UREMIA THERAPY USING THE ALPHA-KETO ANALOGUE OF PHENYLALANINE

By

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UREMIA THERAPY USING THE ALPHA-KETO ANALOGUE OF PHENYLALANINE

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CHAPTER I

INTRODUCTION

Significance of the Study

The protein portion of the daily dietary intake represents the most important dietary problem in the patient with chronic renal disease (12). Since 1836, many dietary regimens have been advocated in an attempt to deal effectively with this problem. Early attempts at dietary management were based on the principle of restricting the daily protein intake in an effort to diminish the accumulation of end-products of protein metabolism, which is chiefly urea (9). Although the exogenous protein intake is closely related to the degree of uremia, even in the absence of dietary protein intake, endogenous tissue catabolism will raise blood urea levels and result in the accumulation of nitrogenous end-products which adversely affect the patient's health (9).

Because of this latter consideration, researchers have become interested in the substitution of alpha-keto analogues of essential amino acids for the amino acids as a therapy for uremia. According to Walser et al. (18), the rationale for this type of therapy is based on several earlier observations:

> . . . a.) urea is continually degraded to ammonia and carbon dioxide by intestinal urease in normal subjects, as well as in patients with uremia; b.) uremic patients respond favorably to restriction of dietary protein; c.) urea or ammonia can be utilized for protein synthesis, particularly when dietary protein is restricted; and d.) alpha-keto

> analogues of most of the essential amino acids can promote growth in rats fed diets devoid of the corresponding amino acid. (18, p. 678)

The utilization of the keto acid analogues of essential amino acids has important practical implications in the treatment of uremia. If these compounds could, in appropriate amounts, replace all or most of the essential amino acids, a synthetic diet could be produced which would be virtually nitrogen-free. Such a diet would have an advantage over current or natural diets in that it would maximize reutilization of the patient's available ammonia nitrogen, thereby reducing the amount of urea nitrogen formed for excretion (1).

For the growing rat, the essential amino acids can, indeed, be replaced by their alpha-keto acid analogues without reducing the animal's rate of growth if adequate non-specific nitrogen is provided (21). Given the carbon skeleton, the rat by amination or transamination converts the analogue to the essential amino acid and utilizes nitrogen from the ammonia pool in the process. In most studies, the non-essential nitrogen to be used for the conversion of the keto analogues to amino acids has been furnished in the form of dietary ammonium salts. If not supplemented in the diet, the reserve of the ammonia pool would be used.

In uremia, this surplus of ammonia can be used for synthesis of essential amino acids. Richards et al. (13) found that in patients suffering with chronic renal failure, the amount of nitrogen urea cycled through ammonia was sufficient to supply the nitrogen required for synthesis of essential amino acids. Since the amount of urea recycled is a function of blood urea concentration, it would appear then that the patient would benefit not only from the uptake of ammonia for

amino acid synthesis but also from reducing recycling of urea (12).

Purpose of the Study

Hence, the benefit of alpha-keto acid analogue therapy may be two-fold: (1) in reducing blood urea levels and thus ameliorating the uremic syndrome associated with high blood urea nitrogen levels and (2) in improving the nutritional status of the individual in regard to protein synthesis. The purpose of this study was to observe the effectiveness of substitution of the alpha-keto acid analogue of phenylalanine for tyrosine and phenylalanine in the diet of uremic rats as measured by weight gain and blood urea nitrogen.

Objectives of the Study

The objectives of this study were:

1. To establish a time line for the development of uremia in rats following a five-sixths partial nephrectomy.
2. To determine if surgically-induced uremic rats could convert an alpha-keto acid analogue of an essential amino acid to the amino acid sufficiently to gain weight rapidly.
3. To determine if the conversion of the alpha-keto acid analogue to the essential amino acid would occur rapidly enough to reduce the urea nitrogen in the bloodstream, which would indicate that this clinical syndrome of uremia was being alleviated by alpha-keto analogue use.

