From another perspective, in vivo studies of McFarlane and von Holt (1969) on rats fed 2% casein diet for eight weeks showed that the overall oxidative degradation of leucine and phenylalanine is markedly decreased compared to the controls. The overall oxidative degradation of glutamate and alanine was unaffected. In the present studies, the ratios obtained were: 1.82 for rats fed 25% WG protein diet in Experiment 1, 2.84 for those fed 16% LA protein HE diet and 1.66 for rats fed 16% LA protein LE diet in Experiment 2, and 2.95 for rats fed 8% LA protein diet for three weeks in Experiments 3 and 4. These differences in the amino acid ratios suggest that satisfactory levels of different types of protein may also produce different amino acid ratios.

#### Hematocrit

Rats fed 0.5% LA, 2% WG, and 4% WG protein diets had significantly lower ( $P < .01$ ) hematocrit values than rats fed 8% LA and 8% GL protein diets (Table 29). The highest average hematocrit value was observed among rats fed the control LA protein diet. The hematocrit of these rats was not significantly different from rats fed 8% GL protein. If protein deficiency caused decreased RBC synthesis, then the 8% GL diet must have caused lowered blood volume also. Low hematocrit does not necessarily result from protein deficiency, although there are reports (Edozien and Switzer, 1977; Edozien et al., 1976) that anemia occurs in

PCM. The changes in hematocrit can also be affected easily by changes in plasma volume and water content of the blood among kwashiorkor rats.

#### Urinary urea and creatinine

Analysis of variance indicates significant differences in urea excretions among rats fed the five different diets (Table 30). Kwashiorkor rats fed low concentrations of dietary protein consumed much less protein than those fed 8% LA protein and had a much higher total protein intake (15.36 g) but excreted only the same amounts of urea as those fed 8% GL protein diets which had a total protein intake of 5.6 g only. These results reflect the inefficient utilization of amino nitrogen from gelatin by rats for anabolic processes during growth compared to lactalbumin protein. Gelatin protein which was fed at the same level as lactalbumin was metabolized and excreted largely as urea nitrogen.

The results on urinary creatinine analysis show that creatinine excretions are practically the same for the kwashiorkor models produced by feeding 0.5% LA, 8% GL, 2% and 4% WG protein diets. The data do not reflect protein intakes, but growth or body size. Rats fed 8% GL, with higher total protein intakes, had similar creatinine excretions as those consuming smaller amounts of protein from 0.5% LA and 2% WG protein diets. These results confirm the findings in the previous three experiments that the amount of creatinine excreted in the urine seems to be more closely associated with the body weight and tail length of rats and, therefore, to lean body mass and not in direct relation with the level or kind of protein in the diet. Body weights of rats fed 0.5% LA, 8% GL, and 2% WG protein diets were practically the same at the end of the 21-day

Table 30. Urinary urea, creatinine, urea/creatinine (U/C) ratio, and creatinine/tail length (C/TL) index in rats fed lactalbumin (LA), gelatin (GL), and wheat gluten (WG) for 21 days, Experiment 4

Dietary protein (%)	Urinary urea (mg/12 hr)	Urinary creatinine (mg/12 hr)	U/C ratio	C/TL index <sup>a</sup>
8 LA	16.93 <sup>b</sup>	1.34	12.39	10.76
0.5 LA	6.80	0.37	22.51	4.29
8 GL	17.36	0.37	49.29	4.22
2 WG	10.29	0.34	31.40	3.89
4 WG	7.42	0.47	15.70	5.06
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F-test	**	**	**	**
LSD .01	9.13	0.23	22.42	1.86
.05	6.80	0.17	16.71	1.38
Overall S.E.	6.70	0.17	16.46	1.36

<sup>a</sup>  
Calculated as:  $\frac{\text{creatinine (mg/12 hr)}}{\text{final tail length (cm)}} \times 100.$

<sup>b</sup>  
<sub>n</sub> = 8.

period and were significantly lighter than those rats fed 8% LA protein diets.

