Relationship Between Plasma Amino Acids and Composition of the Ingested Protein

II. A Shortened Procedure to Determine Plasma Amino Acid (PAA) Ratios

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IT has been the desire of nutritionists for many years to develop a direct and short procedure to determine amino acid deficiencies and, in turn, the nutritional quality of a protein. An interrelationship between the concentration changes of the plasma-free amino acids and the amino acid composition of the protein ingested has been demonstrated by an *in vivo* plasma amino acid (PAA) ratio procedure.[1] This interrelationship was suggested by earlier work from this laboratory[2] and by previous reports in the literature.[3–9]

The PAA ratios are determined by expressing the relative changes in concentrations for each of the free essential amino acids of the plasma after the ingestion of a protein meal as a function of the animals' amino acid requirements. This *in vivo* chemical score is feasible by postulating that the free essential amino acids are removed from the plasma by the body tissues at rates proportional to the amino acid requirements of the test animal. Therefore, it is logical to assume that the most limiting amino acid of a dietary protein would give the smallest positive or largest negative ratio, and that the reverse would be true for the least limiting amino acid.

For our feeding studies the general procedure followed has been to take a blood sample immediately before the test meal and at hourly intervals for five hours after its ingestion. The PAA ratio for each amino acid is calculated by subtracting the fasting level amino acid concentration from the average plasma amino acid concentration after the meal. This difference or postabsorptive change is then divided by the amino acid requirement for the test subject and multiplied by 100 to make the ratio a small whole number.

In these initial studies[1] the nutritional limiting orders obtained from the PAA ratios for the essential amino acids of wheat gluten, gelatin and casein for the dog were found to agree quite well with the limiting orders obtained by the chemical score method of Block and Mitchell.[17] By this *in vivo* procedure, lysine was found to be the most limiting amino acid for wheat gluten, tryptophan for gelatin and arginine and methionine for casein.

These experiments demonstrate that amino acid deficiencies of a protein, and proper supplementation levels to overcome these deficiencies can be evaluated by this technic. Opportunities for the quantitative evaluation of amino acids in nutrition by this procedure appear quite promising. It is believed that, with further improvements and advances, this *in vivo* technic will aid in solving a few of the many problems still facing nutritionists.

To increase the utility of this PAA ratio method,[1] modifications which reduce the time and blood volume required for each experiment have been studied and are presented here. The applicability of this procedure for human studies is demonstrated. Preliminary data are also presented which show the general

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TABLE I

Comparison of Plasma Amino Acid Ratios Determined by Long and Short Procedure*

	Methionine	Cystine†	Arginine	Lysine	Phenylalanine‡	Isoleucine	Leucine	Valine	Threonine	Histidine
Long Procedure, Experiment 1 (Part 1)										
Hr. 1	1.04	2.29	3.00	3.09	2.94	1.57	2.80	2.77	4.34	1.81
Hr. 2	1.31	2.67	3.45	2.92	3.05	1.60	2.88	2.96	5.08	1.78
Hr. 3	1.39	2.90	3.49	3.31	3.61	2.00	3.58	3.51	5.30	2.99
Hr. 4	1.12	1.98	2.97	2.90	3.40	1.74	3.16	2.97	5.59	2.77
Hr. 5	1.16	1.76	3.13	3.03	2.84	1.52	2.76	3.05	5.00	1.62
Average (1 to 5)	1.20	2.32	3.21	3.05	3.17	1.69	3.04	3.05	5.06	2.19
Fasting level	0.91	1.67	2.56	2.15	1.98	0.79	1.62	1.81	3.41	1.20
Average minus fasting level	0.29	0.65	0.65	0.90	1.19	0.90	1.42	1.24	1.65	0.99
PAA ratio§	5.7	9.2	11.4	12.7	12.9	14.5	16.7	19.4	41.3	49.5
Limiting order	...	1	2	3	4	5	6	7	8	9
Short Procedure, Experiment 1 (Part 2)										
Pooled (1 to 5)‖	1.13	2.38	3.11	2.98	3.38	1.71	3.21	2.93	4.83	2.33
Fasting level	0.91	1.67	2.56	2.15	1.98	0.79	1.62	1.81	3.41	1.20
Average minus fasting level	0.22	0.71	0.55	0.83	1.40	0.92	1.59	1.12	1.42	1.13
PAA ratio¶	4.3	10.0	9.7	11.7	15.2	14.8	18.7	17.5	35.5	56.5
Limiting order	...	2	1	3	5	4	7	6	8	9

