PROTEIN QUALITY IN HUMANS:

ASSESSMENT AND IN VITRO ESTIMATION

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Protein Quality in Relation to Estimates of Essential Amino Acids Requirements

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According to the FAO/WHO Expert Committee on Energy and Protein Requirements convened in 1971 (FAO/WHO, 1973) the quality of a protein can be estimated from its essential amino acid composition and the safe protein intake at any age with the aid of a scoring pattern derived from amino acid requirement values. Since the requirements for total nitrogen and essential amino acids decline with age (Cohn *et al.*, 1976; Waterlow and Stephen, 1967), the "quality" of a protein must also be related to the age of the subject who consumes it (Arroyave, 1975a; 1975b). This emphasizes the need for precise estimates of amino acid requirements at different ages. Until now, experimental data on amino acid requirements existed only for infants, 10–12 year-old children and adults. No experimental data were available for children of preschool age.

For infants, the data of Holt and Snyderman (1967) and of Fomon and Filer (1967), who used synthetic diets or a variety of infant formulas, provided information on the amounts of essential amino acids required to maintain expected growth rates and nitrogen retentions. The FAO/WHO Committee decided to combine the lower values in the two sets of data to estimate the amino acid requirements for infants under 6 months of age. From these data, an amino acid scoring pattern was calculated in terms of mg of amino acid per g of protein. A protein with an equivalent amino acid composition to that of the scoring pattern would satisfy the infant's amino acid needs if ingested at a "safe level of intake" of 2 g/kg/day (FAO/WHO, 1973).

For children 10–12 years of age, the essential amino acid requirements used are those proposed by Nakagawa *et al.* (1960, 1961a, 1961b, 1962, 1963). These values were derived from nitrogen balance data and were based on the lowest intake required to produce a positive nitrogen balance in all subjects studied (3 to 4 children per amino acid). The children ate mixtures of pure essential amino acids with glycine or mixtures of non-essential amino acids as the source for non-essential nitrogen. However, there is some uncertainty about whether to consider the proposed figures as requirement values since the intake increments used in the studies were large and, therefore, the levels which gave positive nitrogen balances were not necessarily the lowest possible.

Information on amino acid requirements for adults is available from several sources. Rose (1957) determined the amounts necessary to maintain positive nitrogen balance, using diets composed of pure essential amino acids with glycine or urea as non-essential N sources. Since the variability among subjects was large, the level selected was that of the man with the highest requirement. Leverton's studies with young women (1959) used as the discriminating criterion the attainment of nitrogen equilibrium by all subjects. Using the data from women available until 1963, Hegsted (1968) published values derived by regression analysis. Although these figures have been used extensively, the FAO/WHO Committee (1973) suggested figures which were lower than Rose's but higher than Hegsted's.

In summary, experimental data related to essential amino acid requirements were available only for certain age groups. Furthermore, the criteria used in the interpretation of these data were based only on nitrogen balance studies or on adequate weight gain of infants. There was no information from studies in which other indicators were evaluated simultaneously to attain a more precise estimate of amino acid requirements.

The FAO/WHO Expert Committee concluded that a standard amino acid scoring pattern could be established to assess a protein's nutritive quality relative to the pattern. It was accepted that breast milk was the appropriate food for infants; and, therefore, their amino acid requirements "should be excluded from the application of any tentative guide to protein scoring that might be developed for older children and adults" (FAO/WHO, 1973). The Committee also agreed that any such reference pattern should satisfy the needs of preschool children since a protein that was adequate for them would also be adequate for adults, whereas the reverse might not be true. With these points in mind, a provisional reference amino acid scoring pattern was devised for application to all ages beyond infancy, based on *theoretical estimates* of the amino acid requirements of preschool children which were derived from the information available for other age groups and a safe level of protein intake of 1.2 g/kg/day. It is important to emphasize that, until now, there was no direct experimental evidence on the amino acid requirements of young growing children beyond infancy (i.e., children of preschool age).

Since the provisional pattern is still used without change, it is important to establish whether the estimates of amino acid requirements used to recommend that pattern are adequate. The similarity of the provisional pattern with that recommended to fulfill infants' requirements (FAO/WHO, 1973) suggests that the needs for certain essential amino acids may be overestimated in the former. If this were the case, the result would be an underestimation of protein quality based on the reference amino acid scoring pattern.