Analysis of variance indicates that significant differences in U/C ratios exist among rats fed the five different protein diets. Results, however, were contradictory to reports of other workers that the U/C ratio decreases in human subjects consuming low protein diets and increases when dietary proteins are high (Arroyave et al., 1962; Dugdale and Edkins, 1964; Sauberlich, 1974). Simmons (1972) noted that when

excretion of urea increased, creatinine excretions remained fairly constant, resulting in an increase in U/C ratio. In this experiment, since urea excretions were found not to increase in proportion to the increments in total protein intake, the ratio did not increase in a predictable manner.

The results of this experiment indicate that C/TL index is a useful measure of growth and protein nutriture. The increase in tail length is closely associated with increases in creatinine excretion, as influenced by protein nutriture (Table 30). Rats fed 8% LA protein diets weighed more, grew faster, and excreted more creatinine in the urine than those fed low protein diets. These rats fed 8% LA protein diets weighed more and had significantly higher C/TL ratios indicating greater lean body mass in proportion to length. Tails of deficient rats continued to grow despite losses in weight during rapid period of growth. Kwashiorkor rats fed 2% WG protein had the lowest C/TL index. The value was not significantly different from those for rats fed 8% GL and 0.5% LA protein diets. The index improved when the protein in the diet was increased from 2 to 4% WG and, of course, from 0.5 to 8% LA protein.

#### Urinary hydroxyproline

Data indicate that urinary OHP<sub>r</sub> excretions are decreased in states of severe protein deficiency (Table 31). Rats fed 0.5% LA and 2 and 4% WG protein diets are in the preclinical stages of developing kwashiorkor, and they have significantly lower ( $P < .01$ ) OHP<sub>r</sub> excretions than rats fed control 8% LA protein diets. Analysis of variance failed to show any differences in OHP<sub>r</sub> excretions among the kwashiorkor rats fed different

Table 31. Urinary hydroxyproline (OHP<sub>r</sub>), hydroxyproline/creatinine (OHP<sub>r</sub>/C) ratios, and hydroxyproline (OHP<sub>r</sub>) index in rats fed lactalbumin (LA), gelatin (GL), and wheat gluten (WG) for 21 days, Experiment 4

Dietary protein (%)	Urinary OHP <sub>r</sub> (μg/12 hr)	OHP <sub>r</sub> /C ratio	OHP <sub>r</sub> index <sup>a</sup>
8 LA	278 <sup>b</sup>	20.61	2.30
0.5 LA	40	11.00	0.48
8 GL	52	14.96	0.67
2 WG	46	14.02	0.63
4 WG	50	10.68	0.55
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F-test	**	**	**
LSD .01	55.35	4.51	0.34
.05	41.25	3.36	0.25
Overall S.E.	40.64	3.31	0.25

<sup>a</sup> Calculated as:  $\frac{\text{hydroxyproline } (\mu\text{g}/12 \text{ hr}) \times \text{body wt (kg)}}{\text{creatinine (mg/12 hr)}} \times 100.$

<sup>b</sup><sub>n</sub> = 8.

diets. The small differences in the rates of growth among rats fed different kinds and levels of protein to produce kwashiorkor were not to be reflected by differences in OHP<sub>r</sub> excretion rates. The test was unable to detect better weight maintenance in rats fed 4% WG protein than in those fed the other deficient diets. Failure to grow during protein deprivation results in decreased collagen metabolism and reduced OHP<sub>r</sub> regardless of the specific dietary protein used to generate the condition.

The OHP<sub>r</sub> excretions do not necessarily parallel creatinine excretions in these rat models of kwashiorkor. Rats fed 8% GL and 2% WG protein diets have higher OHP<sub>r</sub> excretions in relation to creatinine excretions than those fed the 4% WG protein diets. The results with the 0.5% LA protein diet were intermediate and not different from those with the 2% WG protein diet but were significantly lower than those fed the 8% GL protein diet.

With the use of weight as another factor to calculate the OHP<sub>r</sub> index, the treatment comparisons retained practically the same trend as for comparisons of the OHP<sub>r</sub>/C ratios. Rats fed 8% GL and 2% WG protein diets had similar OHP<sub>r</sub> indices to those fed 0.5% LA and 4% WG protein diets.

## SUMMARY AND CONCLUSIONS

Four experiments were conducted to investigate the effects of an intermediate quality protein, wheat gluten (WG), fed with varying energy levels on the symptomatology and selected biochemical indicators of experimental protein-calorie malnutrition (PCM) in rats.

