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UTILIZATION OF AMINO ACIDS FROM PROTEIN

MANUAL OF PROCEDURES

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UTILIZATION OF AMINO ACIDS FROM PROTEIN MANUAL OF PROCEDURES

Herman De Haas and Ellen H. Morse, compilers

INTRODUCTION

The importance of protein in the diet is well established. Proteins differ with respect to their constituent amino acids, both qualitatively and quantitatively. The nutritive value of protein is dependent not only upon these features but also on the availability of the amino acids. To evaluate the quality of a given protein, comparisons have been made of its amino acid content, determined by chemical means, with the amino acid content of a "standard" protein such as egg albumen, which is recognized as a high quality protein. The Food and Agriculture Organization suggested a provisional amino acid pattern for a high quality protein based on amino acid requirements for human subjects. This pattern consisted of those amino acids essential for man and also included cystine and tyrosine. In this pattern, 75% of the nitrogen was undefined (1).

The nutritive value of protein was formerly assumed to be related only to the essential amino acid content. However, the content of these amino acids alone has not provided an adequate explanation for the marked differences in nutritive values of proteins. Also, the essential amino acids predicted to be most limiting from a chemical determination of the amino acid composition of the protein have not always proved to be physiologically limiting. Furthermore, recent work indicates that nitrogen from nonessential amino acids may have a significant effect on protein quality.

To meet the protein needs of the newly developing countries and the increasing world population, plant proteins and protein mixtures will be required in larger and larger amounts. The proteins studied in this investigation included an animal and a vegetable protein which are important agricultural products of the United States. While general information about the nutritive quality of these proteins is available, this regional study was designed to contribute to an understanding of the reasons for the quality differences and to provide a basis for formulating protein mixtures of high quality using various protein sources.

The project was directed toward providing information to advance the understanding of several unresolved questions concerning nitrogen requirements and utilization:

1. Does the quality and quantity of the nitrogen from different sources influence the requirement of essential amino acid nitrogen?

2. Is the nonessential amino acid pattern of a protein one of the factors which contribute to its nutritive value?
3. How do certain factors that determine the nutritive value of proteins exert their influence?
4. What are reliable methods for the assessment of the nutritive value of proteins in human nutrition?
5. What amino acid pattern would constitute an ideal reference pattern?

These questions are not new, but so far the answers are incomplete or contradictory.

Initially, it was decided to determine if selected food proteins would have comparable nutritive values when their essential amino acid patterns were adjusted (or equalized) with crystalline amino acids to a defined pattern and secondly to investigate any observed differences in terms of the proportions and availability of the individual amino acids.

Methods were needed for assessing the nutritive value of various proteins for humans. Current methods are limited primarily to the determination of nitrogen balance. Only limited interpretation can be made from such findings as they measure over-all nitrogen metabolism, giving no information about the various mechanisms involved in this elaborate biochemical process. There are inherent difficulties involved in conducting studies with human subjects being held to deficient diets under rigorously controlled conditions for a length of time sufficient to yield results that reflect the deficiency. Quicker and more precise methods of evaluation, as well as a more completely defined standard for comparisons, were needed which would not be too costly in time or money for routine evaluation of protein quality.

With these objectives in mind, the Northeast Regional Technical Committee proposed to conduct rigorous experiments with different species of animals and compare these findings with human studies conducted with similar, but less rigorous conditions. The technical committee then defined certain experimental conditions within which all stations were to operate. The composition of the nitrogen portion of the diet was very carefully defined as well as the levels of nitrogen to be fed. Other components of the diet were also specified as to amounts and source for procurement. The length of the experimental periods were subject to delineation as well as the mode of administration of the diets. The way in which these criteria were established is set forth in this bulletin.

DIETARY COMPOSITION

Preliminary Considerations. Originally three different isolated, intact proteins were to be employed, but because of the unavailability of one

of these, kidney bean, only two, wheat gluten and a casein-lactalbumin mixture, were employed. Wheat gluten was chosen because of (a) previous evidence of poor utilization, (b) wheat and other cereals are important sources of protein and (c) it was available commercially. Milk protein, which is a protein of high nutritive value, was used for comparison. It was prepared by mixing purified casein and lactalbumin in a 5:1 ratio by weight.

Sufficient quantities of the proteins were obtained so that all stations used proteins from the same commercial source, prepared by the same process and from the same lot. The proteins were as near the natural state as possible with a minimum of denaturation and heat treatment during preparation.

Initially the level of each protein to be fed per gram of nitrogen in the diet was determined by the essential amino acid present in the greatest percent excess with respect to the defined standard pattern (see below). Crystalline amino acids were added to bring each of the other essential amino acids to the quantity specified in the standard pattern. A defined mixture of nonessential amino acids was added to supply the remaining nitrogen. Thus the resulting pattern of essential amino acids and also the ratio of total essential to total nonessential amino acid nitrogen were constant but the proportions of each supplied by the test protein and the pattern of the nonessential amino acids differed. Since many factors affect the availability of amino acids from intact protein, crystalline amino acids in the standard pattern were used as a reference rather than an intact protein.

NE-52 Amino Acid Pattern. The amino acid pattern selected as the reference standard employed the essential amino acids in the proportions of the FAO pattern (1) and the nonessential amino acids in the proportions in which they occur in milk protein (2) with the following modifications:

1. The essential amino acids were used in the FAO proportions based on 90 mg of tryptophan per gram of total nitrogen but with values of 126 mg of L-cystine and 144 mg DL-methionine per gram of total nitrogen.
2. Arginine and histidine were provided at a minimum of 230 mg and 170 mg per gram of dietary nitrogen, respectively. Adjustment of these two amino acids to this minimum proved unnecessary for the two proteins selected.
3. The remaining nitrogen was supplied by a mixture of nonessential amino acids based on the proportions of these amino acids as found in milk protein. The final values were adapted from those found in Orr and Watt (2).

Using the above criteria, two amino acid mixtures were formulated as indicated in tables 1 and 2.

TABLE 1
NE-52 Amino Acid Mixture A[1]—Essential Amino Acids Based on the FAO Pattern.

Amino acid	Mg amino acid per gram nitrogen[2]
L-Cystine	126
L-Isoleucine	270
L-Leucine	306
L-Lysine	270[3]
DL-Methionine	144
L-Phenylalanine	180
L-Threonine	180
L-Tryptophan	90
L-Tyrosine	180
L-Valine	270

[1] 11.351% N calculated from molecular weights including hydrochloride.
[2] Mg of each amino acid per gram of nitrogen in the amino acid diet. All diets contained this pattern of essential amino acids.
[3] Added as an equivalent amount of the monohydrochloride.

TABLE 2
NE-52 Amino Acid Mixture B[1]—Nonessential Amino Acids Based on Milk Protein.

Amino acid	Mg amino acid per gram nitrogen[2]
L-Alanine	329
L-Arginine	349[3]
L-Aspartic Acid	697
L-Glutamic Acid	2,242
Glycine	189
L-Histidine	269[4]
L-Proline	1,066
L-Serine	563

[1] 13.006% N calculated from molecular weights including hydrochlorides.
[2] Mg of each amino acid per gram of nitrogen in the amino acid diet only. A mixture of these amino acids in this ratio was used to provide nonessential nitrogen in the protein-containing diets.
[3] Added as an equivalent amount of the monohydrochloride.
[4] Added as an equivalent amount of the monohydrochloride n

Nitrogen Sources. All stations obtained their protein and amino acid dietary components from the same commercial sources. The proteins, amino acids and amino acid mixtures were drawn from the same lots by all stations. The 5:1 casein-lactalbumin, the amino acid mixtures A and B, and the individual amino acids were procured from General Biochemicals, Chagrin Falls, Ohio. The wheat gluten, General Mills Pro-80 Vital Wheat Gluten prepared by a variation of the Martin process and dried in high vacuum at mild temperatures, was procured in one 200-lb. lot. This was separated into smaller lots of 3-5 lbs. each and stored under nitrogen in polyethylene bags placed in metal containers. All proteins were stored in freezers until used.

