

## **A Comparative, Double-blind, Triple Crossover Net Nitrogen Utilization Study Confirms the Discovery of the Master Amino Acid Pattern**

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### **Abstract**

This comparative, double-blind, triple crossover Net Nitrogen Utilization study, has determined the percentage of Anabolized Amino Acids (UAA), also known as Net Nitrogen Utilization (NNU) and of Catabolized Amino Acids (UCA), in 66 subjects during a twelve-week period. The subjects were alternatively given the amino acid formula of Master Amino acid Pattern (Diet A), amino acid formula of the hen whole-egg protein (Diet B), and hen whole-egg protein (Diet C). The comparative results showed that Diet A (MAP) induced a UAA (NNU) equivalent to a 99%, that means that the 99% of its constituent Amino Acids acted as precursors of body's protein synthesis, and that Diet A induced a UCA equivalent to 1%, that means that only 1% of its constituent amino acids originated nitrogen catabolites. The results also showed that the UAA in the 66 subjects, while receiving Diets B and C, was a 28% and 32% lower respectively than while receiving diet A (MAP). These results confirm the discovery of MAP, meaning the ideal amino acid formula for human nutrition, capable of inducing a UAA (NNU) higher than the hen whole-egg protein, officially considered the protein with the highest biologic-value, according to the Chemical Protein Score Tables.

Key words: Essential Amino Acids, nutritional rehabilitation, high biologic-value proteins, utilization of anabolized amino acids, utilization of catabolized amino acids.

### **Introduction**

The scientific discoveries related to proteins and their constituent amino acids began in 1820 when Braconnot discovered the essential amino acid Leucine. In 1838, Mulder identified and classified for the first time, proteins and remarked its importance for human life. In 1881, Schultze and Barbieri discovered Phenylalanine and in 1889 Drechsel discovered Lysine. Between 1892 and 1897, Atwater calculated the energetic value of the proteins (extracorporeal), through the use of the calorimeter Atwater-Rosa, and estimated, also, its energetic metabolic value (corporeal). In 1901, Fischer discovered Valine and Hopkins and Cole discovered Tryptophane. In 1904, Ehrlich discovered Isoleucine and in 1922, Muller discovered Methionine. Finally in 1935, Rose concludes the identification of the eight essential amino acids with the discovery of Threonine. In 1946, the same Rose estimates for the first time the daily requirements of essential amino acids for humans (Table I). Also in 1946, Block and Mitchell suggested that the biological value of the dietary proteins depends on its constituent amino acids and show that if the eight essential amino acids are not available simultaneously at the time of the protein synthesis, the intracellular deficit, even thought of only one amino acid, would limit the protein synthesis of the body (1). Since 1947 there have been numerous unsuccessful attempts to discover a protein that could induce a Utilization of anabolized amino acids (UAA, also known as Net Nitrogen Utilization (NNU) higher than the one induced by the hen whole-egg protein, officially considered the protein with the highest biologic-value, with an hypothetical value of 100 according to the "Chemical Protein Score Tables" (2).

This study has determined the UAA and the Utilization of catabolized amino acids (UCA), in 66 subjects while receiving diet A (Amino acid formula of MAP); of diet B (amino acid formula of whole-hen egg protein; and diet C (hen whole-egg protein).

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Table I. Daily Requirement of Amino Acids, W. Rose (1946)

Amino Acid	Minimum Amount	Recommended Amount
Isoleucine	0.70	1.40
Leucine	1.10	2.20
Lysine	0.80	1.60
Methionine	1.10	2.20
Phenylalanine	1.10	2.20
Threonine	0.50	1.00
Tryptophane	0.25	0.50
Valine	0.80	1.60
TOTAL	6.35 g	12.70 g

### Study population

The study population comprised 66 healthy subjects, 33 men and 33 women, with a mean age of 27.0 years (SD = 3.4) (range 22-34). The subjects were selected if they satisfied all inclusion and none of the exclusion criteria.

- Inclusion criteria were: (a) good health, (b) age between 21 and 40 years, (c) either male or female subjects.
- Exclusion criteria were: (a) Being underweight. (b) Pregnancy or lactation, (c) Current disease, which could alter the N balance, (d) phenylketonuria.

All 66 subjects provided informed consent to participated in the study. The 33 men and 33 women selected were randomly integrated according to sex and number into 3 matched groups of 11 men and 11 women, identified as group 1, 2 and 3. The ages, heights and ideal weights are shown in Table II. Table III shows the characteristics of all 66 subjects.

