Assessment of Factors Influencing Estimation of Availability of Threonine, Isoleucine, and Valine in Cereal Products

H. J. H. DE MUELENAERE,[1] M-L. CHEN,[2] AND A. E. HARPER

The availability of threonine, isoleucine, and valine in corn and rice was determined. Measurements of threonine availability were influenced by changes in the composition of the basal diet and in the amounts of protein and calories in the experimental diets. An interaction between the calorie and protein levels of the diet in relation to measurements of growth was eliminated by calculating availability values from a standard curve based on amount of amino acid consumed instead of amino acid content of the diet. The low availability values for isoleucine in zein and corn gluten, when assessed by the growth method, were found to be mainly due to leucine-isoleucine antagonism in the experimental diet. Elimination of the antagonism resulted in higher availability values. Although a considerable amount of valine was excreted in the feces of rats fed zein, the values for availability assessed by the growth method increased when the leucine content of the experimental diet was decreased.

Factors influencing the measurement of availability of lysine in corn and rice and the general background of the problem have been discussed (*4*). The present paper is a report on factors that influence measurement of the availability of threonine, isoleucine, and valine in these cereals.

Few values have been published for the availability of threonine, isoleucine, and valine in cereal products and those reported do not agree well. Linkswiler, Fox, and Crooke-Fry (*10*) and de Muelenaere and Feldman (*6*) reported high availability of isoleucine in maize for both man and rat. Deshpande *et al.* (*7*) on the other hand found that only 30% of isoleucine in zein was available to the rat. Geiger, Courtney, and Geiger (*8*) reported poor availability of valine in zein, while Linkswiler *et al.* (*11*) found it to be highly available in maize for man.

Materials and Methods

The samples and basal diet, care and feeding of animals, and methods for determining water and nitrogen content of carcasses have been described (*4*). For this study 1.36% of L-lysine was added to the lysine-deficient basal diet (*3*). Threonine, isoleucine, and valine contents of corn gluten, reconstituted corn gluten, rice, rice protein, and zein were determined by microbiological assay using *Leuconostoc mesenteroides*, except for threonine which was assayed with *Streptococcus faecalis* (Table I).

Availability was estimated by the growth method from changes in weight, empty weight, water content, and nitrogen content of the carcasses (*4*). Unless indicated otherwise, availability values were calculated using a standard curve in which weight gain was plotted against the amount of amino acid consumed.

Availability determination by fecal analysis has been described (*4*).

Experimental and Results

The observations from which availability values were calculated are presented in Tables II, III, and IV.

Availability of Threonine. The availability of threonine in corn gluten, reconstituted corn gluten, rice, rice protein, and zein as determined by growth and fecal analysis methods, and the true digestibilities are given in Table V. The availability values listed for the growth method are over-all means for four criteria. Two availability values determined independently of each other with two different basal diets are listed.

Values for availability of threonine in maize products as determined by the growth method are considerably lower than those for rice. The composition of the basal ration had a marked effect on availability, in that lower values were obtained with ration B. If this were due to a greater degree of amino acid imbalance or to the larger amount of protein source used in experiment B,

Table I. Amino Acid Content of Products Studied (G./100 g.)

	Lysine	Threonine	Valine	Isoleucine
Corn gluten	0.880	1.798	2.540	2.181
Reconstituted corn gluten	0.880	1.771	2.599	2.188
Zein	...	3.640	4.330	5.070
Rice	0.245	0.231	0.473	0.319
Rice protein	2.773	3.000	...	2.853

Department of Biochemistry, University of Wisconsin, Madison, Wis., and Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass.

[1] Present address, Department of Biochemistry, Natal Agricultural Research Institute, Pietermaritzburg, Natal, South Africa.

[2] Present address, Department of Biochemistry, National Defense Medical Center, Taipei, Taiwan (Formosa), China.