Dietary Calculations.

1. The amino acid content of the proteins (mg of amino acid per gm of nitrogen) was determined. See table 3.
2. A sample calculation of the protein and amino acid contents of a diet is given on the next page.

TABLE 3
Amino Acid Analyses of Proteins

Amino acid	Ion Exchange Chromatography[1] 5:1 Casein-lactalbumin[2]	Wheat gluten[2]
	Mg amino acid per gm nitrogen	
Alanine	239	178
Arginine	231	251
Aspartic Acid	525	215
Cystine[4]	47	161
Glutamic Acid	1,448	2,598
Glycine	121	250
Histidine	174	161
Isoleucine	375	242
Leucine	614	484
Lysine	515	118
Methionine	170	106
Phenylalanine	343	402
Serine	375	330
Threonine	295	186
Tyrosine	334	229
Valine	447	258
Proline	658	1,148
NH_4+	104	186
	Microbiological Assay[3]	
Cystine[4]	57.6	141.1
Tryptophan	103.8	66.9

[1] H. H. Williams, New York (acid hydrolysis).
[2] Casein-lactalbumin, 13.87% N; wheat gluten, 13.14% N as determined by the New York Agricultural Experiment Station, Cornell.
[3] Pela Braucher, Maryland (acid and alkaline hydrolysis).
[4] The microbiological assay values for cystine were used in the calculations.

a. Distribution of dietary nitrogen.

1) Nitrogen from protein (casein-lactalbumin—CL).

As leucine was in the largest percent excess above the amount needed to supply the quantity called for in the NE-52 essential amino acid pattern (table 4), this amino acid was used to determine the total amount of nitrogen supplied by the protein (CL).

(Eq. 1) N from protein (CL) =

$$\frac{\text{306 mg leucine/gm N NE-52 pattern}}{\text{614 mg leucine/gm N CL}} \times 1000 \text{ mg N} =$$

498.371 mg N/gm dietary N to be supplied by casein-lactalbumin.

This ratio also provides the figure of 49.84% used to determine the amounts of essential amino acids provided by the protein (CL) per gm of dietary nitrogen, column D of table 4.

2) Nitrogen from added crystalline essential amino acids.

In order to bring the level of the essential amino acids other than leucine to that of the NE-52 pattern, additional amounts of crystalline amino acids—see column E, table 4—were added. The nitrogen of these amino acids was calculated from their molecular weights and is given in column F, table 4. The total nitrogen supplied by these crystalline essential amino acids was 44.929 mg nitrogen per gm of dietary nitrogen.

3) Nonessential amino acid nitrogen.

Nonessential amino acid nitrogen was supplied by the protein and by added amounts of NE-52 mix B (table 2) to provide 75% of the dietary nitrogen. Equation 2 was used to calculate the nitrogen to be provided by the addition of mix B.

(Eq. 2). Remainder of N = 1000 mg N – (mg of N provided by protein + mg N from added EAA)

1000 mg N – (498.371 mg N from CL + 44.929 mg N from EAA) =
456.700 mg N/gm of dietary N to be supplied by NE-52 mix B of nonessential amino acids (table 2)

4) Protein to provide 100% of leucine.

The protein (CL) provided 100% of the leucine of

TABLE 4
Sample Calculations of the Essential Amino Acid Components of Diets.

	NE-52 EAA[1] Pattern Mg AA[1]/gm	Mg AA per gm N of CL[1]	Source and Amt. of EAA to Make NE-52 EAA Pattern		Mg Nitrogen from Added AA
			Amt. from Protein Mg AA/gm N	Amt. from Cryst. AA Mg AA/gm N	
Amino Acids A	B	C	D (49.84% of C)	E (B-D)	F
L–Cystine	126	57.6	28.71	97.29	11.343
L–Isoleucine	270	375.0	186.89	83.11	8.875
L–Leucine	306	614.0[2]	306.00	0	0
L–Lysine	270	515.0	256.67	13.33[4]	2.555
DL–Methionine	144	170.0	84.72	59.28	5.565
L–Phenylalanine	180	343.0	170.94	9.06	0.768
L–Threonine	180	295.0	147.02	32.98	3.878
L–Tryptophan	90	103.8	51.73	38.27	5.250
L–Tyrosine	180	334.0	166.46	13.54	1.047
L–Valine	270	447.0	222.77	47.23	5.648
					44.929

[1] Abbreviations: EAA—essential amino acids; AA—amino acids; CL—casein-lactalbumin.

[2] Leucine was in largest percent excess above the NE-52 pattern value, therefore, this amino acid was the only one supplied completely by the protein.

[3] Calculated from molecular weights of individual amino acids as found in *Handbook of Chemistry and Physics*, 44th ed., The Chemical Rubber Co. (1963-1964).

[4] Added as equivalent amount of lysine monohydrochloride.

the NE-52 EAA pattern and this also determined the amount of nitrogen supplied by the protein.

(Eq. 3) $\frac{306}{614}$ = fraction of N supplied by casein-lactalbumin

$$\frac{306}{614} \times \frac{1}{0.1387 \text{ gm N/gm CL}} = \frac{3.5932 \text{ gm CL/gm}}{\text{dietary N}}$$

5) Amount of mix B.

(Eq. 4) $\frac{456.700 \text{ mg N from mix B/gm dietary N}}{0.13006 \text{ gm N/gm mix B} \times 1000 \text{ mg/gm}} =$
3.5115 gm NE-52 mix B/gm dietary N.

None of the values given are accurate to more than three significant figures, but to avoid accumulative errors,

additional figures were carried through the calculations and the final values rounded off to three significant figures.

Although the nitrogen content of the proteins varied with the moisture content, the figure of 13.87% nitrogen, which was the value found by one station for the casein-lactalbumin, is used in this manual. Each station determined its own value for use in its calculations.

Similar calculations were done for wheat gluten using the value 13.14% nitrogen and the three rat test diets of table 6 were then formulated. Again, percent nitrogen in wheat gluten was determined by each station prior to calculations similar to equation 4.

Diet Compositions. The diets, including the pre-experimental diet, for the rat experiments were rigidly defined to ensure uniformity at all stations. The diets for the other species studied (pigs, *Tetrahymena*, and man) were patterned after the rat diets, but certain modifications in the non-nitrogenous constituents were made to meet the requirements of those species. The human diets included a small amount of nitrogen supplied by low-nitrogen foods that were included for palatability. All stations working with a given species used the same procedure to formulate their diets.

Methods of Feeding. In the rat growth experiments controlled amounts of diets containing either 1.2% or 1.6% nitrogen were fed. Pigs and human subjects were fed restricted amounts of nitrogen based on body weights. Energy sources were provided *ad libitum* for the pigs and were adjusted to maintain body weight in the human studies. In the stress studies rats were depleted by feeding a protein-free diet, then repleted by feeding diets containing controlled amounts of nitrogen.

In test meal studies with rats, a slurry of the rat growth diet containing a definite level of nitrogen in relation to body weight was administered or the rats were trained to consume the test meal in a two-hour period. In human test meal studies, the test meal provided 1/5 of the day's total nitrogen and energy intake. Two levels of nitrogen were tested with each source of protein. In *Tetrahymena*, several concentrations of the nitrogen-containing components were incorporated into the growth media.

The following experimental procedures were established by the Technical Committee of NE-52 based on the preceding guidelines.

EXPERIMENTAL PROCEDURES FOR RAT STUDIES

Growth Studies.