MEASUREMENT OF AMINO ACID REQUIREMENTS FOR CHILDREN OF PRESCHOOL AGE

Investigations were carried out at the Institute of Nutrition of Central America and Panama (INCAP), designed to provide *experimental* information on essential amino acid requirements for 2-year old children using several criteria of evaluation. We studied a total of 42 children ranging in age from 21 to 27 months. These children had been malnourished but they had fully recovered at least one month prior to the beginning of the investigation. During that time they were healthy and growing at adequate rates. At the beginning of the studies, their weight was over 95% of that expected for their height, their creatinine height index was above 0.90 (Viteri and Alvarado, 1970) and they had normal serum total proteins, albumin and amino acid patterns and normal hematologic indices. During the course of the study, the children were maintained under close medical supervision to ensure that their health and growth were not impaired.

Throughout the study, all children received dietary nitrogen (N) in amounts equivalent to 1.2 g of protein/kg/day. The diets consisted of a core of 0.3 g of cow's milk protein per kg per day plus a mixture of purified amino acids in the same proportions and amounts found in 0.9 g of milk protein/kg/day. Table 3.1 shows the amino acid contents of this semi-synthetic diet.

TABLE 3.1. CALCULATIONS FOR THE PREPARATION OF THE EXPERIMENTAL DIETS

			Amino acid composition of the basal semi-synthetic diet	
Amino acid	mg/g of milk protein[1]	mg/1.2 g of milk protein	Milk core: mg/0.3 g of milk protein	+ mg of purified amino acids equivalent to 0.9 g milk protein
Isoleucine	47	56	14	42
Leucine	95	114	28	86
Lysine	78	94	23	71
Total sulfur a.a.[2]	33	40	10	30
Methionine	25	30	8	22
Cystine	8	10	2	8
Total aromatic a.a.[3]	102	122	31	91
Phenylalanine	54	65	16	49
Tyrosine	48	58	14	44
Threonine	44	53	13	40
Tryptophan	14	17	4	13
Valine	58	70	17	53
Histidine	27	32	8	24
Alanine	35	42	10	32
Aspartic Acid	77	92	23	69
Glutamic Acid	222	266	67	199
Glycine	20	24	6	18
Proline	91	109	27	82
Serine	58	70	17	53

[1]FAO/WHO (1973) and FAO (1970).
[2]Met/Cys = 3.05
[3]Phe/Tyr = 1.13

The composition of the basal diet is that shown in the last two columns. The essential amino acid under study was partially replaced by glycine in order to provide the essential amino acid at five different levels of intake. These levels varied between "maximum" (equivalent to that in 1.2 g of milk protein/kg/day) and "core" (equivalent to that in 0.3 g of milk protein/kg/day). For example, in the case of lysine, the following levels were provided by the semi-synthetic diets:

Milk	Lysine	Total Lysine
1) Core	+ 71 mg	= 94 mg, equivalent to that in 1.2 g milk protein/kg/day
2) Core	+ 43 mg	= 66 mg, equivalent to that in 0.85 g milk protein/kg/day
3) Core	+ 30 mg	= 53 mg, equivalent to that in 0.68 g milk protein/kg/day
4) Core	+ 16 mg	= 39 mg, equivalent to that in 0.50 g milk protein/kg/day
5) Core	+ 0 mg	= 23 mg, equivalent to that in 0.30 g milk protein/kg/day

A similar procedure was followed with five other essential amino acids. Table 3.2 shows the amounts of each amino acid fed at the five levels of intake. The amount of total nitrogen was kept constant with changes in the dietary glycine using isonitrogenous equivalents as shown in Table 3.3.

All diets provided 100 kcal/kg/day with 30% of the energy from vegetable oil. They were complemented with vitamins, minerals, and electrolytes to satisfy the children's needs. The diets were fed as 5 identical meals per day.

TABLE 3.2. ESSENTIAL AMINO ACID INTAKES FOR EACH AMINO ACID UNDER STUDY

Amino acid	mg of amino acid per g of milk protein	Amino acid intake levels, mg/kg/day[1] A	B	C	D	E
Isoleucine	47	14	24	32	40	56
Lysine	78	23	39	53	66	94
Methionine + Cystine	33	10	16	22	28	40
Threonine	44	13	22	30	37	53
Tryptophan	14	4	7	10	13	17
Valine	58	17	29	39	49	70

[1] Equivalent to the amino acid contents of 0.3 (A), 0.5 (B), 0.68 (C), 0.85 (D) and 1.20 (E) g of milk protein/kg/day.