CHAPTER II

REVIEW OF LITERATURE

Urea is the chief nitrogenous waste product of protein metabolism. It is formed in the liver by the deamination of amino acids and carried to the kidneys by the blood (11). The concentration of urea in the urine depends on 1.) the concentration of urea in plasma, 2.) the rate of glomerular filtration, 3.) tubular secretion, and 4.) tubular reabsorption (9). In the case of chronic renal disease, where there is a progressive loss of functional glomeruli, there will be a decrease in urea filtration with a subsequent rise in blood urea nitrogen (BUN) levels. As urea nitrogen concentration increases in the blood, toxic substances, i.e., ammonia, associated with protein catabolism will also accumulate. Eventually a point is reached when the individual cannot tolerate the excessive levels of these toxins in the blood stream and the individual will become comatose (9).

The underlying mechanisms by which elevated urea levels are sharply lowered through elimination of protein from the diet of the azotemic patient are not well understood. Certainly, the amount of excess nitrogen intake is reduced. However, the use of urea nitrogen as a direct substitute is not possible. Yet it is postulated that utilization of urea for anabolic purposes is responsible for most of the improvement (9). Evidence of the ability to incorporate nitrogen from urea into body protein has been found in several species. Davies and Kornberg (3)

found that liver protein of the cat was labeled with N^{15} after being fed labeled urea with a low protein diet. Lui et al. (10) showed that liver, kidney and blood protein were labeled with the isotope when N^{15}-labeled urea was fed to growing pigs with a low protein diet. Rose and Dekker (14) also demonstrated that urea nitrogen was utilized for synthesis of non-essential amino acids by the growing rat when no protein nitrogen was available.

Using these earlier animal studies as guidelines, Giordana (6) postulated that if urea, under certain conditions, were used for protein synthesis, patients suffering from chronic renal disease could utilize their own urea for anabolic purposes. This assumption led to the development and clinical application of diets restricted to proteins of high biologic value for the dietetic alleviation of the uremia. The results of his study suggest that urea nitrogen can be utilized for synthesis of non-essential amino acids if urea is either given exogenously to non-uremic subjects or if derived endogenously from retained urea in patients with renal failure.

The results of the Giordano study were corroborated by Giovannetti and Maggiore (8) the following year (1964). Again, a diet which provided only the minimal amount of essential amino acids plus adequate energy was found to alleviate the symptoms of azotemic patients while resulting in a positive nitrogen balance. All of these studies are based on the utilization of endogenous urea by the patient as a source of nitrogen for synthesis of non-essential amino acids.

Alpha-Keto Acid Analogue Therapy

As early as 1954, studies were under way to modify the concept of

indispensability of "essential amino acids". It has been reported that young rats grew as well when five different keto acid analogues were used in a diet to replace their corresponding amino acids (21). In 1971, Gallina et al. (5) reported that a young woman maintained a positive nitrogen balance when alpha-ketoisovaleric acid was added to a diet deficient in valine. Later that same year, Richard et al. (13) reported that at least two of the eight essential amino acids could be replaced by the alpha-keto analogue in the diet of healthy and uremic patients. The administration of this diet resulted in either a positive nitrogen balance or a reduced negative nitrogen balance. Also, in 1970, Rudman (15) reported that man could convert alpha-ketoisovaleric acid and phenylpyruvic acid to the essential amino acids, valine and phenylalanine, even though the efficiency of these conversions was considerably less than 100 percent.

Significance of Blood Urea Nitrogen Levels

The significance of urea in the clinical syndrome of uremia remains somewhat controversial (18). Urea itself is non-toxic, so the symptoms of uremia are not caused by its accumulation. Thus a substance capable of reducing blood urea may or may not be beneficial for the uremic patient if this is its only action (20).