1. Male weanling rats of the CFN strain were purchased from Carworth Farms at a weight of 50±5 grams.
2. Eight or more rats per diet were recommended.
3. Plan of experiments:
   a. Experimental design. (See table 5.)

TABLE 5

Distribution of Animals for Nitrogen Level 1
(Repeat for N Level 2)

Groups using wt. of rats	Carcass analysis[1]	Diets		
		Amino acid (AA)	Casein-lactal-bumin (CLAA)	Wheat gluten (WGAA)
1	2 rats	2 rats	2 rats	2 rats
2	2 rats	2 rats	2 rats	2 rats
3	2 rats	2 rats	2 rats	2 rats
4	2 rats	2 rats	2 rats	2 rats

[1] Animals sacrificed at beginning of experimental period for determination of initial composition.

1) Rats were divided into four evenly spaced weight groups between 57 and 67 grams (62±5) and assigned randomly, two rats from each group to each treatment, in order to nullify differences that might be attributed to different starting weights.

2) If it was not possible to do all animals at once, one animal was used at each nitrogen level for each diet and the test repeated until eight animals had been fed each diet at each level of nitrogen.

b. During the three or more days preceding the experimental period, a pre-experimental diet designed to accustom the animals to the experimental diets was fed. See 4a page 14, for preparation of this pre-experimental diet.

c. The experimental period was started when the rats weighed 62±5 grams.

d. On the day that the experimental period was started, one group was sacrificed for carcass analysis (total nitrogen).

e. The experimental period was of two weeks duration.

f. After the experimental period, carcass analysis was done as at the start of this period.

g. The rats were on a controlled dietary intake.

1) Studies were conducted by feeding at a level governed by the group that consumed the lowest amount of food on the preceding day. All animals were given 10 grams of food the first day. If the lowest consuming group ate from 8.0 to 8.4 grams, the amount fed the second day was 9.0 grams. If the amount consumed by this group was between 8.5 and 9.0 grams, the amount fed the second day was 10 grams. The amount fed on a given day was from 0.6 to 1.5 grams over the amount consumed on the preceding day by the group that ate the least. Whole gram weights of diets were employed.

2) In other studies, the rats were pair fed to those on the amino acid diet. This differed from the preceding experiment, section 1), in that the diets were fed at a slightly lower level to ensure complete ingestion of all diets.

3) Experiments in which the nitrogenous components of the diet were fed once a day and all other dietary components combined and made available *ad libitum* were also carried out. The quantity of nitrogenous components fed was determined on the basis of the daily consumption of the complete diets as found in the previous experiment, section 2).

4. Diets.

a. Pre-experimental diet for rat growth studies. This diet contained 1.2% nitrogen consisting of 3/4 of the nitrogen as casein (not casein-lactalbumin) and 1/4 of the nitrogen as amino acids from a 1:3 mixture of amino acid mixtures A and B (25% of the nitrogen from mixture A and 75% of the nitrogen as mixture B). This was supplemented with DL-methionine (0.3% of the diet) resulting in a total nitrogen content of 1.228%. Other constituents were as described for the experimental diets in the following section. Each 100 grams of pre-experimental diet for rats contained 0.9 gm of nitrogen supplied by casein, 0.6607 gm amino acid mixture A, 1.730 gm amino acid mixture B, and 0.3 gm DL-methionine. The remaining components, corn oil, cellulose, vitamins, minerals and carbohydrates, were as given below for the experimental diets.

b. Experimental Diets.

1) Protein and amino acid components. The protein and amino acid components of the three experimental diets are given in table 6. The dietary components in table 6 were fed at 1.2% and 1.6% nitrogen levels. For each 100 grams of diet, the quantities in table 6 were multiplied by the percent nitrogen in the diet.

2) Fat—5% corn oil. Mazola, Corn Products Co., New York, New York.

3) Bulk—2% cellulose. Alphacel Non-nutritive Bulk, Nutritional Biochemicals Corp., Cleveland, Ohio. A measured amount of agar may be added or substituted.

4) Minerals — 4% Jones-Foster salt mix (3). Nutritional Biochemicals Corp., Cleveland, Ohio. Composition, grams: NaCl, 292.5; KH_2PO_4, 816.6; $MgSO_4$, 120.3; $CaCO_3$, 800.8; $FeSO_4.7H_2O$, 56.6; KI, 1.66; $MnSO_4.2H_2O$, 9.35; $ZnCl_2$, 0.5452; $CuSO_4.5H_2O$, 0.9988; $CoCl_2.6H_2O$, 0.0476.

5) Vitamins — 2.2% vitamin mix. Vitamin Diet Fortification Mixture in Dextrose, Nutritional Biochemicals Corp.,

TABLE 6
Protein and Amino Acid Components of The Experimental Rat Diets.

Component	Diets		
	AAD[1]	CLAA	WGAA
	Mg per gram of dietary nitrogen		
Casein-lactalbumin	—	3,593.00[2]	—
Wheat gluten	—	—	3,408.00[3]
Amino acid mix A[4]	2,083	—	—
Amino acid mix B[5]	5,871	3,511.00	3,255.00
L-Tryptophan	—	38.27	60.04
L-Threonine	—	32.98	96.72
L-Isoleucine	—	83.11	161.64
L-Leucine	—	—	89.28
L-Lysine monohydrochloride	—	16.65	271.32
DL-Methionine	—	59.28	96.54
L-Cystine	—	97.29	62.82
L-Phenylalanine	—	9.06	—
L-Tyrosine	—	13.54	77.46
L-Valine	—	47.23	154.48
	7,954	7,501	7,733

[1] Abbreviations: AAD—amino acid diet
CLAA—casein-lactalbumin supplemented with amino acids diet
WGAA—wheat gluten supplemented with amino acids diet

[2] Based on 13.87% nitrogen.
[3] Based on 13.14% nitrogen.
[4] For composition of amino acid mix A, see table 1.
[5] For composition of amino acid mix B, see table 2.

Cleveland, Ohio. Composition per kilo: vitamin A, 900,000 IU; vitamin D, 100,000 IU; alpha tocopherol, 5.0 gm; ascorbic acid, 45.0 gm; inositol, 5.0 gm; choline chloride, 75.0 gm; menadione, 2.25 gm; p-aminobenzoic acid, 5.0 gm; niacin, 4.5 gm; riboflavin, 1.0 gm; pyridoxine hydrochloride, 1.0 gm; thiamine hydrochloride, 1.0 gm; calcium pantothenate, 3.0 gm; biotin, 20 mgm; folic acid, 90 mgm; vitamin B_{12}, 1.35 mgm; dextrose to make one kilogram.

6) Carbohydrate—The remainder of the diet was made up with 50% sucrose and 50% cornstarch. Argo Cornstarch, Corn Products Co., New York, New York.

7) Conditions of storage:

a) Diets were refrigerated.

b) No antioxidant was added.

Note: Calculations were based on the theoretical nitrogen values of amino acid mixtures A and B and of individual amino acids and on determined nitrogen values of the proteins. Notation was made of materials used.

5. Parameters:

a. Growth as measured by weight gains. Rats were weighed at least at 0, 1 and 2 weeks. Weighing on days 0, 2, 4, 7, 9, 11 and 14 was recommended. Weighing was done in the morning.

b. Feed—consumption records were kept individually for animals throughout the experiment.

c. Carcass analysis. Animals were sacrificed in the morning. The rats were sacrificed using ether and the gastrointestinal tract removed, cleaned, rinsed with distilled water and replaced in the abdominal cavity. The weight of the rat for analytical purposes was taken at this time. The rats were placed individually in plastic bags or wrapped in aluminum foil and stored frozen until carcass analysis was done.

1) Method employed at Maine.