Table II. Subject's Characteristics by Group

Group	Characteristics	Mean	SD	Range
1	Age (years)	27.1	3.9	22 – 32
	Height (cm)	164.3	6.4	152 – 176
	Ideal Weight (Kg)	54.9	8.4	41.5 – 68.5
2	Age (years)	27.9	3.3	22 – 34
	Height (cm)	162.7	7.4	150 – 171
	Ideal Weight (Kg)	53.6	8.9	40.0 – 64.0
3	Age (years)	25.9	2.8	22 – 30
	Height (cm)	161.8	7.6	147 – 172
	Ideal Weight (Kg)	52.8	9.2	37.5 – 65.0

SD = Standard Deviation

Table III. Characteristics of the 66 Subjects of the Study

Characteristics	Mean	SD	Range
Age (years)	27.0	3.4	22 – 34
Height (cm)	162.9	7.1	147 – 176
Ideal Weight (Kg)	53.8	8.7	37.5 – 68.5

SD = Standard Deviation

### Study Design and Diet Definition

The study was carried out during a 114-day-period in double-blind conditions, using a triple crossover technique (3). This technique allowed each subject to receive as an N source, diets A, B and C in different sequences. The study was divided into the following two phases:

- a) The preliminary phase was conducted during a 30-day period, to equalize and stabilize subjects' protein and energy metabolism, thus avoiding metabolism differences that could affect their N balance. To achieve this, the 66 subjects received the Metabolism Equalizing & Stabilizing Diet (MESD) before giving diets A, B and C (Table IV).
- b) The main phase was conducted during three consecutive four-week periods (84 days), at which time the subjects' N balances were assessed to determine their UAA y la UCA during diets A, B and C.

Group 1, 2 and 3 received diets A, B and C following the obligatory sequence (Table IV).

Table IV. Sequence of Diets by Group and Period

Diet	Period	Group 1	Group 2	Group 3
Preliminary Diet	30 days	MESD	MESD	MESD
First Diet	28 days	A	B	C
Second Diet	28 days	B	C	A
Third Diet	28 days	C	A	B

MESD = Metabolism Equalizing & Stabilizing Diet.

Diets A, B and C consisted in identical composition of equal amount of protein or amino acids, carbohydrates, fats, vitamins, minerals and trace elements, and had the following characteristics:

- a) Diet A provided each subject an amino acid intake of 0.4 g/kg per day (equivalent to 64 mg/kg per day of N) through the amino acid formula of MAP (Table V), plus an energy intake of 50 kcal/kg per day, through essentially protein-free carbohydrates and fats (Table VI).

Table V. Amino Acid Formula of the Master Amino Acid Pattern (g)

L-Isoleucine	1.483
L-Leucine	1.964
L-Lysine	1.111
L-Valine	1.657
L-Methionine	1.429
L-Phenylalanine	0.699
L-Threonine	1.289
L-Tryptophane	0.368
Total	10.000

Table VI. Essentially Protein-free Carbohydrate and Fat Foods

Food	Protein (g) x 100 g	Energy (kcal) x 100 g
Sugar	0.0	384
Corn oil	0.0	884
Apricot	0.8	57
Pineapple	0.4	52
Peach	0.8	52
Strawberry	0.8	36
Annona	0.4	52
Tangerine	0.7	43
Mango	0.5	59
Apple	0.3	58
Cantaloupe	0.5	25
Orange	0.7	50
Medlar	0.2	44
Papaya	0.5	32
Pear	0.3	56
Watermelon	0.5	22
Celery	0.8	19
Eggplant	1.0	27
Zucchini	0.5	14
Lettuce	1.0	13
Cucumber	0.7	15
Tomato (mature)	0.8	21
Manioc	1.0	132
Carrot	0.8	41

- b) Diet B provided each subject an amino acid intake of 0,4 g/kg per day (equivalent to 64 mg/kg per day of N) through the amino acid formula of the hen whole-egg protein (4), plus an energy intake of 50 kcal/kg per day, through essentially protein-free carbohydrates and fats. (Table VI).
- c) Diet C provided each subject an amino acid intake of 0,4 g/kg per day (equivalent to 64 mg/kg per day of N) through hen whole-egg protein (5), plus an energy intake of 50 kcal/kg per day, through essentially protein-free carbohydrates and fats (Table VI).