The most common cause for an increased concentration of blood urea nitrogen is impaired kidney function. Blood urea nitrogen levels are certainly indicative of a disease process in which kidney function is hampered so that harmful substances accumulate in the body (17). Consequently, the blood urea concentration is a useful indication of the

course of kidney disease. Clinical improvement almost always results when blood urea nitrogen levels decrease and are brought within the normal range. Generally, alleviation of uremic symptoms (as indicated by BUN levels) is by the restriction of dietary protein. If an agent could be added to the diet that would promote nitrogen anabolism by directing ammonia derived from intestinal ureolysis to protein synthesis, clinical improvement can also be achieved. Reduction of urea nitrogen levels in the blood would continue to serve as an indicator for such a reaction (20).

CHAPTER III

MATERIALS AND METHODS

Male weanling (approximately three weeks old) rats of the Sprague-Dawley strain,[1] weighing 50 to 75 grams were used in the study. Upon arrival they were immediately transferred to randomly assigned individual stainless steel cages. The animals were held three days to compensate for any dehydration or starvation effects of shipping before taking initial weight and BUN measurements. During this period the animals were fed commercial rat ration and water ad lib.

Eight animals were used for surgical practice and for determining rapidity of the onset of uremia following nephrectomy. The surgical procedure (Appendix A) was modified when six of the nephrectomized rats died due to hemorrhage and surgical trauma. In the surviving rats, the uremic syndrome appeared within 36 hours, as determined by BUN levels (Appendix B). Consequently, it was decided to place the rats on the test diets immediately following surgery.

Sixteen of the initial twenty test rats survived the surgical procedure and were paired for dietary assignment. Test Diets A and Test Diet B (Appendices C and D) were randomly assigned within pairs. In the evening of Day 0, Day 5, Day 10 and Day 15, each rat was weighed and bled by the tail-clipping method (Appendix E). Analysis of variance

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[1]The rats were purchased from the Sprague-Dawley Company, P. O. Box 4220, Madison, Wisconsin 53711.

(AOV) was made on the data obtained for significant differences in performance. Following measurements on Day 15, all animals were euthanized.

CHAPTER IV

RESULTS AND DISCUSSION

The results are shown in Figure 1. In both diet treatments, the weight gain was rapid for the first five days and then essentially plateaued for the next ten days. Gains did not differ due to treatment ($P > 0.5$), as shown in Table I.

TABLE I

F SCORE REFLECTING DIFFERENCES IN WEIGHT GAINS BETWEEN TREATMENT METHODS

Treatment Methods	$\bar{X}$ gm.	F	Level of Significance
Diet A (10 EAA)	149.89	3.09	NS
Diet B (9 EAA + analogue)	146.84		

The BUN levels rapidly declined during the first five days then stabilized for the next ten days. The mean urea-nitrogen level was lower ($P < .001$) with Diet B (8.84 mg%) than Diet A (13.5 mg%) as shown in Table II. These data would suggest that the amount of urea synthesis was reduced by the uptake of ammonia for the synthesis of amino acids from the keto analogues in Diet B.