Experiment 1 was designed to produce experimental PCM in rats fed wheat gluten diets and determine certain biochemical indices of protein-calorie status in these animals. Forty weanling rats were allotted to diets containing 0.4, 1, 2, 10, and 25% wheat gluten protein. The diets were fed for 4-5 weeks or until symptoms of PCM were produced. The clinical condition of kwashiorkor and marasmic-kwashiorkor with the characteristic edema and growth failure was produced in rats fed 2% or less WG protein. Growth in terms of weight gain and tail length increase was fastest in rats fed 25% WG protein. Rats fed 0.4, 1, and 2% WG protein diets lost weight but their tails continued to grow for three weeks. Rats fed 10% WG protein maintained original weight but appeared undernourished; their tails grew throughout the experiment. Food intake increased with increasing level of dietary protein. During edema development in the fourth or fifth week, rats fed 1 and 2% WG protein regained some weight because of fluid accumulation. Liver weights were almost the same for both undernourished and PCM rats. Serum total protein, albumin, and albumin/globulin (A/G) ratios were lowest in rats fed 0.4, 1, and 2% WG proteins. The decrement in serum albumin level was proportionately more than the decrease in serum total protein concentrations as protein nutriture worsened. No significant differences



in nonessential/essential (N/E) amino acid ratios (leu, iso, val, met/gly, gln, tau, ser) were observed among rats in any of the treatments, although the ratios tended to increase in malnourished rats. Urinary urea excretions reflected the level of total protein consumption. Significantly higher urea/creatinine (U/C) ratios were observed among rats receiving higher levels of protein. On the other hand, creatinine excretions were higher for the healthy (fed 25% WG protein) and somewhat undernourished (fed 10% WG protein) than for PCM rats (fed 0.4, 1, and 2% WG protein). The three most protein deficient diets produced practically the same low creatinine/tail length (C/TL) indices. Similarly, low hydroxyproline (OHPr) excretions reflected the slow growth or lack of it in PCM rats, compared to rats that were slightly better- or well-nourished. Hydroxyproline/creatinine (OHPr/C) ratio and hydroxyproline index were also low for both undernourished and PCM rats. These three parameters appeared reliable in detecting growth failure during the early stages of PCM.

Experiment 2 was designed to determine whether kwashiorkor can be produced separately from marasmus by feeding low wheat gluten protein diets with energy levels varied through dilution with cellulose. Eighty weanling rats were allotted to ten diet treatments. The ten diet treatments included five protein levels (2, 4, 8, 16% from wheat gluten and 16% from lactalbumin) and two energy levels (high, 5% cellulose, and low, 55% cellulose). The feeding period lasted from 4-5 weeks. Rats fed low energy diets consumed almost twice as much food as those fed high energy diets. The clinical symptoms of kwashiorkor but not marasmus were produced in rats fed 2% WG protein high and low energy diets; the gross edematous feature accompanied by increased fluid

accumulation in the peritoneal cavity was more severe in rats fed 2% WG protein low energy diets. The differences in edema in the rats may be attributed to a consequence of increased water intake due to high fiber content of the low energy diet. As protein intake among rats fed wheat gluten protein diets decreased with diminishing dietary protein concentrations, the growth parameters decreased proportionately. Liver weights were almost the same for rats fed 2, 4, and 8% WG protein high and low energy diets. Serum protein, albumin, and A/G ratios decreased as levels of dietary protein were lowered. Serum albumin levels were significantly lower for the more edematous rats fed 2% WG protein low energy diet than those fed the same diet at high energy level. This finding indicates a possible usefulness of serum albumin levels in assessing the severity of the kwashiorkor condition. No significant differences in serum albumin levels were observed between rats fed high and low energy diets, except in the 2% WG protein groups. The differences in serum total protein concentrations with changing dietary protein levels were smaller than the differences in serum albumin levels and A/G ratios. Plasma/serum N/E amino acid ratios appeared to possibly increase during undernourishment, although the effect was not significant. The ratios were similarly low in both severe PCM and healthy nutritional states. Urea excretions responded to the quality and quantity of protein intake. As dietary wheat gluten protein levels increased from 2 to 16%, urea excretions also increased both at high and low energy levels in the diet. Rats consuming 16% lactalbumin (LA) protein consumed more food and protein, and grew better than those fed 16% wheat gluten protein diets. The level of urea excretion was, however, almost the same for both groups