The MESD, A, B y C diets were supplemented with vitamins and minerals in accordance with the U.S. recommended daily allowance (RDA).

**Methods:**

**Determining daily protein requirements**

The daily protein requirement of each subject was determined subtracting a factor of 100 from the subject's height in cm., then multiplying the result for a factor of 0.9 (men) or 0.8 (women) in accordance with the subject's sex. The following formulas were applied:

$$\text{Men's daily protein requirement} = [(H - 100) \times 0.9] \text{ g}$$

$$\text{Women's daily protein requirement} = [(H - 100) \times 0.8] \text{ g}$$

The result was rounded off to the nearest 0.5 g.

**Determining Weight**

The subjects' weight in kg was determined daily in the early morning before breakfast, after the subjects' evacuation and urination. The result was rounded off to the nearest 100 g.

### Determining N Balance

To determine the UAA and the UCA of each one of the 66 subjects while given the diets A, B y C, it has been utilized the N balance methodology, which has being the fundamental analytical technique for nutrition studies during the last 150 years (6,7). The following formulas were applied:

$$B = I - O$$

$$O = (U + F + S)$$

$$B = I - (U + F + S)$$

where:

**B** = N balance; **I** = N intake; **O** = N Output; **U** = N loss in urine; **F** = N loss in feces; **S** = N dermal losses.

N balance represents the difference between the Intake (I) and output (O) of N. The N balance could be positive ( $I > O$ ), when the N intake is higher than its output (N retention), as in active growth and during pregnancy; it could be negative ( $I < O$ ), when N intake is less than its output (N loss), as it could occur while an inadequate protein intake or an excessive catabolism; or it could be in equilibrium ( $I = O$ ), when the difference between the N intake and its output is equal to zero (neither retention nor loss of N), as it happen while an adequate protein intake.

Due to the fact that the methodology of the nitrogen balance is valid only when it is carried out in a accurate manner (8,9), the following precautions were taken during the study:

- a) To avoid or to reduce to a minimum the possible differences in the retention of N, caused by a determined dietetic sequence, it was utilized the triple crossover technique (3), considering the fact that the N retention increases after a negative nitrogen balance (10). This fact allowed that each subject received as an N source, the same three diets in different sequences.
- b) To avoid error common to the energetic intake, which could influence upon the nitrogen balance (11-13) and having into consideration than carbohydrates have a protein saving effect (5), the diets MESD, A, B and C gave each subject a constant energy intake, equivalent to 50 kcal/kg per day during the whole period of the study.
- c) To achieve an energy intake equivalent to the fat and carbohydrates contained in the hen egg (14) (diet C), it was added 0,9 g of fat and 0,07 g of carbohydrates per each gram of MAP (diet A) and for each gram of the amino acid formula of the hen egg protein (diet B).
- d) To avoid errors in the N intake which could influence upon the N balance, the carbohydrates and fat of the diets MESD, A, B and C where chosen from a list of essentially protein-free foods. (Table VI).
- e) To avoid an excessive N intake in mg/kg per day, which could influence upon the N balance (15), the protein requirements were calculated according to the ideal weight of each subject.
- f) To avoid an overvaluation of the N intake, due to an incomplete ingestion of the diet MESD, A, B y C, the total consumption of each designated portion was reached.
- g) In order to maintain the double-blind condition of the study, the diets A, B and C were given with the same fruit shake from foods selected from Table VI.
- h) To avoid a possible error of interpretation, the N daily output of each subject, which usually it is not lineal, was averaged during a four-week period corresponding to each diet.

### Determining UAA and UCA

The UAA and the UCA of an specific protein can be determined through the N balance in a subject while that particular protein is given. To better understand UAA and UCA are, in relation to an specific protein, it is necessary to analyze the following:

When a protein is digested, namely, it is enzymatically hydrolyzed during the digestive tract, the protein releases its constituent amino acids, which after their absorption in the small intestine, they can follow one of the following two metabolic pathways:

- a) **Anabolic pathway**, in which the amino acids act as precursors of body protein synthesis (primary function of amino acids) without providing either energy nor nitrogen catabolites.
- b) **Catabolic pathway**, in which the amino acids are deaminated, which means that their amino groups (NH<sub>2</sub>) are separated from their carboxylic groups (COOH) providing energy and originating energy and nitrogen catabolites (secondary function of amino acids).