For analysis, the rats were removed from the freezer, allowed to thaw in the plastic bag, cut into pieces with a large pair of scissors and dried to constant weight in a vacuum oven at 80-90°C. The dried rat was ground in an intermediate size Wiley mill and the ground carcass was extracted with ether in a Soxhlet extractor. The extracted, ground rat tissue was converted to a fine powder with a mortar and pestle. Triplicate analyses for nitrogen were done by a microkjeldahl method (4).

2) Method employed at Delaware.

At the time of analysis, the frozen carcass was placed in a glass jar with a loose-fitting lid and autoclaved at 15 lbs. pressure and 120°C for two hours. After removal from autoclave, the jar was allowed to cool to room temperature. The contents of the jar were transferred to a weighed Waring Blendor jar. The blender jar and its contents were weighed and an amount of distilled water equal to the weight of the rat added. The jar and its contents were weighed again and the contents blended until homogeneous (about 10 minutes in a regular kitchen-type Waring Blendor after the blades move freely; about three minutes in a heavy-duty Waring Disintegrator.)

A portion of the homogenate was immediately transferred to a beaker and kept in constant motion on a magnetic stirrer for sampling. For Kjeldahl analyses, 10 gm samples were weighed out and transferred quantitatively to 100 ml volumetric flasks. Concentrated H_2SO_4 (15 ml) was added and the flask was allowed to stand for several hours. After agitating the contents of the flask, an additional 25 ml of concentrated H_2SO_4 was added and the flask allowed to stand for at least 24 hours before diluting to volume with distilled water and mixing thoroughly. Using a 5 ml volumetric pipette, triplicate aliquots of this acidified and diluted homogenate were delivered into Kjeldahl flasks for nitrogen determination (4). This procedure is a modification of procedures for carcass analysis outlined by Mickelsen and Anderson (5).

Stress: Depletion-Repletion Studies.

1. Male rats of the CFN strain were purchased from Carworth Farms at a weight range of 200-250 gm. A starting weight of approximately 220 gm was found most satisfactory (CFE strain was used when CFN strain was unavailable.)

2. Plan of experiments:

a. Experimental design. Rats were divided randomly into groups according to the design found in table 5.

b. Pre-experimental period. A pre-experimental diet, 3a page 18, was fed to the rats for at least one week prior to the beginning of the depletion period. This pre-experimental period was extended, when necessary, until the rats reached 250 ± 5 gm.

c. Depletion. The rats were depleted of protein by feeding

them a protein-free diet of the same composition as the other rat diets (table 6) except with carbohydrate replacing the protein and/or amino acids. This diet was fed *ad libitum* for 14 days with an average weight loss of 21%. (Extending the depletion period for a third week increased the loss to only 22% and the original recommendation of a 25 to 30% weight loss was abandoned.)

d. Repletion.

1) The experimental diets were fed *ad libitum* for a controlled time period. A time period of 14 days was used for the higher nitrogen diets and 21 days for the lower nitrogen diets. All animals were in repletion stage when sacrificed.

2) Controlled intake studies. (See also 3g, page 14).

a) A study was conducted by feeding at a level governed by the group that consumed the lowest amount of food on the preceding day. (See 3g 1), page 14.)

b) The controlled feeding of the protein components of the diet separate from the non-protein portion of the diet was also done. The protein components of the diet were fed to the rats at 8 a.m. and 5 p.m. with one half the daily nitrogen ration being supplied at each feeding. It was not necessary to increase the palatability of this portion of the diet for these adult rats. The non-protein portion of the diet was fed *ad libitum*.

3. Diets:

a. Pre-experimental diet for rat stress studies. This diet contained 1.2% nitrogen supplied by casein and was supplemented with 0.3% methionine. The remaining components of the diet were the same as those in the other rat diets. (See 4b, page 14.) Note: This diet did not contain free amino acids for pre-conditioning of the rats to the experimental diets because the depletion diet did not contain free amino acids (or protein). Also, casein not casein-lactalbumin was the source of nitrogen for economy reasons. This diet differed from the pre-experimental diet 4a, page 14, fed for growth experiments of rats and pigs.

b. Depletion diet. A protein-free diet of the same composition as the other diets with carbohydrate (50% sucrose, 50% cornstarch) replacing the protein was used. (See 4b, page 14.)

c. Repletion diets.

1) For the controlled intake study a) page 18, the complete rat diets, 4b, page 14, were fed with the levels of nitrogen the same as in the diets for the growth experiments (1.2 and 1.6% nitrogen).

2) In the controlled intake study b), page 18, protein components were fed at levels of 0.15 and 0.225 gm of nitrogen per rat per day with the remainder of the diet fed *ad libitum*. The ratios of protein and amino acid components were the same as those for the growth experiments b1), page 14, and the protein-free portion of the diet was the same as the depletion diet described above.

4. Parameters:

a. Weight gain. Rats were weighed 2, 2, and 3-day intervals, i.e., Monday, Wednesday, Friday. Weighing was done in the morning.

b. Food consumption records were kept individually for animals throughout the experiment.

c. Total plasma protein concentration changes were determined in study b).

1) Blood was collected two hours after feeding the morning protein meal by heart puncture after anesthetizing the rat and opening the chest.

2) The plasma was analyzed by either the autoanalytical method of Weichselbaum (6) or the colorimetric method of Lowry et al. (7).

d. Carcass nitrogen analysis was done essentially by the method employed at Delaware, c2), page 17. To ensure better sampling, several large aliquots of the homogenate were taken and combined. This combined sample was pre-digested with HCl, and samples were taken from this digested sample for determination of nitrogen by the Kjeldahl procedure (4). The carcass analysis procedure was based on that of Michelsen and Anderson (5).

Test Meal Studies.

1. Male rats of the CFN strain were purchased from Carworth Farms at a weight of 250-300 gm.

2. Plan of experiments:

a. Experimental design. Each test meal required five or six animals—one to be sacrificed at time of administration of test meal and the others serially thereafter. See section 4 below for number of animals and time intervals for sacrifice used with the two methods of administering the test meals. The three treatments, amino acid (AAD), wheat gluten supplemented with amino acids (WGAA) and casein-lactalbumin supplemented with amino acids (CLAA) diets with five or six animals per treatment required 15 or 18 animals in one replication. Initially each replication was re-

peated three times for a total of 45 or 54 rats so that there were three rats per time interval for each treatment. Treatments were run serially and then repeated in order that differences in time of day for sacrifice would be similar for each treatment. This procedure was repeated for each level of nitrogen studied.

3. Diets:

a. The pre-experimental diet was the same as that used for growth studies, 4a page 14, and was fed for one week prior to the test meal studies (West Virginia) or throughout a training period for requiring the rats to consume their daily ration in a period of two hours (Pennsylvania).

b. The test meal composition was the same as the 1.6% nitrogen diet used for rat growth studies, 4b, page 14.

4. Administration of test meals and sampling:

a. Pennsylvania.

The rats were trained to consume their daily rations in a period of two hours. After a 22-hour fast, the animals were given their test meal containing 0.065 gm nitrogen per kilogram of body weight. Animals were sacrificed at intervals of 1/2, 1, 2 and 3 hours after the two-hour eating period for sample collection.

b. West Virginia.

The diets were slurried with a 0.5% agar solution so that the resultant test-meal mixture contained 0.8 gm of nitrogen per 100 ml. After a minimum of 15 hours of fasting, test meals were administered by stomach tube in amounts corresponding to 2 ml (16 mg N) per 100 gm of body weight. Animals were anesthetized with ether and sacrificed for sampling at 10, 20, 30, 60 and 120-minute intervals after administration of the test meals.