* Dog 96 (23 kg.) fed 84.9 gm. wheat gluten, 93.8 gm. sucrose, 1.28 gm. L-lysine monohydrochloride, and 20 gm. corn oil mixed with 150 ml. H_2O. Amino acid concentrations expressed as mg. per 100 ml. plasma.

† Includes methionine.

‡ Includes tyrosine.

§ Line 8 divided by dog amino acid requirement times 100.[1] Cystine + methionine requirement equals 7.1.[15]

‖ Analysis of pooled sample containing equal volume of five hourly samples after meal.

¶ Line 3 divided by dog amino acid requirement times 100.[1] Cystine + methionine requirement equals 7.1[15]

magnitude of the experimental error encountered in determining the PAA ratios when ion exchange chromatography is employed for amino acid analyses.

EXPERIMENTAL

The general procedure previously reported[1] to determine the PAA ratios has been modified slightly. The outline for a feeding test employing the "long procedure" and the "short procedure" is illustrated by experiment 1, part 1 and experiment 1, part 2, respectively. For experiment 1 (Table I), Dog 96 (23 kg.) ate the wheat gluten test meal after an overnight fast (16 to 18 hours). Immediately before the meal, and each hour for five hours after the meal, 20 ml. of blood was drawn with a heparinized syringe via the jugular vein. Results are nearly identical if blood is drawn via the cephalic vein; however, use of the jugular vein is preferred for large blood samples. The blood samples were centrifuged promptly, and 5 ml. of the plasma for each of the six samples in experiment 1 (part 1, long procedure) was prepared for chromatographic analysis by treating with picric acid.[10] For experiment 1 (part 2, short procedure) the pooled plasma sample was obtained by combining 1 ml. of plasma from each hourly sample in a test tube which was kept in an ice bath. After the fifth-hour sample, the pooled plasma was brought to room temperature and prepared for chromatographic analysis. Keeping the plasma cold in this manner permitted the quantitative recovery of all the amino acids including cystine.

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TABLE II
Reference Amino Acid Standard for Adult Human Being

Amino Acid	Ovalbumin* / Threonine	Rose† / Threonine = 3.0	Average Reference Standard
Lysine	5.8	4.8	5.3
Methionine	3.3	...	3.3
Cystine‡	4.9	6.6	5.8
Tryptophan	1.1	1.5	1.3
Phenylalanine§	7.7	6.6	7.2
Threonine	3.0	3.0	3.0
Isoleucine	4.2	4.2	4.2
Leucine	6.4	6.6	6.5
Valine	4.8	4.8	4.8

* Triplicate chromatographic analysis. Ovalbumin obtained from Joe Lowe Corp., New York.
† Rose minimum requirements.[13]
‡ Includes methionine.
§ Includes tyrosine.

A recently developed chromatographic method of Hamilton[11] is now being employed for the amino acid analyses. This method is faster and eliminates repacking of the columns, which was necessary in the procedure previously employed.[12] An additional saving of time has been realized by modifying the photometric ninhydrin procedure of Moore and Stein.[16] After the ninhydrin color is developed and each fraction is diluted with the alcohol-water mixture, the tubes for each peak are poured into an amber Erlenmeyer flask and mixed thoroughly. A Beckman Model DU Spectrophotometer is employed to determine the optical density for each individual amino acid peak. With the use of standards it has been found that this procedure is as accurate as reading each individual fraction.