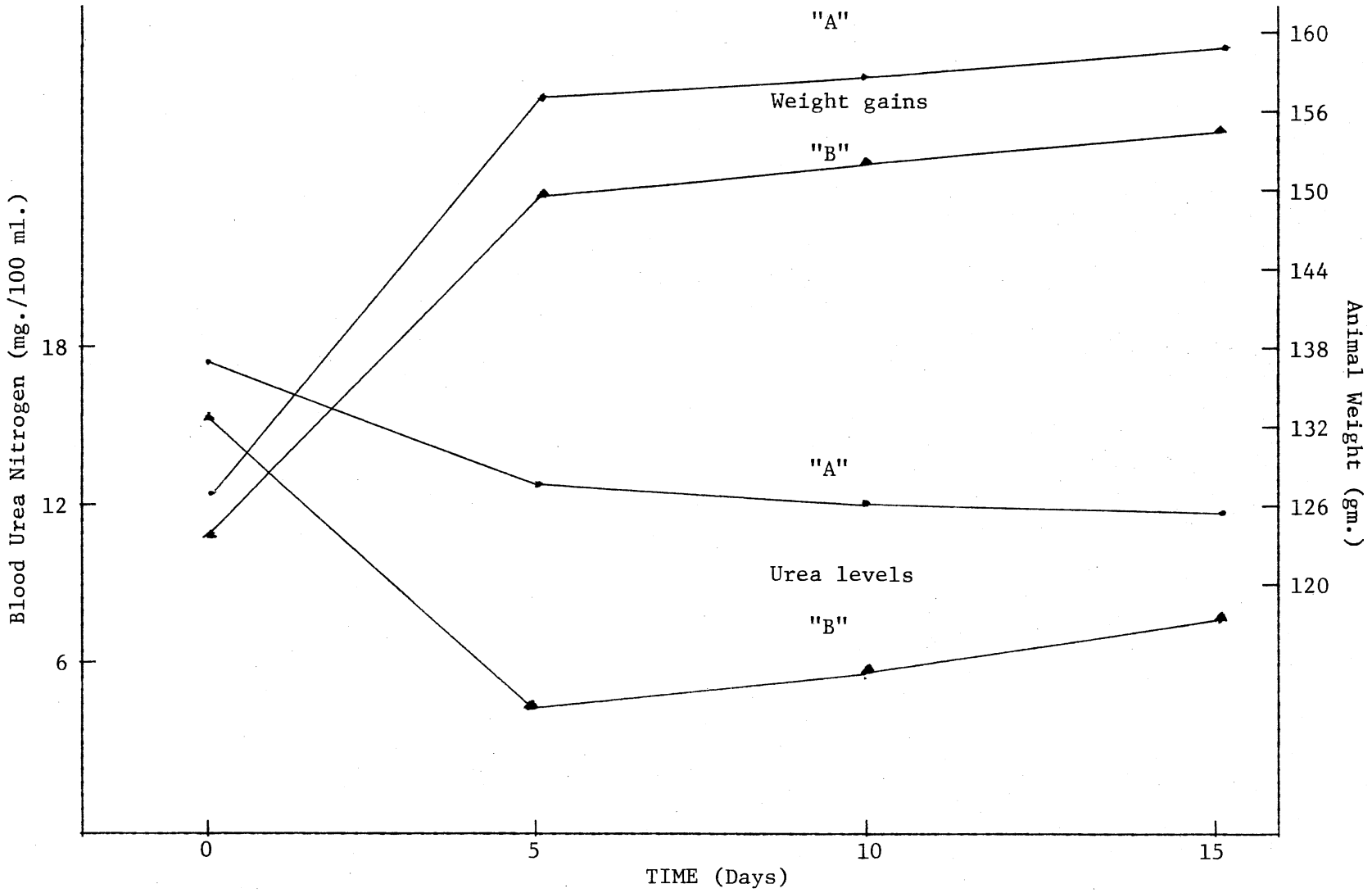

Figure 1. Influence of Diet on Weight Gain and Blood Urea

TABLE II

F SCORES REFLECTING DIFFERENCES IN BUN LEVELS BETWEEN TREATMENT METHODS

Treatment Methods	$\overline{X}$ mg%	F	Level of Significance
Diet A (10 EAA)	13.50	28.74	.001
Diet B (9 EAA + analogue)	8.84		

Results of this study confirmed previous work cited, showing that the alpha-keto analogue of phenylalanine can replace phenylalanine and tyrosine in the diet of rats. It was also indicated that urea recycling is reduced by the uptake of ammonia for amino acid synthesis.

The reason for leveling-off effect of BUN levels is unclear. However, several possibilities have been suggested in previous studies. Wood and Cooley (21) suggest that keto acids are unstable and partially unusable, so that they cannot be considered equivalent to the amino acids on a molecular basis. It may be necessary to supply an analogue at a level greater than equimolar amounts.