Table II. Performance of Rats Used in Threonine Availability Study

(14-day experimental period)

Experimental Diet	Food Consumed, G./Rat	Change in Weight, G./Rat	Change in Empty Weight, G./Rat	Nitrogen Content, Mg./Rat	Water Content, G./Rat	Threonine Content, Mg./Rat
Basal A						
+0.0% L-threonine	34.0	−13.2	−17.8	1078	26.6	240
+0.1	43.9	−8.6	−12.7	1175	29.7	273
+0.2	59.2	1.0	−3.3	1440	36.0	856
+0.3	96.1	25.4	18.6	1853	50.4	479
+0.4	140.9	57.2	47.8	2456	70.8	626
+0.5	144.7	70.2	60.14	2700	80.2	730
+10% corn gluten	43.3	−5.2	−13.8	1218	29.4	317
+10% reconstituted corn gluten	42.9	−8.0	−11.9	1228	30.6	310
+70% rice	52.4	−3.6	−7.7	1302	33.4	344
Basal B						
+0.0% L-threonine	102.1	26.8	19.4	1802	51.0	496
+0.05	142.8	57.2	48.6	2445	68.5	572
+0.10	161.6	67.2	58.3	2620	75.5	658
+0.15	158.1	65.8	59.8	2665	77.8	746
+5% corn gluten	137.5	50.8	43.4	2330	66.9	553
+35% rice	148.6	57.4	51.9	2570	71.7	643

Table III. Performance of Rats Used in Isoleucine Availability Study

(14-day experimental period)

Experimental Diet	Food Consumed, G./Rat	Change in Weight, G./Rat	Change in Empty Weight, G./Rat	Nitrogen Content, Mg./Rat	Water Content, G./Rat	Isoleucine Content, Mg./Rat
Basal						
+0.0% DL-isoleucine	34.6	−18.0	−19.2	975	23.9	288
+0.2	42.8	−12.8	−15.0	...	27.0	...
+0.4	50.6	−1.8	−5.6	1290	33.4	333
+0.6	78.3	17.8	11.1	1660	45.0	473
+0.8	128.1	51.6	43.3	2274	66.7	643
+1.0	141.6	65.8	56.4	2614	75.4	810
+5% zein	44.6	−10.6	−13.0	1194	29.0	327
Minus ½ leucine + 5% zein	43.0	−9.2	12.3	1170	29.6	356
Minus leucine						
+5% zein	42.3	−4.4	−9.6	1240	31.1	360
+10% corn gluten	42.9	−9.2	−13.9	1120	27.9	355
Minus leucine						
+10% corn gluten	52.8	−6.0	−11.7	...	...	...
+70% rice	58.9	−3.0	−5.0	1200	34.1	418
+7.5% rice protein	48.7	−5.6	−8.5	...	32.1	...

the opposite would be expected. Calhoun *et al.* (*3*) reported that the availability of lysine in wheat products determined by the growth method, using a basal ration containing amino acids as the sole nitrogen source, was consistently higher than when a basal ration containing wheat gluten supplemented with amino acids was used. They attributed the difference to higher fecal excretion of lysine by rats fed the basal ration containing wheat gluten. In the present study the amounts of threonine excreted by rats fed ration B are also higher than the amounts excreted by those fed ration A. The higher availability values obtained with basal ration A could also be due to growth stimulation from the addition of the test protein to a basal ration containing exclusively amino acids. Recent studies (*14*) which resulted in substantial improvements in the basal amino acid diet sug-

Table IV. Performance of Rats Used in Valine Availability Study
(14-day experimental period)

Experimental Diet	Food Consumed, G./Rat	Change in Weight, G./Rat	Change in Empty Weight, G./Rat	Nitrogen Content, G./Rat
Basal				
+0.0% L-valine	34.5	−15.0	−18.1	1028
+0.1	43.8	−10.0	−12.5	1125
+0.2	54.3	−1.4	−4.5	1342
+0.4	107.0	41.8	35.3	2091
+0.6	138.7	70.6	64.6	2684
+0.7	149.2	75.0	66.6	2856
+70% rice	66.4	6.8	3.0	1637
+10% corn gluten	51.1	−8.4	−7.0	1280
+10% reconstituted corn gluten	41.4	−8.3	−10.7	1178
+5% zein	44.5	−11.0	−12.8	1085
Minus leucine + 5% zein	45.7	−8.4	−10.2	1126