In utilizing the "long" or "short" procedures a minimum of 30 ml. and 10 ml., respectively, of plasma is required for a feeding test. If one also desired to determine tryptophan, an additional 40 per cent plasma volume is required in each case since microbiological assay is employed for this analysis.

For the human feeding tests, the method outlined for the dog studies was followed as closely as possible. All human subjects were normal, healthy, adult males. No restriction was placed on the subject's diet before the test, except that he consumed nothing but water sixteen hours prior to the experiment. For easy consumption of the test meal, the test protein (wheat gluten) and corn oil were slurried in small portions of orange juice and consumed as rapidly after mixing as possible. For each experiment a total of 150 gm. of a frozen orange juice concentrate diluted to 500 ml. was consumed. Usually, the meal was ingested in two to three minutes. Blood was obtained at the specified times by venous puncture in the cubital region, and the plasma obtained and analyzed as described for the dog. The long procedure was employed for experiments 2, 3 and 4 (Table III) and the short procedure was used for experiments 5 and 6 (Table IV). Replicate experiments with the same subject were conducted with a three- to six-week period between tests to be certain that no stress due to blood volume loss could be placed upon the subject. This precaution has been followed since it has been observed during the course of our studies (unpublished data), that a stress apparently due to blood volume loss may cause a general lowering of the PAA ratios. For all the experiments reported here, the PAA ratios for methionine alone and for methionine plus cystine are given, with the latter being used in estimating the nutritional limiting order. Both are presented since there is some question as to which method of calculation should be preferred.

RESULTS AND COMMENTS

The results of a feeding test with wheat gluten supplemented with L-lysine monohydrochloride for Dog 96 are given in Table I. In part 1 of this experiment, the long procedure employed previously[1] with certain modifications (see Experimental section) was used to determine the PAA ratios and the *in vivo* nutritional limiting order. The same values were determined in part 2 by the short procedure (see Experimental section). It can be seen that variations between the PAA ratios obtained by the two procedures are within the experimental error of the method employed for the amino acid analyses.

These findings demonstrate that the short

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TABLE III

Triplicate Human Studies with Wheat Gluten* Giving Standard Deviations for PAA Ratios