5. Parameters:

a. Free amino acids were analyzed in one or more of the following:

1) Portal blood plasma
2) Intestinal contents
3) Systemic blood plasma

The rats were anesthetized with ether and opened ventrally. Blood was collected from the portal vein or heart in syringes rinsed in a physiological saline solution containing 1,000 USP units of sodium heparin per ml. Plasma was removed after centrifuging at 2,000 RPM for 15-20 minutes. When it was necessary to pool samples, an equal amount of plasma from each rat was used.

Plasma proteins were removed by precipitation with sulfosalicylic acid (two volumes of plasma to three volumes of 10% sulfosalicylic acid) and centrifuged as above. The supernatant solutions were stored frozen until analyzed.

Before being analyzed by automated ion exchange chromatography the thawed samples were adjusted to pH 1.5-2.5 with 1.0 N NaOH.

EXPERIMENTAL PROCEDURES FOR PIG STUDIES

These studies have been published (8).

Growth Studies.

1. Five-week old Yorkshire pigs were procured from the New Jersey Agricultural Experiment Station.
2. Plan of Experiments:
   a. Statistical design.

TABLE 7
Distribution of Pigs

	Diets[1]		
Litter Nos.	WAA	CLAA	ADD
1	2 pigs	2 pigs	2 pigs
2	2 pigs	2 pigs	2 pigs
3	2 pigs	2 pigs	2 pigs

[1] ADD—amino acid diets; CLAA—casein-lactalbumin supplemented with amino acids diet. WGAA—wheat gluten supplemented with amino acids diet.

1) Six littermate male pigs were divided into two weight groups. The three heavier pigs were assigned randomly to the three treatments (diets) and the three lighter-weight pigs also. Thus there were two replicates (one heavy and one light pig) per treatment. The experiment was repeated three times giving a total of six pigs per treatment.

2) Analysis of variance was determined with the variance partitioned between treatments, experiments and replicates.

b. The pigs were maintained in individual metabolism cages (28" x 36") at 19-25°C with constant illumination.

c. The pigs were weaned to the pre-experimental diet, fed this diet for one week and then fed the test diets for two weeks.

d. The nitrogen-containing portion of the diet was fed as an agar gel at 9 a.m. and 3 p.m. This portion of the diet contained non-nitrogenous dietary components which were not included in this portion of the rat diets for the similar experiments of paragraph g 3), page 14.

e. The nitrogen-free diet and water were fed *ad libitum*.

f. Urine and feces were collected over a six-day period during the last week.

g. Urine samples were collected hourly during the nights of the seventh and fourteenth days.

3. Diet:

a. Nitrogen sources and proportions were the same as for rats (see table 6). The other constituents were similar with the exception of the replacement of Alphacel by agar. See table 8 for composition of pig diets.

b. The nitrogen intake was adjusted daily according to the formula: Grams nitrogen = 1.7 (kg average body weight of six pigs in an experiment)$^{0.63}$ The exponent 0.63 was derived from values for the protein requirements of pigs weighing 10, 25 and 50 pounds (9).

4. Parameters:

a. Food consumption. The agar gel diet containing the nitrogen source was always completely consumed within five minutes.

b. Weight gain. Calculated from daily weights (taken about 8:30 a.m.) by the method of least squares or the method of averages.

c. Nitrogen balance.

d. Urine analyses. Urine was collected daily from the eighth to the thirteenth days, inclusive. Each day's urine was collected in a bottle that contained 10 ml of 10% sulfuric acid. Water was added to make a volume of 4,000 ml and an aliquot was frozen. The six daily aliquots made up the composite sample which was analyzed as follows:

1) Nitrogen. Kjeldahl (4).

2) Urea. Pellerin's adaptation (10) of the method of Coulombe and Favreau (11).

3) Ammonia. Microdiffusion, using Obrink modified Conway units (12).

4) Creatine and creatinine. Clark and Thompson method (13) automated, using a 20:1 molar ratio of sodium hydroxide to picric acid.

e. Fecal analysis. Feces were collected from the eighth to

TABLE 8

Pig Diet Compositions

Components	Pre-experimental[2] %	Test Diet[1] Components AAD[2] %	CLAA[2] %	WGAA[2] %	Nitrogen-free supplements[3] %
Casein	15.6	—	—	—	—
Casein-lactalbumin[4]	—	—	10.8	—	—
Wheat gluten[4]	—	—	—	10.2	—
Amino acids[5]	6.7	23.9	11.7	13.0	—
Cornstarch	33.0	32.2	32.9	32.6	—
Sucrose	33.0	32.2	32.9	32.6	87.3
Corn oil[6]	5.0	5.0	5.0	5.0	5.0
Cellulose[6]	—	—	—	—	2.0
Agar[7]	1.0	1.0	1.0	1.0	—
Mineral mix[8]	3.5	3.5	3.5	3.5	3.5
Vitamin mix[6]	2.2	2.2	2.2	2.2	2.2
Nitrogen, % dry matter	3.0	3.0	3.0	3.0	—
Gm water added[9] / Gm dry matter	0.5	0.5	0.5	0.5	—

[1] For abbreviations, see footnote 1, table 7.
[2] Intake restricted.
[3] Available *ad libitum* to all pigs in all test periods.
[4] As described on page 9.
[5] Formulated as given in table 6. The total quantities of each amino acid are listed by Babcock and Markley (8).
[6] Brands and composition as specified in 4b, page 14.
[7] Oxoid Ionagar #2, Calab Laboratories, Chicago Heights, Ill. Nitrogen content was 0.04%.
[8] Composition (%): $CaHPO_4$, 57.50; KH_2PO_4, 7.50; NaCl, 16.60; K_2CO_3, 12:00; $MgCO_3$, 4.00; $FeSO_4.7H_2O$, 1.50; $MnSO_4.H_2O$, 0.36; $CoCl_2.6H_2O$, 0.12; $CuSO_4$, 0.10; NaF, 0.02; $ZnCO_3$, 0.30; KI, 0.001.
[9] For the diets containing the nitrogen sources, the water, corn oil and agar were heated to boiling, cooled to 60-65°C and added to the other pre-mixed ingredients. All diets were stored in air-tight plastic containers at 3°C and used within a few days.

the thirteenth days, inclusive. Each day's collection was frozen. The six-day composite was made up to 1,500 grams with water containing 1 ml concentrated sulfuric acid, homogenized, and an aliquot frozen for analysis.

1) Nitrogen. Kjeldahl (4).

f. Carcass analysis. At the end of each experiment, all pigs were sacrificed at about 9 a.m. They were stunned electrically (110 v. AC ear-to-ear for 20 seconds), suspended by the hind legs, and bled from the neck. The following organs were removed, weighed and frozen for subsequent analysis: adrenals, heart, kid-

neys, liver, pancreas, spleen and testes. Other viscera were discarded. The eviscerated carcass was allowed to hang in a refrigerator for 18-36 hours; the carcass was then bisected and length measurements were made between the 6th and 27th vertebrae. The eviscerated carcasses, including the skin, head, and feet, were dried to constant weight in a forced-air oven (101°C), and ground in a meat grinder and Waring Blendor; samples were frozen for analysis. Dry matter, ether extract and crude protein (N x 6.25) were determined by A. O. A. C. methods (4).

g. Liver analyses.

1) Dry matter, ether extract, and crude protein (N x 6.25) were determined by A.O.A.C. methods (4).

2) Nucleic acids. Wannemacher, et al. (14).

h. Energy calculations.

1) Diets. The caloric content was calculated from gross energy values of the dietary components, with cellulose, agar, and minerals excluded. Energy values of the nitrogen sources were determined by bomb calorimetry; other values were taken from handbooks. The values, in kilocalories per gram, employed for these calculations are listed in table 9.

2) Carcasses. These values were calculated on the basis of 9.5 kcal/gm of ether extract plus 34.2 kcal/gm of body nitrogen (15).

TABLE 9
Energy Values Employed in Calculations.