TABLE 3.3. ISONITROGENOUS EQUIVALENTS OF GLYCINE TO COMPENSATE FOR CHANGES IN THE DIETARY ESSENTIAL AMINO ACIDS

Amino acid	Nitrogen content, %	mg of glycine used to substitute for each mg of the essential amino acid
Isoleucine	10.68	0.57
Lysine	19.16	1.03
Methionine	9.39	0.50
Cystine	11.66	0.62
Threonine	11.76	0.63
Tryptophan	13.72	0.74
Valine	11.96	0.64
Glycine	18.66	1.00

Experimental Periods and Sequences

The requirements for total sulfur amino acids, isoleucine, lysine threonine, tryptophan, and valine were studied, each with six children. In the investigation of each amino acid, three children followed a "descending" and three an "ascending" experimental design as described below. Prior to the beginning of the study, all the children ate a milk-based diet which provided between 2 and 3 grams of milk protein and 100 kcal per kg/day.

Descending Design

The children received a milk formula which provided 1.2 g protein/kg/day for nine days. They were then progressively adapted to the milk plus amino acid diet (basal diet) described above. This adaptation period lasted 6 days during which the milk diet was gradually replaced by the basal diet; thus, by the 6th day, the children ate exclusively the basal diet. The children remained with this diet for 9 days. During the last 4 days of the 9-day period, excreta were collected for analyses of nitrogen and determination of nitrogen balance and for measuring other biochemical components in urine (see below). After that, the level of the specific amino acid under study was decreased every 9 days in four sequential steps until the content of the amino acid was equivalent to that of 0.3 g of milk protein/kg/day. Studies were conducted during the last 4 days of each period. In total, five 9-day periods were involved per essential amino acid.

Ascending Design

This was similar to the descending design but the children started with the lowest level of intake of the amino acid being studied (i.e., the level contained in 0.3 g of milk protein/kg/day) which was then increased every 9 days until the diet contained an amount of the amino acid under study equivalent to that of 1.2 g milk protein/kg/day. Except for the specific amino acid under study, the concentration of all other essential and non-essential amino acids was kept constant based on the composition shown in Table 3.1.

Evaluation Variables

1. Nitrogen Balance: Nitrogen excretion was measured in a 4-day fecal collection and in individual 24-hour urine collections throughout the same 4 days. The nitrogen content of the diet was also measured in each balance period. Balance was calculated as intake minus urinary and fecal excretions and minus 8 mg N/kg/day to account for integumental and other miscellaneous losses (Viteri and Martinez, 1981).
2. Urinary excretion of urea and creatinine: These were determined in the four 24-hour specimens collected during each balance period.
3. Creatinine-Height Index (CHI): CHI was calculated from the child's

height, which was measured at 14-day intervals (Viteri and Alvarado, 1970), and from the mean daily creatinine excretions.

4. Blood amino acids: Free amino acids in plasma and erythrocytes were determined after 12 hours of overnight fast and again 4 hours after breakfast, by gas liquid chromatography of the trimethylsilyl derivatives (Gehrke and Leimer, 1970; Gehrke and Takeda, 1973).

RESULTS AND DISCUSSION

A detailed assessment of the evaluation variables, which included the analysis of various ratios of the free amino acids in plasma and erythrocytes and the changes from fasting to post-prandial values, indicated that only the plasma concentration of the free amino acid under study after an overnight fast, the nitrogen balance and the urinary urea/creatinine ratio changed in relation to the amino acid intake. The criteria used to determine amino acid requirements were:

1. Free amino acid concentration in plasma after an overnight fast: the lowest level of amino acid intake *before* the plasma concentration of that amino acid showed a consistent tendency to decrease, which suggests a decrement in dietary amino acid adequacy.

2. Nitrogen balance:

a) Lowest level of amino acid intake which allowed *all* children to retain at least 16 mg N/kg/day, which is the nitrogen requirement for growth in this age group (FAO/WHO, 1973).

b) Lowest level of amino acid intake *before* the mean N balance showed a consistent tendency to decrease, which suggests a decreased utilization of dietary amino acids and/or an increased catabolism of endogenous protein.