	Lysine	Methionine	Cystine†	Tryptophan	Phenylalanine‡	Threonine	Isoleucine	Leucine	Valine
Experiment 2 (Subject A)									
Hr. 1	2.48	0.75	1.88	1.32	3.23	2.30	2.12	3.57	4.29
Hr. 2	2.86	0.85	2.06	1.32	3.85	2.90	2.72	4.59	5.69
Hr. 3	2.32	0.74	1.92	1.30	3.81	2.21	2.38	4.02	5.59
Hr. 4	2.58	0.71	2.03	1.24	2.68	2.35	2.41	4.10	5.56
Hr. 5	2.00	0.51	1.70	1.24	3.03	1.95	1.77	3.01	4.89
Average (1 to 5)	2.45	0.71	1.92	1.28	3.32	2.34	2.28	3.85	5.20
Fasting level	2.46	0.57	1.40	1.20	2.25	1.52	1.15	2.00	3.00
Average minus fasting level	−0.01	0.14	0.52	0.08	1.07	0.82	1.13	1.85	2.20
PAA ratio§	−0.2	4.2	9.0	6.2	14.9	27.5	26.9	28.5	45.9
Limiting order	1	...	3	2	4	6	5	7	8
Experiment 3 (Subject A)									
Hr. 1	2.39	0.58	2.38	1.56	2.89	2.28	2.15	3.44	4.70
Hr. 2	2.43	0.63	1.59	1.76	3.29	2.33	2.30	3.86	4.87
Hr. 3	2.34	0.76	2.12	1.75	4.16	2.45	2.38	4.08	5.68
Hr. 4	1.87	0.74	1.99	1.48	2.77	2.55	2.11	3.52	4.76
Hr. 5	1.93	0.52	1.46	1.18	2.55	1.69	1.60	2.99	3.75
Average (1 to 5)	2.19	0.65	1.91	1.55	3.13	2.26	2.11	3.58	4.75
Fasting level	2.34	0.40	1.55	1.37	1.71	1.65	1.00	1.88	3.07
Average minus fasting level	−0.15	0.25	0.45	0.18	1.42	0.61	1.11	1.70	1.68
PAA ratio§	−2.8	7.6	7.8	13.9	19.7	20.3	26.4	26.2	35.0
Limiting order	1	...	2	3	4	5	7	6	8
Experiment 4 (Subject A)									
Hr. 1	2.52	0.73	2.48	1.49	3.84	2.22	2.06	3.24	3.96
Hr. 2	3.28	0.60	1.10	1.85	2.78	2.88	2.07	3.40	4.04
Hr. 3	2.38	0.73	2.28	1.79	4.70	2.27	2.24	3.69	4.52
Hr. 4	2.29	0.51	1.80	1.33	3.00	2.14	1.73	3.02	6.10
Hr. 5	1.94	0.49	1.89	1.37	3.50	2.33	1.44	3.28	4.18
Average (1 to 5)	2.48	0.61	1.91	1.57	3.56	2.37	1.91	3.33	4.70
Fasting level	2.56	0.43	1.45	1.38	2.16	1.76	1.09	1.93	3.15
Average minus fasting level	−0.08	0.18	0.46	0.19	1.40	0.61	0.82	1.37	1.55
PAA ratio§	−1.5	5.5	7.9	16.1	19.4	20.3	19.5	21.1	32.3
Limiting order	1	...	2	3	4	6	5	7	8
Average PAA ratio	−1.5	5.8	8.2	12.1	18.0	22.7	24.3	25.3	37.7
± standard deviation	±1.3	±1.7	±0.7	±5.2	±2.7	±4.4	±2.6	±3.8	±7.2
Limiting order	1	...	2	3	4	5	6	7	8
Chemical score wheat gluten‖	35.9	48.5	59.5	69.2	131.0	96.7	105.0	117.0	91.5
Limiting order	1	...	2	3	8	5	6	7	4

* Subject A (80 kg.) fed 84.9 gm. wheat gluten and 20 gm. oil slurred in 500 ml. of orange juice. Amino acid concentrations expressed as mg. per 100 ml.

† Includes methionine.

‡ Includes tyrosine.

§ Line 8 divided by amino acid reference standard (Table II) times 100.

‖ Scored against amino acid reference standard (Table II). Amino acid and protein content of wheat gluten previously given.[1]

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TABLE IV
Variation of PAA Ratios for Human Subjects A* and B*

Experiment No.	Lysine	Methionine	Cystine†	Tryptophan	Phenylalanine‡	Threonine	Isoleucine	Leucine	Valine
				PAA Ratio§					
5‖ (Subject B).	−1.1	9.4	13.8	40.2	33.8	40.0	31.9	35.2	47.6
6‖ (Subject B).	2.3	13.6	15.0	29.4	35.0	45.7	33.8	35.1	49.8
5 and 6.....	0.6	11.5	14.4	34.8	34.4	42.9	32.9	35.2	48.7
Average ± standard deviation...	±2.2	±3.0	±0.9	±7.6	±0.9	±4.0	±1.3	±0.2	±1.6
2, 3 and 4¶ (Subject A).	−1.5	5.8	8.2	12.1	18.0	22.7	24.3	25.3	37.7
Average ± standard deviation...	±1.3	±1.7	±0.7	±5.2	±2.7	±4.4	±2.6	±3.8	±7.2
Weighted standard deviation 2 through 6...	±1.5	±2.3	±0.8	±6.3	±2.2	±4.1	±2.2	±2.9	±5.7

* Both subjects weighed 80 kg. and consumed 84.9 gm. wheat gluten and 20 gm. corn oil slurried in 500 ml. of orange juice.
† Includes methionine.
‡ Includes tyrosine.
§ Calculated as shown in Table III.
‖ Short procedure employed.
¶ Data from Table III.

procedure can be utilized to determine PAA ratios with a 60 to 70 per cent saving in time and blood volume required for each test. The use of this short method will also reduce the possibility of stress due to blood volume loss being placed upon the subject. Development of this method should permit its use on small animals. Preliminary tests on individual animals or groups of animals would be necessary to establish the proper timing for taking the blood samples after the meal. Having established this, the short procedure could be employed to conduct the complete test.