	Kilocalories per gram		Kilocalories per gram
Minerals, Alphacel and agar	0.0	Leucine	6.516
Vitamin mix	4.043	Lysine HCl	6.361
Corn oil	9.483	Methionine	5.417
Cornstarch	4.20	Cystine	3.929
Sucrose	3.936	Phenylalanine	6.698
Amino acid mix A	5.742	Tyrosine	5.850
Tryptophan	6.589	Valine	5.955
Threonine	4.192	Amino acid mix B	3.939
Isoleucine	6.507	Casein-lactalbumin	5.456[1]
		Wheat gluten	5.685[2]

[1] 5.817 kcal/g dry matter. 93.8% dry matter in original casein-lactalbumin.
[2] 5.843 kcal/g dry matter. 97.3% dry matter in original wheat gluten.

EXPERIMENTAL PROCEDURES FOR TETRAHYMENA STUDIES

Species.

1. *Tetrahymena pyriformis W.* were used in these studies.

Plan of Experiments.

1. Two proteins, supplemented with amino acids, and the control crystalline amino acid diet were tested at several levels of nitrogen.

2. Statistical design:

a. Part A. Using six tubes per test as shown in table 10, casein-lactalbumin versus control at 150, 200 and 250 mcg nitrogen per ml of culture media with three replicates constituted one experiment. Wheat gluten was run in identical fashion with its own set of controls.

TABLE 10

Statistical Design for Tetrahymena Experiments, Part A.

		Nitrogen level, mcg N/ml media					
		150		200		250	
		AAD[1]	CLAA	AAD	CLAA	AAD	CLAA
Casein-lactalbumin	Replicate 1	—[2]	—	—	—	—	—
	Replicate 2	—	—	—	—	—	—
	Replicate 3	—	—	—	—	—	—
		150		200		250	
		AAD	WGAA	AAD	WGAA	AAD	WGAA
Wheat gluten	Replicate 1	—[2]	—	—	—	—	—
	Replicate 2	—	—	—	—	—	—
	Replicate 3	—	—	—	—	—	—

[1] For abbreviations, see footnote 1, table 7.
[2] Represents six tubes, one blank and five experimental.

b. Part B. All treatments AAD, CLAA and WGAA were compared at two levels of nitrogen, N_1 and N_2, at one time. This was replicated as shown in table 11.

TABLE 11

Statistical Design for Tetrahymena Experiments, Part B

	N_1			N_2		
	AAD[1]	CLAA	WGAA	AAD	CLAA	WGAA
Replicate 1	—[2]	—	—	—	—	—
Replicate 2	—	—	—	—	—	—
Replicate 3	—	—	—	—	—	—

[1] For abbreviations, see footnote 1, table 7.
[2] Represents six tubes, one blank and five experimental.

3. Growth media:

See table 12.

a. Components other than protein and amino acids were ke constant.

b. The carbohydrate employed was glucose, 1,000 mcg/m

c. Other constituents.

1) A constant mineral mixture containing a full con plement of salts was used.

2) A constant vitamin mixture containing a full comple ment of water soluble vitamins with the exception of ascorbi acid was employed.

3) Medium included guanylic acid, uracil, cytidylic acid adenylic acid and alpha-lipoic acid.

TABLE 12

Composition of the Tetrahymena Basal Medium.

Constituents	Mcg/ml of Basal Mediun
Nucleic Acid Mix A	
Guanylic Acid	30.0
Uracil	10.0
Cytidilic Acid	25.0
Adenylic Acid	20.0
Vitamin Mix B	
Thiamine HCl	6.0
Pyridoxine HCl	6.0
Vitamin Mix C	
Pteroylglutamic Acid	0.06
Calcium pantothenate	0.60
Riboflavin	0.60
Nicotinic Acid	0.60
Pyridoxal HCl	0.60
Pyridoxine di HCl	0.60
Mineral Mix	
$MgSO_4.7H_2O$	250.00
$CaCl_2.2H_2O$	50.00
$Fe(NH_4)_2(SO_4)_2.6H_2O$	25.00
$CuCl_2.2H_2O$	5.00
$FeCl_3.6H_2O$	1.25
$ZnCl_2$	0.05
$MnCl_2.4H_2O$	0.50
K_2HPO_4	100.00
Alpha-Lipoic Acid	0.0033
Biotin	0.003
Choline	6.00
Sodium Acetate	1000.00
Dextrose	1000.00

4) No fat or fat soluble vitamins were added.

5) The pH was adjusted to 7.0 prior to start of incubation.

4. Parameter:

a. Growth of the *Tetrahymena pyriformis W.* was measured by the colorimetric method of Anderson and Williams (16).

EXPERIMENTAL PROCEDURES FOR HUMAN STUDIES

laintenance Studies Utilizing A Crossover Design.

1. Level and Distribution of Nitrogen Intake:

a. The nitrogen level for each dietary period of the human studies was:

1) Adults. 0.065 gm or 0.08 gm nitrogen/kg body weight daily (approximately 0.4 gm or 0.5 gm protein/kg daily).

2) Adolescents. 0.10 gm nitrogen/kg body weight daily (approximately 0.6 gm protein/kg daily).

b. Three meals per day were fed, supplying 1/5, 2/5 and 2/5 of the total daily nitrogen intake, respectively.

2. Plan of Experiments (Connecticut, Maryland, Rhode Island, Vermont).

a. Crossover. The amino acid control or test protein diet was assigned to each subject at random for the first test period of ten days (comprising two five-day nitrogen balance periods); then followed a second test period of ten days when each subject received the other treatment.

b. A minimum of eight subjects with four on each diet at one time was used; each subject served as his own control and analysis of variance was done as follows:

Crossover	*Replications*							
	1	2	3	4	5	6	7	8
First test period	A	A	B	B	A	B	A	B
Second test period	B	B	A	A	B	A	B	A

3. Diets.

a. Preliminary diet.

1) The preliminary diet consisted of foods with a total nitrogen content (determined by analysis) equivalent to the specified levels per kg of body weight.

2) Menus were planned by each station individually. A five-day pattern was used to conform with specified fecal composite days.

3) Each station analyzed its own preliminary diet for nitrogen by the macro- or microkjeldahl method (4).

b. Basal diet.

1) The basal diet contained low-protein foods fed in conjunction with both the control and experimental diets.

2) The daily nitrogen intake of the basal diet was fed as a constant amount, all subjects receiving the same amount, and was planned to provide as low an intake of nitrogen as possible. By analysis, values averaged about 700 mg nitrogen daily.

3) By definition, the nitrogen was classified as nonessential amino acid nitrogen and was subtracted from the amount of nitrogen assigned to mix B. See c 2), below.

4) The diet used in ARS contract research projects (referred to as the USDA diet) served as a guide for planning the basal diets (17) (see table 8).

a) Each subject received the USDA diet or similar low-protein foods in the amounts and kinds as indicated. Other fruits of equivalent nitrogen content were substituted, provided that the total nitrogen content of each day's diet remained the same within experimental error.

b) Foods providing non-protein calories were chosen from those listed in the USDA diet or from other sources as desired and were not necessarily the same from station to station.

5) Dietary fat approximated 30% of the total daily non-protein calories. Any nitrogen-free fat was acceptable.

6) Dietary carbohydrate approximated 70% of the total daily non-protein calories and consisted primarily of unmodified cornstarch, sucrose and simple sugars but might include limited amounts of wheat, arrowroot or tapioca starches.

c. Control diet.

1) The control diet consisted of two mixtures of crystalline amino acids with the basal diet.

a) NE-52 Mix A: Essential amino acids in proportion (gm AA/gm N) to the FAO pattern (see table 1).

b) NE-52 Mix B: Nonessential amino acids in the proportion (gm AA/gm N) as found in milk (see table 2).