Table V. Availability of Threonine as Determined by Growth and Fecal Analysis Methods

Protein Source in Test Diet	Availability, %				Digestibility of Protein (True), %
	Growth Method		Fecal Analysis		
	A[a]	B[b]	A[a]	B[b]	
10% corn gluten	61.8	...	99.4	...	98.8
5% corn gluten	...	45.3	...	87.5	...
10% reconstituted corn gluten	64.8	...	93.5	...	97.0
70% rice	93.7	...	100.0	...	102.1
35% rice	...	85.4	...	94.3	...
5% zein	52.2[c]	...	...	...	...
5% zein hydrolyzate	70.0[c]	...	...	...	...

[a] Basal diet A contained complete amino acid mixture except threonine.
[b] Basal diet B contained 10% wheat gluten, 5% gelatin, 0.3% L-histidine, 0.8% L-lysine·HCl, 0.15% DL-tryptophan, 0.1% DL-phenylalanine, and 0.6% DL-valine.
[c] Empty weight gain used as criterion.

gest that this probably is so; nevertheless comparisons based on results obtained with the diet used in this study should be valid.

The high availabilities obtained by the fecal analysis method indicate that although the threonine of corn gluten is well absorbed from the digestive tract, it is not fully utilized by the animal body. This is in agreement with earlier reports by Geiger *et al.* (*8*) and de Muelenaere *et al.* (*5*).

Availability values for threonine in corn gluten and reconstituted corn gluten by the fecal analysis method correspond well with the true digestibilities of the respective proteins. Threonine availability as determined by growth is considerably higher for the zein hydrolyzate than for zein itself. The high availability of threonine in corn gluten by fecal analysis suggests that threonine is liberated and absorbed in the digestive tract at a rate slower than that required for optimum utilization for protein synthesis. This is in agreement with the suggestion made by Geiger *et al.* (*8*) that certain threonine-containing peptides are enzyme-resistant. The fact that only 70% of the threonine in the acid hydrolyzate of zein is available must be attributed to growth-influencing factors other than the limiting amino acid.

The high value for availability of threonine in rice deserves comment. From growth studies and calculations based on the generally accepted amino acid requirements of the rat, threonine of rice appeared to be poorly available (*9*, *13*). Subsequent calculations based on more recent studies of rice (*16*) and of the amino acid requirements of the rat (*15*) suggest that, as found in the present study, threonine in rice is highly available.

The effects of threonine and zein content of the diet on availability as determined by gain in empty weight are illustrated in Table VI. Zein was added to four basal diets that differed in L-threonine content. No consistent effect on availability of threonine was noted in determinations at different points on the standard curve, but the value for availability decreased with increasing amounts of zein in the diet.

Table VI. Per Cent Availability of Threonine[a] in Zein

(Determined by growth method at different zein contents of experimental diet and at different heights of standard growth curve)

Zein in Diet, %	Threonine in Diet, %	Free Threonine Supplement in Experimental Diet			
		0.0 %	0.1 %	0.2 %	0.3 %
2.5	0.09	49.3	49.4	54.9	46.1
5.0	0.18	52.2	41.2	...	...
7.5	0.27	33.2	32.9	27.4	...
10.0	0.36	...	26.4	...	...

[a] Availability values calculated from empty weight gains.