of rats, indicating the positive influence of high quality protein on utilization efficiency and nitrogen balance. In contrast to Experiment 1, U/C ratios decreased as dietary protein intakes increased. Creatinine excretions were positively associated with levels of protein intake, protein nutriture, and tail length growth. PCM rats fed 2% WG protein and having more severe edematous condition had lower creatinine excretion and C/TL indices than their counterparts with less severe edema. C/TL indices increased with improvement of protein level and quality. OHPr excretions were significantly lower for PCM rats than for those fed marginal or adequate protein. OHPr/C ratios and OHPr indices were low among PCM and marginally undernourished rats, and these parameters can be useful indicators of protein-calorie status.

Experiment 3 was designed to produce experimental kwashiorkor separately from marasmus by feeding different dietary concentrations of wheat gluten protein with energy varied by restriction of food intake. Forty-eight weanling rats were fed diets containing 2% and 4% WG and 8% LA protein diets in ad libitum or 50% ad libitum amounts. Clinical kwashiorkor and marasmus were produced separately. Rats fed 2% WG protein diets ad libitum developed kwashiorkor and those fed 2 and 4% WG proteins in restricted amounts developed marasmus. By the third week of the feeding period, 75% of the rats fed 2% WG protein diet in restricted levels had died. Marasmic rats were more stunted in growth, had shorter tail lengths, and were more severely emaciated with no signs of edema than those with kwashiorkor. Serum proteins were higher for rats fed LA than those fed WG protein diets. No significant differences in serum total protein were observed between rats fed ad libitum and

those given restricted amounts of food. Serum albumin levels were lowest for kwashiorkor rats fed 2% WG protein. Marasmic rats had significantly higher serum albumin levels than kwashiorkor rats. When compared to those fed 8% LA diets, serum albumin was lower for marasmic rats fed 2% WG protein diets in restricted amounts, but A/G ratios were normal or near normal. Serum albumin and A/G ratios appear to be useful indicators for differentiating between kwashiorkor and marasmus, and between all PCM and healthy rats. N/E amino acid ratios were practically the same for rats fed ad libitum and in restricted amounts. Rats fed 4% WG protein diets had the highest ratios regardless of ad libitum or restricted feeding. Kwashiorkor rats had significantly lowered hematocrit values. Marasmic rats had intermediate values which were not significantly lower than the controls. Urea excretion and U/C ratio did not consistently reflect level of protein-calorie nutriture. Creatinine excretion reflected the degree or severity of protein-calorie deficiency. Marasmic rats which suffered more growth stunting and more muscle wasting had lower creatinine excretions than kwashiorkor rats. As the quality and levels of dietary protein and the calorie intake decreased, C/TL index also decreased. Kwashiorkor rats had higher OHP<sub>r</sub> excretions than marasmic rats but similar OHP<sub>r</sub>/C ratios. Urinary hydroxyproline seems to be useful in differentiating between marasmic and kwashiorkor rats; hydroxyproline/creatinine ratio and hydroxyproline index can help in detecting growth failure early during malnutrition.

Experiment 4 was designed to make comparisons of the PCM produced by three different types and levels of dietary protein, wheat gluten (2% and 4%), lactalbumin (0.5%), and gelatin (8%). Rats fed 8% lactalbumin