Triplicate human feeding studies with wheat gluten (experiments 2, 3 and 4) were conducted to determine whether (1) the PAA ratio procedure could be utilized for human studies and (2) the PAA ratios for the same subject and protein were reproducible. For the human experiments an amino acid "reference standard" was obtained by averaging the amino acid pattern for ovalbumin and the minimum amino acid requirements determined by Rose[13] with threonine set at 3.0 (Table II). Using this reference standard for the human studies, the PAA ratios were calculated as reported for the dog.[1]

A tabulation of all the data for these three experiments is shown in Table III. A striking similarity between the plasma amino acid changes after the meal and the PAA ratios for the three experiments is shown. Two-way analyses of variance[14] and calculated 95 per cent confidence limits of the experimental data presented here revealed that the general nutritional limiting order determined from the PAA ratios is statistically sound. Of greatest interest are the most limiting amino acids, and analyses reveal that statistical significance can be given to the PAA ratios for these amino acids. These findings parallel those found for the dog[1] and show the general reproducibility of the PAA ratios and the usefulness of this method for human studies.

Two striking differences are noted between the nutritional limiting order obtained from

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the PAA ratios for experiments 2, 3 and 4 and the chemical score of wheat gluten. From the nutritional limiting order, phenylalanine appears to be more limiting and valine less limiting than indicated by the chemical score. These findings probably reflect differences in availability and rate of absorption.

To compare the variation of the PAA ratios for different subjects with that obtained for the same subject, experiments 5 and 6 were conducted with subject B. Both human subjects (A and B) weighed 80 kg. and were fed the identical wheat gluten test meals. The average PAA ratios with the standard deviations are given in Table IV for the triplicate tests with subject A (experiment 2, 3 and 4) and for the duplicate tests with subject B (experiment 5 and 6). Also given is the weighted standard deviation for each amino acid obtained by pooling the results for the five individual experiments. The variability in the PAA ratios for each amino acid for different subjects is similar even though the ratios may be different.

Comparison of the average PAA ratios for the two subjects shows the ratios to be generally higher for subject B than subject A. In particular statistical significance at the 95 per cent level can be given to the differences in the ratios for the two subjects for tryptophan, phenylalanine plus tyrosine, threonine, isoleucine and leucine. It appears also that significance should be given to the differences in the ratios for the two subjects for cystine plus methionine. However, since the standard deviation found for this amino acid pair is unusually low compared to that found for the other amino acids, reservation will be exercised in claiming significance in this case until additional studies can confirm or reject this standard deviation value. Variations of this nature in the PAA ratios that appear for normal subjects may reflect differences in protein utilization and/or amino acid requirements and/or protein nutritional status. Utilization of this technic with subjects under stress, e.g., illness, malnutrition, may serve to uncover relationships between protein and amino acid metabolism under certain particular stress conditions.

SUMMARY

A previous report[1] presented an *in vivo* plasma amino acid (PAA) ratio technic to evaluate the amino acid adequacy in a food protein for the nourishment of the dog. A modification of the original method is presented which reduces by approximately 60 per cent the time and amount of blood required for each experiment. The applicability of this procedure for human studies is demonstrated. The reproducibility of the PAA ratios is shown with the variation from subject to subject being significantly greater for some amino acids than the variation for the same subject. These findings may reflect individual differences in protein utilization and/or amino acid requirements and/or protein nutritional status. Experimentation with subjects under stress, e.g., illness, malnutrition, should be quite promising.

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