In Table VII are given the analyses of variance of weight gain, weight gain per 100 grams of diet consumed, and weight gain per gram of threonine consumed for an experiment designed to test the effect of calorie level and interaction between calorie and zein content in the experimental diet. The experimental design was that of a randomized block with four blocks. Each block consisted of nine rats, each of which was fed one of nine rations obtained by the combination of three levels of zein (2.5, 5.0, and 7.5%) and three levels of extra corn oil (4, 8, and 12%). The basal diet was identical with that described, except for the amino acid mixture. A diet containing 5% of casein, supplemented with free amino acids to attain total amino acid levels identical to those of the previously described basal diet, was used. The amino acid content of casein was determined by column chromatography (*12*). Increasing the calorie content of the diet depressed growth significantly. This growth depression is also evident when weight gain is expressed per 100 grams of food consumed or per gram of threonine consumed. A significant interaction exists between the zein and calorie levels of the diet when weight gain is used as the criterion. The interaction is eliminated by using either weight gain per 100 grams of food or weight gain per gram of threonine as the criterion of performance.

Availability of Isoleucine and Valine. In Table VIII are given the availability values for isoleucine in cereal products. The availability values listed for the growth methods are the means obtained by using four criteria of growth as described earlier and calculated from a standard curve in which gain was plotted against isoleucine intake.

To test the effect of leucine-isoleucine antagonism on the measurement of availability (*1*) of isoleucine in zein, three different experimental diets were prepared. Al

Table VII. Effect of Increasing Levels of Oil and Zein on Growth of Rats

Level of Zein, %	Level of Oil				Analysis of Variance			
	4%	8%	12%	$\bar{X}$	Source	D.F.	M.S.	F
	Empty Weight Gain, G.							
					Blocks	3	148.0	
2.5	16.5	11.2	8.8	12.2	Oil	2	856.7	30.9[a]
5.0	32.4	19.4	13.2	21.7	Zein	2	1304.4	47.1[a]
7.5	46.7	26.4	25.9	33.0	Oil × zein	4	77.4	2.8[b]
$\bar{X}$	31.9	19.0	16.0		Error	24	27.6	
L.S.D. of means: within table, 10.3 (4 observations); for oil and zein levels, 5.9 (12 observations)								
	Empty Weight Gain, G./ 100 G. Food Consumed							
					Blocks	3	50.6	
2.5	15.9	12.5	10.3	12.9	Oil	2	623.5	16.5[a]
5.0	28.5	17.7	14.5	20.2	Zein	2	245.0	42.0[a]
7.5	31.3	25.8	24.9	27.4	Oil × zein	4	24.7	1.6
$\bar{X}$	25.3	18.7	16.6		Error	24	14.8	
L.S.D. of means: within table, 7.5 (4 observations); for oil and zein levels, 4.4 (12 observations)								
	Empty Weight Gain, G./G. Threonine Consumed							
					Blocks	3	726.0	
2.5	68.7	53.9	44.5	55.7	Oil	2	3267.6	15.3[a]
5.2	100.0	65.1	48.5	71.2	Zein	2	1772.0	8.3[c]
7.5	92.8	72.4	73.3	79.5	Oil × zein	4	312.0	1.4
					Error	24	213.8	
$\bar{X}$	87.2	63.8	55.5					
L.S.D. of means: within table, 28.1 (4 observations); for oil and zein, 16.5 (12 observations)								

[a] Statistically significant at 0.1% level ($P \leq 0.001$).
[b] Statistically significant at 1% level ($P \leq 0.01$).
[c] Statistically significant at 5% level ($P \leq 0.05$).

Table VIII. Availability of Isoleucine and Digestibility of Test Proteins

Changes in Basal Amino Acid Diet	Availability, %			
	Growth Method		Fecal analysis	Digestibility
+5% zein	51.9[a]	(47.9)[b]	84.6	
Minus ½ leucine +5% zein	56.8	(55.2)[b]	86.5	
Minus leucine +5% zein	79.3[a]	(74.7)[b]	94.9	
+10% corn gluten	53.5	(53.1)[b]	79.8	98.8
Minus leucine +10% corn gluten + 1.25% leucine		(74.5)[b]		
+0.45% isoleucine		(84.0)[b]		
+1.25% leucine (total) +0.45% isoleucine		(100.0)[b]		
+70% rice	81.1	(87.8)[b]	102.1	102.1
+7.5% rice protein	86.0	(86.5)	93.7	96.1
+5% acid hydrolyzate of zein		(47.0)		

[a] Ten rats used for experimental group. [b] Values derived from empty weight gain.

contained 5% of zein added to the basal diet; in the second and third either half or all the leucine present in the basal amino acid mixture was omitted. A similar procedure was followed for isoleucine availability in corn gluten.