The data suggest that the analogues are not converted at 100% efficiency as evidenced by the beginning upswing of the BUN levels of the rats on Diet B containing the analogue. Hence it is possible that the level of analogue presented is insufficient to sustain amino acid synthesis for prolonged periods.

The whole mechanism of analogue therapy is dependent on high caloric intake. It is possible that even though these diets were high

enough in calories, food was not consumed in large enough amounts for enough calories to be present for sustained amino acid synthesis as the rat grew larger. Since weanling rats were used, it was expected that an accelerated growth phase would be encountered. However, the diets may not have provided enough calories for the additional stress of the growth phase.

As the project neared termination, space became a problem. The cages became too small for the rats. Literature suggests that overcrowding can decrease feed efficiency due to psychological factors. This may be one consideration for the plateau of weight gain for both diets. Since food intake was not measured, it cannot be stated definitely that this was the problem, but environmental conditions should definitely be considered in future studies.

The whole area of enzyme kinetics and turnover rate was unexplored in this study. Problems involving synthesis rate both in the liver and muscle tissues may account for the plateau of BUN levels. However, further study would be needed before any far-reaching implications could be stated.

CHAPTER V

SUMMARY, IMPLICATIONS, AND RECOMMENDATIONS

The effect of the alpha-keto acid analogue substitution for phenylalanine and tyrosine on uremia was investigated. The experimental units were sixteen surviving surgically-induced uremic rats. After surgery and onset of uremia, the animals were paired and randomly assigned diets within each block. Both diets consisted of a basal diet plus an amino acid mixture, with Treatment A containing phenylalanine and tyrosine and Treatment B containing the alpha-keto acid analogue of phenylalanine.

Weight gains and blood urea nitrogen levels were observed for a fifteen day period at five day intervals. Statistical analysis revealed no statistical significance on weight gain between treatments. However, the BUN levels for Treatment B were significantly lower ($P < .001$) than for Treatment A. The results of this experimental study indicate that alpha-keto acid analogue therapy is an effective therapy for uremia.

Implications

Implications from the data generated in this study are:

1. Alpha-keto acid analogues will be helpful in alleviating the uremic syndrome for short durations.
2. A synthetic, low protein diet containing one or more alpha-keto acid analogues could be used intermittantly with dialysis, thus reducing cost of therapy and discomfort for the patient.

3. Since alpha-keto analogues can be used for the *in vivo* synthesis of essential amino acids, it is within the realm of possibility that diet therapy for inborn metabolic errors involving essential amino acids (particularly phenylalanine) may include the use of alpha-keto analogues in the future.

Recommendations

Recommendations for further research are:

1. Extend the number of days of data collection, using a larger population in a carefully controlled environment. This researcher would suggest a population of 30 weanling rats. Amount of ration consumed daily should also be measured to estimate food intake efficiency.
2. Experiment with varying levels of caloric content of diet to establish optimum caloric intake.
3. Replications or expansions of this study should incorporate the experimental diet in pelletized form.
4. Pariorbital bleeding technique for the collection of blood samples should be used instead of the tail bleeding method used in this study.
5. Substitute two or more alpha-keto analogues at different levels to study nutrient interactions and to establish optimum levels of supplementation.
6. Using perfusion techniques, investigate the enzyme activity and turnover rates in the liver and various muscles for each amino acid analogue under investigation.

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APPENDIX A

PROCEDURE FOR SURGICALLY-INDUCED UREMIA

Procedure for Surgically-Induced Uremia[1]

The rats were anesthetised with nembutal (1 cc. of a 60 mg./ml. solution in 8.13 cc. sterile saline) and the upper and lower poles of the left kidney were removed, leaving the pelvis and hilum intact (Figure 2). Bleeding was controlled by the application of silicone gel foam. One week later, the whole right kidney was removed. Care was taken to preserve the suprarenal glands and their vascular connections. The approach on each occasion was laterally. Before closure, the surgical incision was sprayed with 50 x 10^3 units sodium penicillin on each operation to control urinary infections and possible infection of the surgical site.