Therefore, the percentage of Amino Acids Utilization (AU) of a specific protein, is equal to the sum of its UAA + UCA, according to the following equation:

$$\text{AU} = (\text{UAA} + \text{UCA}) \%$$

Thus, the UAA corresponds to the percentage of the constituent amino acids of a specific protein that follow the anabolic pathway, while the UCA corresponds to the percentage of its constituent amino acids that follow the catabolic pathway.

Consequently, the higher the percentage of UAA of a specific protein, the lesser it would be its energetic supply, and, lesser the percentage of nitrogen catabolites that this would originate. On the contrary, the higher the percentage of UCA of a specific protein, the higher it would be its energetic supply, and higher the percentage of nitrogen catabolites that this would originate.

In order, to determine the UAA, in each of the 66 subjects, during diets A, B and C, it was taken into consideration that:

- a) When the nitrogen balance is negative ( $I < O$ ), or it is in equilibrium ( $I = O$ ), the UAA can be calculated,
- b) When the nitrogen balance is positive ( $I > O$ ), the UAA can not be calculated;

**In order to determine the percentage of UAA and UCA, the following procedure was applied:**

**Knowing the negative nitrogen balance (mg/kg/day) corresponding to each subject, its percentage was determined, utilizing the following equation:**

$$\text{Nitrogen Balance (\%)} = \frac{(I - O) \text{ mg/kg/day}}{I \text{ mg/kg/day}} \times 100$$

**Knowing the negative nitrogen balance percentage corresponding to each subject, the UAA was determined utilizing the following equation:**

$$\text{UAA} = 100 + (\text{nitrogen balance \%})$$

**Knowing the percentage of UAA, corresponding to each subject, the UCA percentage was determined utilizing the following equation:**

$$\text{UCA} = 100 - \text{UAA} \%$$

#### **Determining N intake**

To determine the N intake, the following formula was applied:

$$\text{Quantity of dietary proteins} = \text{Quantity of dietary N} \times 6.25$$

In which the utilization of 6.25 implies that the mean N content of a dietary protein is equivalent to 16%.

#### **Determining N output**

The urine (U) and the feces (F) of each subject were collected daily during the four-week period corresponding to each diet, to determine its N content through the analytic method of Kjeldahl (17). To avoid error in the calculation of the N output, an enema was given to each subject, before and after the MESD, A, B and C diets. Due to the fact that the measurement of the direct N output through the skin and other minor paths (S), is not a common procedure, such output was calculated in each subject with the help of a constant, utilizing the following formula:

$$S = 5 \text{ mg} \times \text{weight (actual) of the subject (kg)} \times \text{day}$$

### Determining loss of lean tissue

The loss of lean tissue in each subject was determined multiplying the N loss of each subject by the 6.25 and then by the 5 factor. The following formula was applied:

$$\text{Lean tissue} = (\text{N} \times 6.25 \times 5)\text{g}$$

Considering that the N content of the different body proteins are equivalent to a 16%, the loss of 1 g of N represents a loss of 6.25 g of body protein. The intracellular protein is found in the lean tissue, in a aqueous solution in a concentration of 20 to 25%, therefore, for each 5 g of hydrated lean tissue, there is 1 g of protein. Consequently, the elimination of 1 g of N represents a loss of 31.25 g of lean tissue.

### Data Analysis

Data were analyzed using the analysis of variance (ANOVA), followed by the Student-Newman-Keuls test.

### Results:

#### Nitrogen Balance

Table VII summarizes the N balance results of the subjects by group, while receiving diets A, B and C. Table VIII shows the results of the N balance of the 66 subjects while receiving such diets.

Table VII. Nitrogen Balance (mg/kg per day) Results by Group and Diet

Group	Diet	N Output		N Balance	
		Mean	SD	Mean	SD
1	A	63.996	0.001	0.003	0.001
	B	82.5	0.2	-18.5	0.2
	C	85.3	0.3	-21.3	0.3
2	B	81.6	0.3	-17.6	0.3
	C	84.3	0.3	-20.3	0.3
	A	63.997	0.001	0.003	0.001
3	C	84.5	0.2	-20.5	0.2
	A	63.997	0.001	0.003	0.001
	B	81.9	0.2	-17.9	0.2

N = nitrogen; SD = Standard Deviation; N Balance = N intake – N Output.