3. Urea/creatinine ratio in urine collected over the 4-day period.

a) Lowest level of amino acid intake which allowed *all* children to maintain a urinary urea/creatinine ratio lower than 2 standard deviations above the mean value observed in all children consuming the basal diet (i.e., lower than 2.49 + 2 (0.90) = 4.29).

b) Lowest level of amino acid intake before the mean urinary urea/creatinine ratio showed a consistent tendency to increase, which suggests an increment in deamination of endogenous amino acids.

These criteria allowed a safety margin above the mean dietary amino acid requirements since they identified the level of intake before deleterious changes took place. That margin of safety was increased by the fact that when the various indicators suggested two different levels as the lowest adequate intake, the higher of the two intake levels was selected on the basis that when dietary requirements are established, it is safer to err by excess than by deficit.

The criteria described under headings 1, 2a, and 3b, which involved the beginning of a definite pattern of change in plasma amino acid concentration, nitrogen balance and urinary urea/creatinine ratio, respectively, might in some instances depend on the subjective assessment of the person who interpreted them. Therefore, the four investigators involved in the study interpreted the results independently. They all agreed almost always and in the few occasions when they did not, the judgement of three prevailed over that of a lone dissenter.

The results obtained with lysine will be presented in detail as an example of the selection criteria. Figure 3.1 shows N balance corrected for integumental and other miscellaneous losses. Only 5 children were studied at the lowest level of intake. Also, the data of one child who had diarrhea during the balance period with an intake of 66 mg lysine/kg/day were not included in the analysis. The figure shows that with lysine intakes below 53 mg/kg/day some children retained less than 16 mg N/kg/day. The trend toward a decrease in N retention could be interpreted to begin with lysine intakes below 66 mg/kg/day based on the mean values. If the individual variability is taken into account, the trend begins with intakes below 53 mg/kg/day. Figure 3.2 shows that the mean concentration of free lysine in plasma began decreasing with dietary intakes below 66 mg/kg/day. Figure 3.3 shows that the mean of the urea/creatinine ratios in urine began increasing with intakes below 66 mg/kg/day, coinciding with the appearance of ratios above 4.19 in some children.

The nitrogen balance data suggested that an intake of 53 mg lysine/kg/day was adequate for all the children studied, whereas the other indicators suggested that some children may need higher intakes. As mentioned above, the criteria which prevailed were those that allowed a greater safety margin, even at the risk of overestimating the requirement level. Therefore, the dietary intake recommended for lysine was defined as 66 mg/kg/day.

The same procedures were followed to interpret the results obtained for the other amino acids under investigation. Table 3.4 shows the recommended levels of intake for the six essential amino acids that were studied, expressed as mg of each amino acid or as milk protein equivalents (i.e., the amount of milk protein which contains the corresponding amount of the essential amino acid). Based on obligatory fecal N losses of 20 mg/kg/day (Torún *et al.*, 1981a) the mean "true" digestibility of the semi-synthetic diet was 97.3%. Table 3.4 also shows the amounts of essential amino acids absorbed at the recommended levels of intake, assuming that all amino acids were absorbed in the same proportion of 97%.

In the case of threonine and tryptophan, the various indicators suggested that the lowest adequate level of intake was either 0.85 or 1.20 g, of milk protein/kg/day. Since the difference between these two levels of intake is large (34%) while the other discrete intervals of intake below 0.85 g/kg/day were smaller, it was decided to use a range rather than a specific value as a first approximation of the recommended intake level for threonine. This range is shown in Table 3.4.