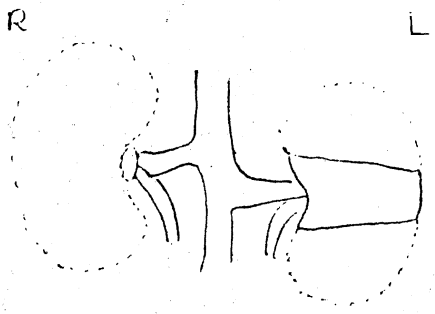

Figure 2. The Extent of the Nephrectomies. The parts of the kidneys which were removed are shown with a broken outline.

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[1]Adapted from Platt, R. Roscoe, M. H. and F. W. Smith. "Experimental Renal Failure." Clinical Science, Vol. 11. (1952), pp. 217-231.

APPENDIX B

PROCEDURE FOR DETERMINATION OF BLOOD UREA BY UREASE AND THE BERTHELOT REACTION (METHOD OF CHANEY AND MARBACH)

Procedure for Determination of Blood Urea

by Urease and the Berthelot Reaction

(Method of Chaney and Marbach)[1]

1. Set up the following in test tube (or cuvets, e.g. Klett tubes, if their capacity if sufficient):

   Blank - 0.2 ml. buffered urease solution.
   Standard - 0.2 ml. buffered urease solution + 20 µl. urea standard.
   Unknown - 0.2 ml. buffered urease solution + 20 µl. serum.

   (The 20 µl. aliquots of standard and unknown are added by TC micro pipets, Sahli hemoglobin pipets being satisfactory if their accuracy has been checked.)

2. Incubate tubes in a water bath at 37°C. for 15 minutes.

3. Add 1.0 ml. phenol color reagent to each tube, mix, then 1.0 ml. alkali-hypochlorite reagent and mix promptly again. It is mandatory that the phenol reagent be added and mixed first!

4. Incubate tubes at 50-60°C for 3 minutes, at 37°C for 20 minutes or at 25°C for 40 minutes.

5. Add water equally to all tubes to bring absorbance readings into desirable absorbance range (preferably 0.2-0.8). For Beckman DU with 1 cm. cuvets, add 8.0 ml. water, and for Klett photometer using No. 54 filter, add 3.0 ml. water. Do not use bare thumb if mixing must be accomplished by inversion; clean Saran Wrap (Dow Chemical) or Parafilm "M" (Marathon) can be used with the thumb.

6. Read absorbances of blank (A_b), standard (A_s) and unknown (A_x) against water at 630 MU or with a filter with nominal wavelength in this region. If A_x is greater than 0.8, dilute the blank and unknown further with water equally, read absorbances again, and make proper corrections in calculations.

7. Calculations:

$$\text{Mg. urea N/100 ml. serum or plasma} = \frac{A_x - A_b}{A_s - A_b} \times 0.004 \times \frac{100}{0.02} = \frac{A_x - A_b}{A_s - A_b} \times 20$$

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[1] Henry, R. J. Clinical Chemistry: Principles and Technics. Harper & Row Publishers, New York. (1965), pp. 266-268.

APPENDIX C

COMPOSITION OF BASAL DIET

Composition of Basal Diet

	gm./kg. diet
Dextrin	675.0
Vitamin mixture[a]	7.5
Choline mixture[b]	7.5
Mineral mix 4164	40.0
Cellulose	20.0
NaCl	10.0
Corn Oil	40.0
Essential amino acid mixture[c]	100.0
Non-essential amino acid mixture[c]	100.0
TOTAL	1000.0

[a]To provide: in mg/kg; thiamin HCl, 15; riboflavin, 15; Ca pantothenate, 75; pyrodixine HCl, 75; nicotinic acid, 30; folic acid, 3; PABA, 75; menadione, 0.15; biotin, 0.15; vitamin B-12 in mannitol (0.1%), 0.3; inositol, 492; Vitamin A-palmitate (250,000), 4,100 IU; ergocalciferol, 12,69 IU.