The comparison of the mean and output differences within groups 1, 2 and 3 between diets A, B and C was statistically significant ( $P \leq 0.001$ ). The comparison of the mean and output differences by each diet (A, B and C), between groups, had a high statistical significance ( $P \leq 0.001$ ) for diets B and C in each case, while having a lower or no statistical significance for diet A (MAP).

#### Effectiveness

Each subject belonging to groups 1, 2 and 3, while receiving MAP (diet A), showed the lowest N output and a UAA significantly higher ( $P \leq 0,001$ ). Also while given diet A, each one of the subjects of such groups kept a nitrogen balance in equilibrium (Table VII y VIII).

Table VIII. Nitrogen Balance (mg/kg per day) by Diet of all 66 Subjects

Diet	N	Mean	SD
A	I	64	-
	O	63.997	0.001
	B	0.003	0.001
B	I	64	-
	O	82.0	0.4
	B	-18.0	0.4
C	I	64	-
	O	84.7	0.5
	B	-20.7	0.5

N = nitrogen; I = N intake; O = N Output; B = N Balance.

The variance related to the mean N output of the subjects while receiving diet A (MAP) points out extremely low and constant (SD = 0,001), this indicate a high UAA during diet A (MAP) in comparison with diets B and C.

### Safety and Tolerance

While receiving diet A (MAP) none of the 66 subjects reported any side effects and none showed adverse effects on blood parameters.

### Discussion

Historically, recommendations for protein intake have been based in the involuntary daily loss of N that result from the catabolism of the body proteins, should be replaced with an equivalent amount of N (proteic), through the ingestion of dietary proteins (18). The 66 subjects of the study maintained a N balance in equilibrium, while receiving 0,4 g/kg per day, of the amino acid formula of MAP (Diet A), equivalent to 64 mg/kg per day of N per subject. The attainment of a nitrogen balance in equilibrium does not itself permit the conclusion that 0,4 g/kg per day of the amino acid formula of MAP (diet A), was nutritionally adequate, this is due to the fact that a nitrogen balance in equilibrium could also be obtained through a decrease of the body protein *turnover* (19, 20). The results of this study have demonstrated that even though diets A, B and C gave each subject the same N intake per kg/day, and the same energy intake, the 66 subjects obtained:

- A UAA equivalent to 99%, while receiving diet A (MAP) (Table IX) in addition to a nitrogen balance in *equilibrium*;
- A UAA 28% lower, compared to diet A (MAP), while receiving diet B, in addition to a *negative* nitrogen balance equivalent to a mean N loss of 18.0 mg/kg/day (SD = 0.4) (Table IX). This means a lean tissue loss of 562.5 mg/kg/day.
- A UAA 32% lower, compared to diet A (MAP), while receiving diet C, in addition to a *negative* nitrogen balance with a mean N loss of 20.7 mg/kg/day (SD = 0.4) (Table IX). This means a lean tissue loss of 646.8 mg/kg/day.

Table IX. Utilization of Anabolized Amino Acids (UAA) by Diet

Diet	UAA (%)	Negative Nitrogen Balance (%)
A	99	1
B	72	28
C	68	32

## Conclusion

The comparative results of this double-blind and triple crossover study, have confirmed the discovery of the Master Amino acid Pattern (MAP), meaning the ideal amino acids formula for human nutrition, the only one capable of inducing a UAA, namely a Net Nitrogen Utilization (NNU) equivalent to 99%, which means that the 99% of its constituent amino acids acted as precursors of the body's protein synthesis. This UAA (NNU) is higher than that of any known dietary protein, even the hen whole-egg protein, until now officially considered the highest biologic-value protein. The comparative results have shown that MAP generated a UCA equivalent to 1%, this means that only 1% of its constituent amino acids generated energy and nitrogen catabolites. This UCA is the lowest compared to that of any known dietary protein. As a result, MAP provides only 0.04 Kcal per gram.

Due to such characteristics, the utilization of MAP could be highly beneficial in the dietary treatment of patients with:

- a) Protein-Energy Malnutrition (PEM);
- b) Anorexia;
- c) Bulimia;
- d) Renal insufficiency;
- e) Hepatic insufficiency;
- f) Anemia due to an insufficient erythropoiesis;
- g) Lack of appetite;
- h) Vomit;
- i) Uncontrollable diarrhea;
- j) Excessive catabolism;
- k) Obesity.

It can also be concluded, that the discovery of MAP allows to calculate, for the first time, the specific UAA (NNU) and UCA of each nutritional protein, in an accurate and specific manner (with an error margin lower than 1%).

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