However, studies on protein requirements of similar children recently carried out at INCAP and discussed in another section of this Conference (Torún *et al.*, Chapter 20) supported our conclusions and allowed us to decide upon a specific value instead of a range of values for the threonine recommendation. Table 3.5 shows the amino acid contents of the safe levels

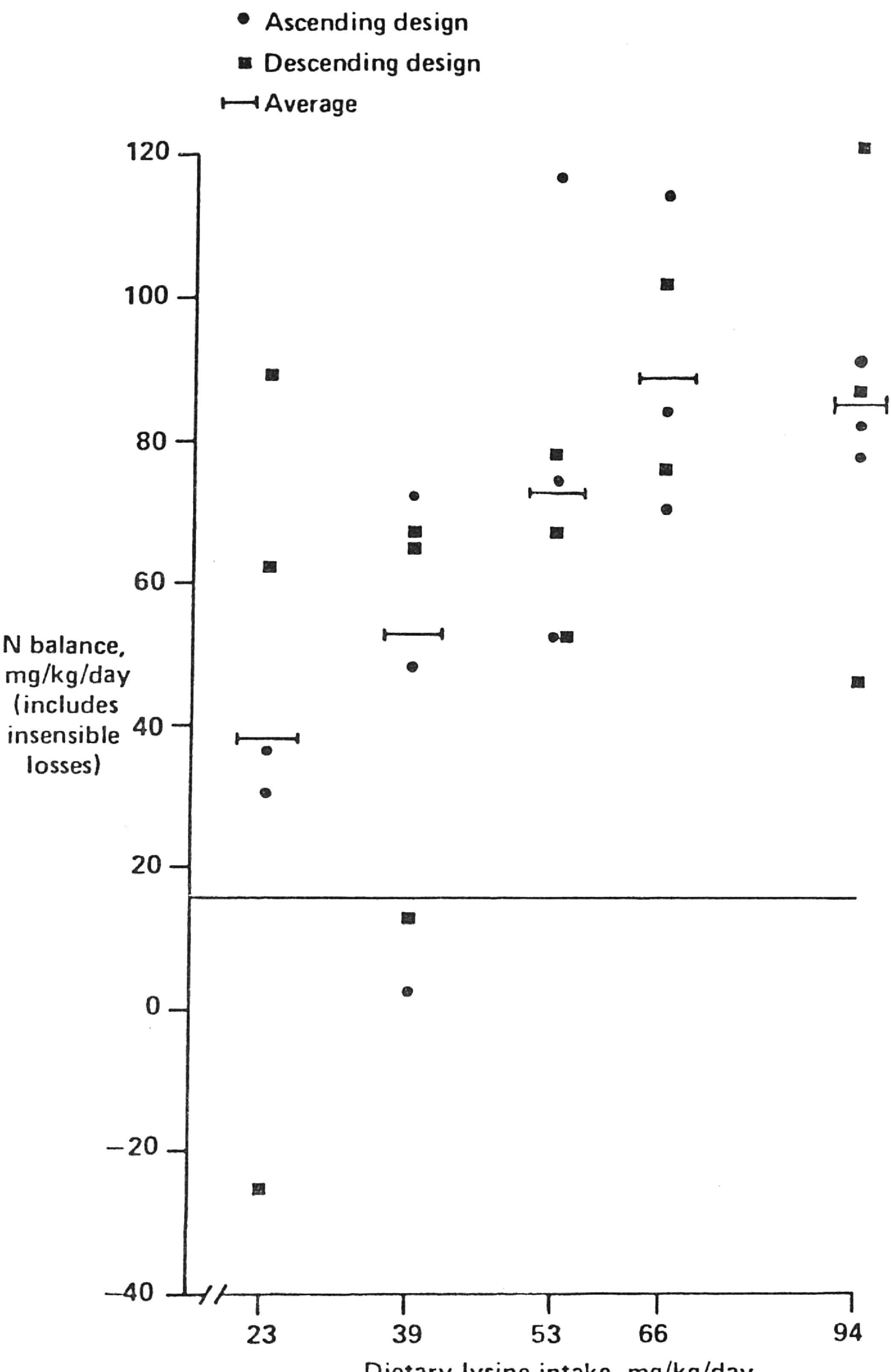

FIG. 3.1. NITROGEN BALANCE DETERMINED IN CHILDREN FED DIFFERENT LEVELS OF LYSINE: SEE TEXT FOR EXPLANATION OF ASCENDING OR DESCENDING DESIGN