[b]Contained choline chloride in sucrose (13.2%), 400 IU/kg. diet.

[c]As listed in appendix table D.

APPENDIX D

COMPOSITION OF BASAL AMINO ACID MIXTURES

Composition of Basal Amino Acid Mixtures

Essential Amino Acids	gm./kg. diet
L-Arginine HCl	4.00
L-Histidine HCl·H_2O	3.27
L-Isoleucine	4.20
L-Leucine	5.85
L-Lysine HCl	7.50
L-Methionine	2.25
L-Threonine	3.87
L-Tryptophan	1.30
L-Valine	5.20
L-Phenylalanine[1]	3.75
Dextrin (from total)	62.64
TOTAL	100.00

Non-essential Amino Acids	gm./kg. diet
DL-Alanine	2.30
L-Asparagine·H_2O	3.95
L-Aspartic acid	2.30
L-Cystine	3.50
L-Glutamic acid	27.30
L-Glycine	13.95
L-Proline	2.30
DL-Serine	2.30
L-Tyrosine[2]	4.00
Dextrin (from total)	38.00
TOTAL	100.00

[1]When phenylalanine is replaced by its analogue (phenylpyruvic acid) on an equimolar basis, dextrin will be added as filler.

[2]When tyrosine is omitted when the phenylalanine is replaced by its analogue (phenylpyruvic acid) on an equimolar basis, dextrin will be added as filler.

APPENDIX E

PROCEDURE FOR COLLECTING BLOOD SAMPLES

Procedure for Collecting Blood Samples[1]

1. Wrap the rat firmly in a small towel with only its head and tail protruding. This procedure serves the double purpose of keeping the rat warm (essential for successful bleeding) and making it easier for the assistant to restrain the rat while the blood is obtained by the operator.

2. Immerse the rat's tail for 1-2 minutes in warm water in a conical flask or other suitable receptable large enough to permit immersion of nearly the whole length of tail. The temperature of the water should be about 45°C. and some antiseptic should be added.

3. Remove the tail from the water and wipe quickly with cotton.

4. Swab the tail in quick succession with alcohol and xylol, thus removing all water from the surface of the tail and dilating the tail vessels.

5. Wipe the tail free from xylol.

6. Place the tail on a pad of clean filter paper on the bench, steady the tip of the tail by holding it between thumb and forefinger of the left hand so that only about a quarter-of-an-inch protrudes; then with a sharp sterilized scalpel (or safety razor blade) deftly remove a tiny slice from the tail tip. It is essential for the success of future bleedings to remove the smallest possible piece of tail.

7. The blood samples can now be sucked into the pipettes or the blood allowed to drip into a heparinized or oxalated tube coated with paraffin wax. It may be necessary to run the forefinger and thumb gently down the tail to expel the blood but this action should never become a vigorous squeezing. If the blood should refuse to flow, repeat the preparatory steps through hot water, alcohol, xylol, etc.

8. When the desired amount of blood has been obtained the tail is thoroughly swabbed with a 0.1 percent solution of acriflavine to prevent infection before returning the rat to its cage. If the tail bleeds excessively, a solution of collodion may be used to cover the excision line.

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APPENDIX F

INDIVIDUAL RECORD

INDIVIDUAL RECORD

Rat No._____ Initial Wt._____ Initial BUN_____

Block_____ L. Kidney removed:_____

Treatment_____ R. Kidney removed:_____

	Day 0	Day 5	Day 10	Day 15
	Date_____	Date_____	Date_____	Date_____
Wt.	_____	_____	_____	_____
BUN	_____	_____	_____	_____

SPECIAL OBSERVATIONS OR NOTES:

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