Isoleucine of rice and rice protein is available to a greater extent than that of the maize products. Availability values for isoleucine in corn gluten and zein assessed by the growth method are considerably lower than values obtained by the fecal analysis method. By reducing leucine in the basal amino acid mixture, the value for availability is substantially increased, indicating that the low values can be attributed to growth retardation resulting from leucine-isoleucine antagonism. An increase in isoleucine availability from 51.9% to 79.3% (47.9 and 74.7% using empty weight gain as the criterion) as a result of omitting leucine from the basal amino acid mixture represents a difference in weight gain of only 3.4 grams. Using 10 rats per group (1 and 3), this difference was highly significant. The availability of isoleucine in corn gluten was increased from 53.1% to 74.5% (groups 4 and 5) by omission of leucine from the basal diet. When free leucine and isoleucine, in the amounts present in 5% of zein, were added to basal diets which contained leucine or no leucine, weight gains and, hence, values for availability were significantly higher if leucine was omitted from the basal amino acid mixture. The observation that the availability of isoleucine in zein and in an acid hydrolyzate of zein was identical is additional evidence that the low value for availability of this amino acid is the result of a leucine-isoleucine antagonism. Availability as measured by the fecal analysis method also increased as a result of the omission of leucine from the basal amino acid mix.

In Table IX are given values for the availability of valine. Valine in rice is more highly available than in maize products, as indicated by both the growth and fecal analysis methods. The high leucine content of the diet affected the measurement of the availability of valine in zein. Benton *et al.* (*2*) observed that a high level of leucine in a 9% casein diet affected both isoleucine and valine utilization. The low availabilities obtained by the fecal analysis method are in agreement with observations by Geiger *et al.* (*8*) that a considerable amount of valine is excreted in feces of rats fed zein diets.

Table IX. Availability of Valine in Cereal Products and Digestibilities of Test Proteins

Changes in Basal Amino Acid Diet	Availability, %		
	Growth method	Fecal analysis method	Digestibility
+5% zein	37.1	62.3	...
Minus leucine +5% leucine	57.4	64.3	...
+10% corn gluten	69.4	84.4	98.8
+10% reconstituted corn gluten	55.1	69.1	97.0
+70% rice	86.4	94.2	102.1

Discussion

Threonine, isoleucine, and valine are in general less available in corn products than in rice products. The present study, like the previous one (*4*), indicates that values for the availability of amino acids from a single product may differ considerably, depending on the method of measurement used.

Measurement of availability of threonine is affected by changes in the protein (Table VI) or in the calorie con-

tent of the diet (Table VII), as is that of lysine (*4*). Calculation of values on the basis of threonine intake rather than dietary level eliminated the interaction between calorie and protein level, but the calorie effect persisted. Such effects would be detected most readily by a slope ratio analysis with statistical tests for deviations from parallelism and linearity, and calculation of the fiducial limits of the estimated potency. A three-dose-level test would increase the number of experimental animals required for availability determinations, but would provide a reliable method for detecting protein and calorie effects.

The higher availability values obtained by the fecal analysis method than by the growth method for threonine, isoleucine, and valine may be partly the result of a slow rate of release of the amino acids in the intestinal tract. The loss of this information is a limitation of the fecal analysis method. There would also appear to be some discrepancy between true digestibility values and values for lysine (*4*) and threonine availability as measured by the fecal analysis method. Lysine is entirely in the soluble, highly digestible fraction of corn gluten; yet availability values below those for true digestibility were obtained (*4*). On the other hand, threonine, which is partly in the zein fraction and about which there is independent evidence of lower availability, was found to be highly available by the fecal analysis method. Synthesis of an amino acid by microorganisms in the intestinal tract would result in high recovery of the amino acid in the feces and hence in a low value for availability; destruction of an amino acid by intestinal microorganisms would have the opposite effect.