served as positive controls. Forty weanling rats were allotted to five protein treatments; the feeding period lasted for three weeks. Food intake and growth rates, in terms of weight gain and tail length growth, were practically the same for the kwashiorkor models produced by feeding 0.5% lactalbumin, 8% gelatin (GL), and 2% wheat gluten protein diets. Because of the shorter feeding period, the kwashiorkor rats were observed to be in the preclinical stages of the disease. Serum protein levels were lowest for kwashiorkor rats, with no apparent differences among the three models. The first symptoms of edema appeared at almost the same time during the third week in some rats fed PCM diets. N/E amino acid ratios were similar for all kwashiorkor rats; marginally undernourished rats fed 4% WG protein diets had highest N/E ratios. Urea excretions showed the influence of protein quality and quantity in the diet. Rats fed low protein diets proportionately had low urea excretions. However, urea excretions for rats fed 8% LA, with higher total food and protein intakes, were almost the same as those fed 8% GL that had lower food and protein consumption. This comparison reflects the more efficient utilization of amino nitrogen from lactalbumin than from gelatin. U/C ratios were low for all kwashiorkor rats, except for those fed 8% GL diets. Creatinine excretions were significantly lower among kwashiorkor rats fed 0.5% LA, 8% GL, and 2% WG protein diets than the undernourished animals, but were not different from each other. Lowered C/TL index consistently reflected growth retardation in PCM and undernourished rats. Similarly, reduced growth and poor physical development, which are typical of PCM and undernourished rats, were indicated in the low hydroxyproline excretion, hydroxyproline/creatinine ratio, and hydroxyproline index.

From these four experiments, it can be concluded that protein quality and quantity influence food intake, growth, and urea excretion in the rats. Energy level in the diet affects food intake and, therefore, protein-calorie nutriture. Biochemical indices that can be recommended as useful for nutritional assessment in field surveys are: serum albumin, urinary creatinine, creatinine/height (tail length) index, urinary hydroxyproline, hydroxyproline/creatinine ratio, and hydroxyproline index. Serum albumin appears to differentiate between marasmus and kwashiorkor, between PCM and normal rats, and detect the severity of the PCM condition. Albumin/globulin ratios distinguish between kwashiorkor and marasmus but not between PCM and normal rats. Urinary hydroxyproline differentiates kwashiorkor from marasmus, while hydroxyproline/creatinine ratio and hydroxyproline index are useful in distinguishing clinical and preclinical PCM from normal animals. The nonessential/essential amino acid ratio, urinary urea excretion, hematocrit, and urea/creatinine ratio are less reliable as indices of PCM and need further studies to validate their usefulness in the nutritional assessment. Urinary urea level reflects quantity and quality of protein intake and not necessarily protein and/or calorie nutriture.

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APPENDIX

Appendix Table A-1. Composition of water-soluble vitamin mixture

Vitamin	Per kg vitamin mix (g)	Per kg diet (mg)
Thiamine HCl <sup>a</sup>	0.5	5.0
Riboflavin	0.5	5.0
Nicotinic acid	2.5	25.0
Pyridoxine HCl	1.5	15.0
Ca-panthothenate	2.0	20.0
Folic acid	0.1	1.0
Biotin	5.0	0.5
Vitamin B <sub>12</sub> <sup>b</sup>	1.0	0.01
Inositol <sup>c</sup>	70.0	700.0
p-Amino benzoate	40.0	200.0
Cornstarch to make 1000 g	896.9	

<sup>a</sup>All vitamins, except inositol, were obtained from General Biochemicals Inc., Chagrin Falls, Ohio.

<sup>b</sup>Commercially available vitamin B<sub>12</sub> in mannitol furnished 0.1 mg B<sub>12</sub> per 100 g of mixture.

<sup>c</sup>Grand Island Biological Company, Grand Island, New York.

Appendix Table A-2. Composition of fat-soluble vitamin mixture

Vitamin	Amount per kg vitamin mix	Composition per kg diet
Vitamin A <sup>a,b</sup>	500 mg	5000 IU
Vitamin D <sup>a,c</sup>	2 g	1000 IU
Vitamin E <sup>d,e</sup>	5 g	50 IU
Vitamin K <sup>f,g</sup>	20 mg	0.2 mg
Corn oil to make 1000 g	992.3 g	

<sup>a</sup>General Biochemicals Inc., Chagrin Falls, Ohio.

<sup>b</sup>Vitamin A Palmitate, 1,000,000 IU/g in oil.

<sup>c</sup>Vitamin D<sub>3</sub> Stock Solution, 1 g = 1.25 mg D<sub>3</sub>.

<sup>d</sup>Roche Chemical Division, Hoffman-LaRoche Inc.

<sup>e</sup>Vitamin E, NF-FCC ( $\alpha$ -tocopheryl acetate), 1 mg = 1 IU.

<sup>f</sup>Nutritional Biochemicals Corporation, Cleveland, Ohio.

<sup>g</sup>Vitamin K, oil soluble, 2 methyl-napthoquinone.