2) Mix A furnished 1/4 and Mix B, including the basal diet nitrogen, 3/4 of the total daily nitrogen intake.

TABLE 13

NE-52 Guide for Planning Basal Diet.[1]

Item	Weight	Protein	Calories[2]
	gm	gm	
Breakfast			
Applesauce, canned	50	0.1	45.0
Lemon juice, canned	25	0.1	5.8
Orange juice, frozen	100	0.8	44.0
Luncheon			
Applesauce, canned	75	0.15	68.0
Lemon juice, canned	25	0.1	5.8
Salad:			
Peach slices, canned	100[3]	0.4	58.0
Pineapple chunks, canned	25	0.1	19.5
Banana	25	0.3	21.0
Lettuce	25	0.2	3.0
Pudding, Danish Dessert	100	tr	154.0
Dinner			
Pear halves, canned	100[4]	0.2	61.0
Tomato juice, canned	100	0.9	19.0
Applesauce, canned	75	0.15	68.0
Lemon juice, canned	25	0.1	5.8
Pudding, Danish Dessert	100	tr	154.0
Total		3.60	731.9
Additional Items			
Wafers (during the day)[5]	1 recipe	tr	
Butter oil[6]			
Jelly[6]		tr	
Fondant[6]			
Sucrose[6]			
Carbonated beverages[6]			
Beverages[6]			
Vitamin capsule[7]	1 each		
Mineral mix[8]	1 serving		

[1] Modified USDA basal diet (17).
[2] Calculated on the basis of data from USDA Agriculture Handbook No. 8, revised 1963.
[3] Drained weight.
[4] Consists of 85 gm of fruit and 15 gm of juice.
[5] See table 14.
[6] Amount adjusted to meet the individual's caloric need.
[7] See table 15.
[8] See table 16.

d. Experimental diet.

1) The experimental diet consisted of a protein-amino acid mixture, mix B and the basal diet.

2) A test protein, casein-lactalbumin or wheat gluten, was adjusted with crystalline essential amino acids so that the final protein amino acid combination was equivalent to the NE-52 Amino Acid Mixture A (gm EAA/gm N) of the control diet.

3) The remaining amount of nitrogen required to provide the specified total daily intake came from:

a) The nonessential amino acid nitrogen of the test protein.

b) Adjusted amounts of mix B.

c) The basal diet.

4) The test proteins were incorporated into human foods as follows:

a) Casein-lactalbumin, mixed raw, in applesauce, was served 1/5, 2/5 and 2/5 at each meal with accompanying amino acid mixture fed in lemon juice (Vermont, Rhode Island).

b) Wheat gluten was incorporated into batches of wafers which were baked separately in advance for each subject. (See table 14.) The wafers were served 1, 2 and 2 at each meal with the accompanying amino acids fed in lemon juice (Vermont, Rhode Island, Connecticut).

c) Wheat gluten was incorporated into wafers at the same minimum levels for all subjects, baked in advance and served one each at noon and night meals. The additional amount of wheat gluten needed for each individual was fed raw in lemon juice at breakfast. Weighed portions of supplementary crystalline amino acids were taken in lemon juice at each of three meals (Maryland).

e. Supplemental Items.

1) Mineral and vitamin supplements were fed with the control diet and experimental diets.

2) Each subject was given one vitamin capsule daily—Upjohn Unicaps, or equivalent (see table 15).

3) Mineral supplementation was equivalent in kinds and amounts to those provided in the USDA or Leverton Mineral and Baking Powder Mixes (18) (see table 16).

4) Method of feeding. Mineral supplements were incorporated in various foods, sometimes raw in juices; calculated amounts baked in wafers became the most satisfactory method for ingestion by the subjects (see table 14).

4. Miscellaneous Dietary Procedures and Suggestions:

a. Food.

1) The preliminary and the basal diets were analyzed in advance for nitrogen by each station as a basis for calculating the nitrogen content of the diets.

2) The nitrogen content of all food fed during the study was analyzed for amino acids and total nitrogen (4).

TABLE 14
Wafer Recipes (Vermont)

Protein-free Wafer

Ingredients	Amount
Fluffo[1]	1600 gm
Cornstarch	3000 gm
Sugar	1300 gm
Water	400 ml
Butter flavor	150 ml
Baking powder mix	110.5 gm
Mineral mix	79.5 gm
	6640.0 gm

Yield: 270 wafters
One wafer, raw weight = 24.59 gm

Method: Use Hobart quantity mixer.

1. Obtain baking powder mix and mineral mix from lab in correctly weighed portions.
2. Cream fat in mixer for three minutes with paddle on speed 3.
3. Combine starch, sugar and minerals in a large bowl.
4. Add dry ingredients to the creamed fat.
5. Add water and butter flavor and combine with spatula before starting mixer to prevent "fly-out."
6. Mix three minutes.
7. Cover mixture to prevent moisture loss during weighing.
8. Weigh raw dough equivalent to one wafer and press evenly with hands to 4-inch diameter on teflon pans.
9. Bake 12 minutes at 350° F.
10. Cool and wrap.

Wheat Gluten Wafer

Ingredients	Amount
Fluffo[1]	640 gm
Cornstarch	1200 gm
Sugar	520 gm
Water	200 ml
Butter flavor	60 ml
Baking powder mix	44.2 gm
Mineral mix	31.8 gm
Wheat gluten	188.4 gm[2]
	2884.4 gm

Yield: 108 wafers
One wafer, raw weight = 26.7 gm

Method: Same as above, except for adding the calculated amount of wheat gluten for one subject for each batch.

[1] Proctor and Gamble, Cincinnati, Ohio. Other vegetable shortenings were permitted.
[2] 17.448 gm of gluten for an 80 kg man is contained in 10 wafers per day.

b. Amino Acids.

1) Ready supply.

a) A two- or three-day supply of amino acids was weighed for each subject in advance, and each subject's

supply was then weighed accurately into separate containers for each meal in the proportion 1/5, 2/5 and 2/5 and clearly marked for each subject.

b) Lemon juice and/or applesauce were the preferred items of the basal diet as carriers of crystalline amino acids.

c) Each subject's quota of amino acids was quantitatively transferred to the carrier material as follows:

Containers holding the weighed amino acids and any equipment used in incorporating the amino acids into the food were thoroughly washed down and the washings transferred to the food or to the liquid which had previously

TABLE 15
Vitamin Supplement[1]

	Weight per capsule
Vitamin A	1.5 mg
Vitamin D	12.5 mg
Thiamine hydrochloride	2.5 mg
Riboflavin	2.5 mg
Ascorbic acid	50.0 mg
Nicotinamide	20.0 mg
Calcium pantothenate	5.0 mg
Pyridoxine hydrochloride	0.5 mg
Cobalamin	2.0 mcg

[1] Unicap, Upjohn Company, Kalamazoo, Michigan. One a day for each subject during the control diet and the experimental diet.

TABLE 16
Mineral Supplement

Leverton Mineral Mix (18)	Gm per subject per day
$CaCO_3$	1.362 gm
KH_2PO_4	1.307 gm
$MgCO_3.Mg(OH)_2.3H_2O$	0.800 gm
$FeC_6H_5O_7.6H_2O$	0.0948 gm
$CuSO_4.5H_2O$	0.0078 gm
KI	0.0002 gm
$MnCl_2.4H_2O$	0.0072 gm
$ZnCl_2$	0.0019 gm
Total	3.58 gm
Baking Powder Mix (18)	
$NaHCO_3$	2.05 gm
$Ca(H_2PO_4)_2.H_2O$	2.86 gm
Cornstarch	2.69 gm
Total	7.60 gm

been weighed into separate labelled containers for each subject.

d) Quantitative transfer methods were used for ingesting the amino acid food mixture.