The significant increases in weight gain with resultant higher values for availability of isoleucine and valine in zein and corn gluten, as a result of alleviation of leucine-isoleucine and valine antagonism, illustrate that such antagonisms must be avoided in experimental diets in order to obtain valid availability values by the growth method.

Effects of amino acid imbalance, antagonism, carbohydrate source, and protein and calorie level must be eliminated if quantitative values for availability are to be obtained with the growth method. Even with such precautions availability values determined by the growth method will probably be only semiquantitative. High, moderate, and low availability can be recognized, but the inherent variability of the animal assay as commonly used is such that values differing by as much as 15 to 20% are seldom statistically significantly different. A multiple-dose-level test with larger groups of animals should improve quantification but as a routine practical assay would be time-consuming and expensive.

The term "availability" is perhaps best defined as that portion of an amino acid present in a protein which is used for growth, development, and maintenance of an animal in so far as it is dependent on the digestibility of the protein; presence of enzyme-resistant peptide linkages; enzyme-inhibiting substances; and rate of release of the amino acid in the intestinal tract. Although it may be debated whether the effect of an amino acid imbalance or antagonism in a protein should be included in the measurement of availability, such effects are not likely to be encountered with practical diets that contain mixtures of proteins but are more likely to be artifacts of the growth method for determining availability. It therefore seems important to separate them as completely as possible in availability measurements.

Literature Cited

(1) Benton, D. A., Harper, A. E., Elvehjem, C. A., *Arch. Biochem. Biophys.* **57,** 13 (1955).
(2) Benton, D. A., Harper, A. E., Spivey, H. E., Elvehjem, C. A., *Ibid.*, **60,** 147 (1956).
(3) Calhoun, W. K., Hepburn, F. N., Bradley, W. B., *J. Nutr.* **70,** 337 (1960).
(4) de Muelenaere, H. J. H., Chen, M-L., Harper, A. E., J. AGR. FOOD CHEM. **15,** 310 (1967).
(5) de Muelenaere, H. J. H., Chen, M-L., Harper, A. E., *J. Nutr.* **74,** 125 (1961).
(6) de Muelenaere, H. J. H., Feldman, R., *Ibid.*, **72,** 447 (1960).
(7) Deshpande, P. D., Harper, A. E., Collins, M., Elvehjem, C. A., *Arch. Biochem. Biophys.* **67,** 341 (1957).
(8) Geiger, E., Courtney, G. N., Geiger, L. E., *Ibid.*, **41,** 74 (1952).
(9) Harper, A. E., Winje, M. E., Benton, D. A., Elvehjem, C. A., *J. Nutr.* **56,** 187 (1955).
(10) Linkswiler, H., Fox, H. M., Crooke-Fry, P., *Ibid.*, **72,** 447 (1960).
(11) Linkswiler, H., Fox, H. M., Geschwender, D., Crooke-Fry, P., *Ibid.*, **65,** 455 (1958).
(12) Moore, S., Spackman, D. H., Stein, W. H., *Anal. Chem.* **30,** 1185 (1958).
(13) Pecora, L. J., Hundley, J. M., *J. Nutr.* **44,** 101 (1951).
(14) Rogers, Q. R., Harper, A. E., *Federation Proc.* **23,** 186 (1964).
(15) Rao, P. B. R., Metta, V. C., Johnson, B. C., *J. Nutr.* **69,** 387 (1959).
(16) Rosenberg, H. R., Culik, R., Eckert, R. E., *Ibid.*, **69,** 217 (1959).

Received for review March 26, 1965. Accepted December 15, 1966. Work supported in part by a grant from the National Livestock and Meat Board, Chicago, Ill.