(1) Plastic wash bottles were provided and each subject washed down the sides of the container until no amino acid crystals were present and drank the wash.

(2) Clear glass containers facilitated inspection.

c. Energy Intake.

1) Energy intake was calculated for each subject on the basis of body weight.

2) Subjects were weighed daily before breakfast, but, in general, energy intake for any one subject was increased or decreased only after establishment of a three-day trend in weight gain or loss.

3) An increase in energy intake of approximately 10 kcal/kg over the amount provided during the preliminary period was satisfactory for maintaining weight in the control and experimental periods.

d. Nitrogen Balance.

1) Nitrogen balance was estimated daily if possible for each subject by using:

a) Previously determined values for dietary nitrogen.

b) Estimated fecal nitrogen values of 1.0 gm nitrogen/subject daily.

c) Analytically determined values of urinary nitrogen.

d) Final calculations were based on five-day balance periods.

2) Negative balances severe enough to be considered harmful did not occur under the conditions of this study.

5. Sample Preparation and Preservation for Analysis:

a. Food.

1) A portion of each food was weighed on a torsion balance at the time of serving. A representative fraction was sometimes used rather than the entire serving.

2) The food item was placed in a weighed container and kept refrigerated until analyzed.

3) Contents of the container were quantitatively trans-

ferred to a weighed Waring Blendor using distilled water and the final product was weighed.

4) After blending until thoroughly homogeneous, convenient amounts of the composite were transferred to glass or plastic containers and frozen until analysis.

5) Appropriate aliquots were weighed for analysis.

b. Urine.

1) A 24-hour urine specimen was defined as that volume of urine voided between the time after breakfast of one day up to breakfast time on the following day (including the urine collected on rising in the morning before breakfast).

2) Urine was collected either directly in wide-mouthed amber or polyethylene containers of convenient size or in a stainless steel cup and then transferred to an amber bottle or polyethylene container. If the collection cup was used, it was rinsed after each use with distilled water and the rinsings added to the urine collection.

3) Urine bottles had tight-fitting lids to prevent leakage and an amount of toluene sufficient to form a thin layer on the surface of the urine (10-20 ml) was added.

4) Urine was kept refrigerated or in as cool a place as possible between voidings until the 24-hour collection was completed.

5) The complete 24-hour collection for one subject was rinsed into a graduated cylinder (glass-stoppered), diluted to the nearest 100 ml with distilled water, volume recorded, convenient amounts transferred to amber or polyethylene bottles and frozen for later analysis. If a sample could be analyzed within 24 to 48 hours, the bottle was kept in the refrigerator.

6) Parameters.

a) Creatinine determinations (13) were done daily.

b) Total nitrogen (4) was done daily when possible.

c) Urea and ammonia determinations (11) were done when convenient.

c. Feces.

1) Fecal collections were marked by means of a carmine dye capsule administered before breakfast on the first day of the collection period. Composites then included all fecal material beginning with that containing the carmine up to, but not including, the next carmine-marked fecal material. The second marker was given before [illegible]

mark the end of a five-day period. In a series of continuous five-day periods, the marker on the sixth day also served to mark the start of the following five-day period.

2) Feces were collected directly in paraffin-coated or waxed freezer cartons (1 pint or 1 quart) or in a receptacle lined with squares of plastic wrap. The plastic square was then folded and placed in a separate container and the container labelled with the subject's identification, and the time of the day, and the number of the collection.

3) Containers were refrigerated or frozen as soon as possible and kept in as cool a place as possible until then.

4) Collections were held in the freezer until time to prepare five-day composites. Marked feces were separated and the five-day composite prepared and weighed.

5) The composite was quantitatively transferred to digestion jars and covered with 2:1 HCl:distilled H_2O, and placed in a hood at room temperature for 48 to 72 hours. After digestion it was either:

a) Transferred quantitatively to a graduated cylinder (glass-stoppered), diluted to the nearest 100 ml with distilled water, mixed well, or

b) Prepared as a slurry using a Waring Blendor and convenient amounts removed from the homogeneous mixture to bottles for storage in freezer.

c) Appropriate aliquots were weighed for analysis of nitrogen (4).

Test Meal Studies (Pennsylvania).

1. General plan of experiments.

a. Each of eight male subjects served as his own control.

b. The preliminary diet of ordinary low-protein (0.065 gm N/kg body weight daily) foods was fed for 10 days.

c. After a 14½-hour fast, a blood sample was drawn for plasma amino acid analysis.

d. In each test, blood was drawn at 1, 2 and 4 hours after the test meals.

e. The plasma amino acid concentration was plotted and analysis of covariance was used to test the slopes of the response lines.

2. First series of test meals:

a. Test No. 1. The amino acid control was fed in applesauce with sugar to provide 1/5 of the total day's requirement for

protein and calories. The nitrogen level was calculated to be 0.013 gm/kg body weight.

b. Tests No. 2 and No. 3.

1) Each test was repeated twice following a weekend of *ad libitum* food selection then a five-day period on the preliminary low-protein diet.

2) Casein-lactalbumin supplemented with amino acids was fed raw in applesauce in Test No. 2.

3) Wheat gluten supplemented with amino acids was fed raw in applesauce in Test No. 3.

3. Second series of test meals.

a. An arbitrary level of 0.039 gm nitrogen/kg body weight was selected.

b. Following another ten-day preliminary period on the low-protein diet, the same three test meals were fed at the level of 0.039 gm nitrogen/kg body weight, and blood samples were taken at the same intervals.

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APPENDIX

Minima of Medical Controls for Human Subjects in the Dietary Studies of NE-52, Utilization of Amino Acids from Protein.

1. *Certification of medical endorsement for the experimental plan.*

   The endorsement by a licensed physician, of health-related features in the experimental plan, seems critical in obviating undue health hazards. Recommendation as to health risks that may be involved would be especially helpful as a part of this certification.

2. *Medical certification of fitness to participate in the medically endorsed plan.*

   This certification for each subject, by a licensed physician, is a meaningful safeguard for participant and researcher. The certification should be based on the physician's full knowledge of the health-related features of the experimental plan and participant's current health status. Recommendations for 1) any limits to participation and 2) intervals for rechecking health status should be a part of the certification.

3. *Certification of consent to participate in the medically endorsed plan.*

   Legal Minors

   The parents or guardians of legal minors should certify this consent based on 1) certification of medical endorsement for the experimental plan; and 2) medical certification of the subject's fitness to participate in the experimental plan.

   Others

   Subjects who are not legal minors should certify consent based on the two types of certification described for minors.

Any recommendations for 1) health risks, 2) limits of participation or 3) rechecking health status should be brought to the attention of consenting persons in either instance. It should be clear who is responsible for rechecking health status at recommended intervals.

4. *Medical certification of health status at advantageous intervals during the experiment.*

   The selected intervals for rechecking health status, for each subject, should be a part of the certification of experimental plan and fitness to participate. A licensed physician should be an integral part of the re-checking.

5. *Procedures deemed pertinent to the institution.*

   Controls, including procedures and records that offer safeguards to the institution and its representatives, are the responsibility of the institution and should be required. Precise records for each subject, as they relate to items 1-4, should be a minimum.

6. The experiment should be conducted only by scientifically qualified persons. The highest degree of skill and care should be required through all stages of the experiment of those who conduct or engage in the experiment.

7. During the course of the experiment the human subject should be at liberty to bring the experiment to an end if he has reached the physical or mental state where continuation of the experiment seems to him to be impossible.

8. During the course of the experiment, the scientist in charge must be prepared to terminate the experiment at any stage, if he has probable cause to believe, in the exercise of good faith, superior skill and careful judgment required of him that a continuation of the experiment is likely to result in trauma, either physical or mental.