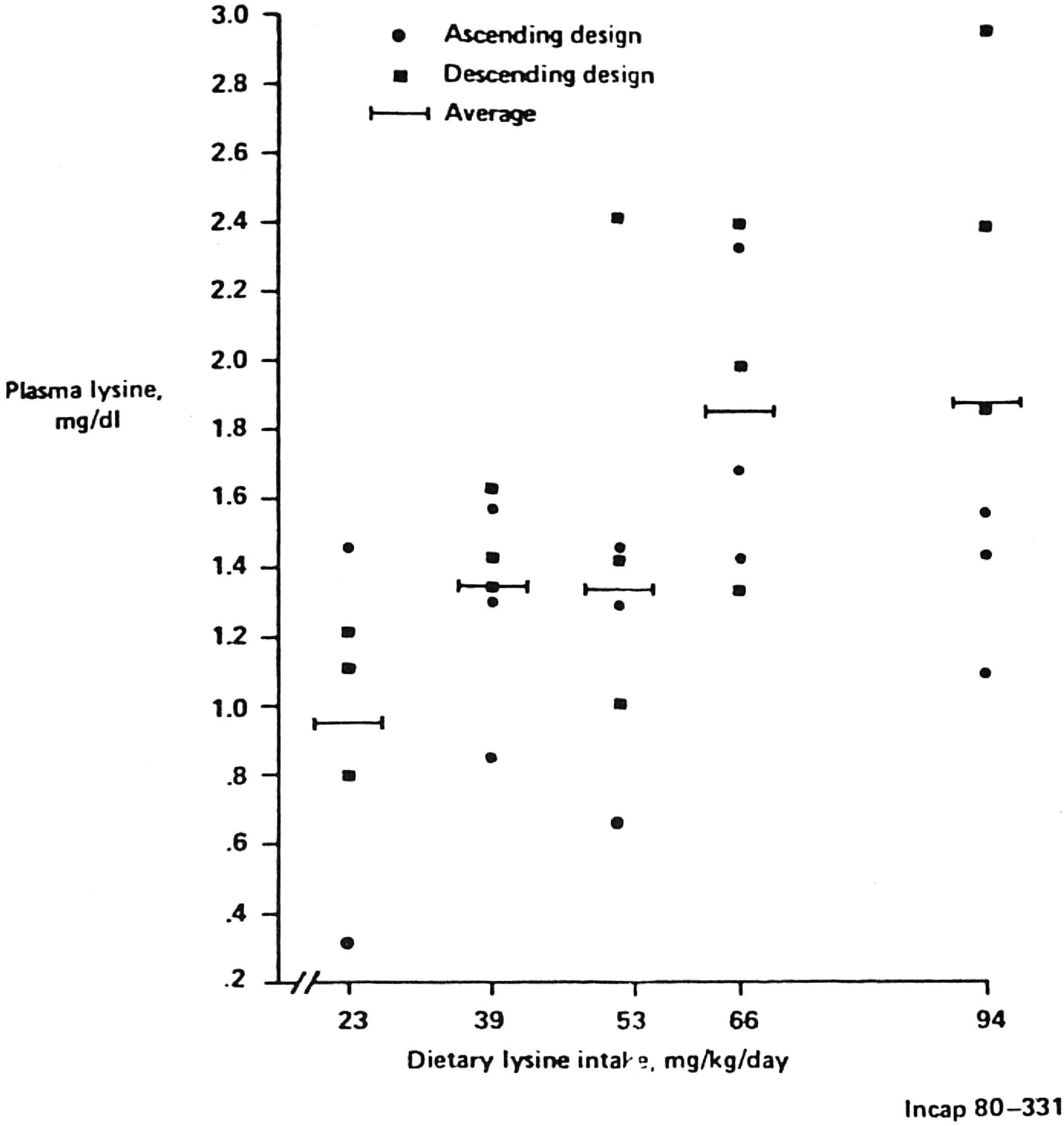

FIG. 3.2. FASTING LEVELS OF PLASMA FREE LYSINE IN CHILDREN FED DIFFERENT LEVELS OF LYSINE

TABLE 3.4. AMINO ACID INTAKES RECOMMENDED FOR PRE-SCHOOL CHILDREN (2-YEARS OLD), INCAP 1980

Amino acid	Amino acid intakes expressed as milk protein equivalents, g kg day	Amino acids (mg/kg/day) ingested	absorbed[1]
Isoleucine	0.68	32	31
Lysine	0.85	66	64
Methionine + Cystine	0.85	28	27
Threonine	>0.85, <1.20	>37, <53	>36, <52
Tryptophan	0.85	13	12.5
Valine	0.68	39	38

[1]Corrected for "true" digestibility of 97%.

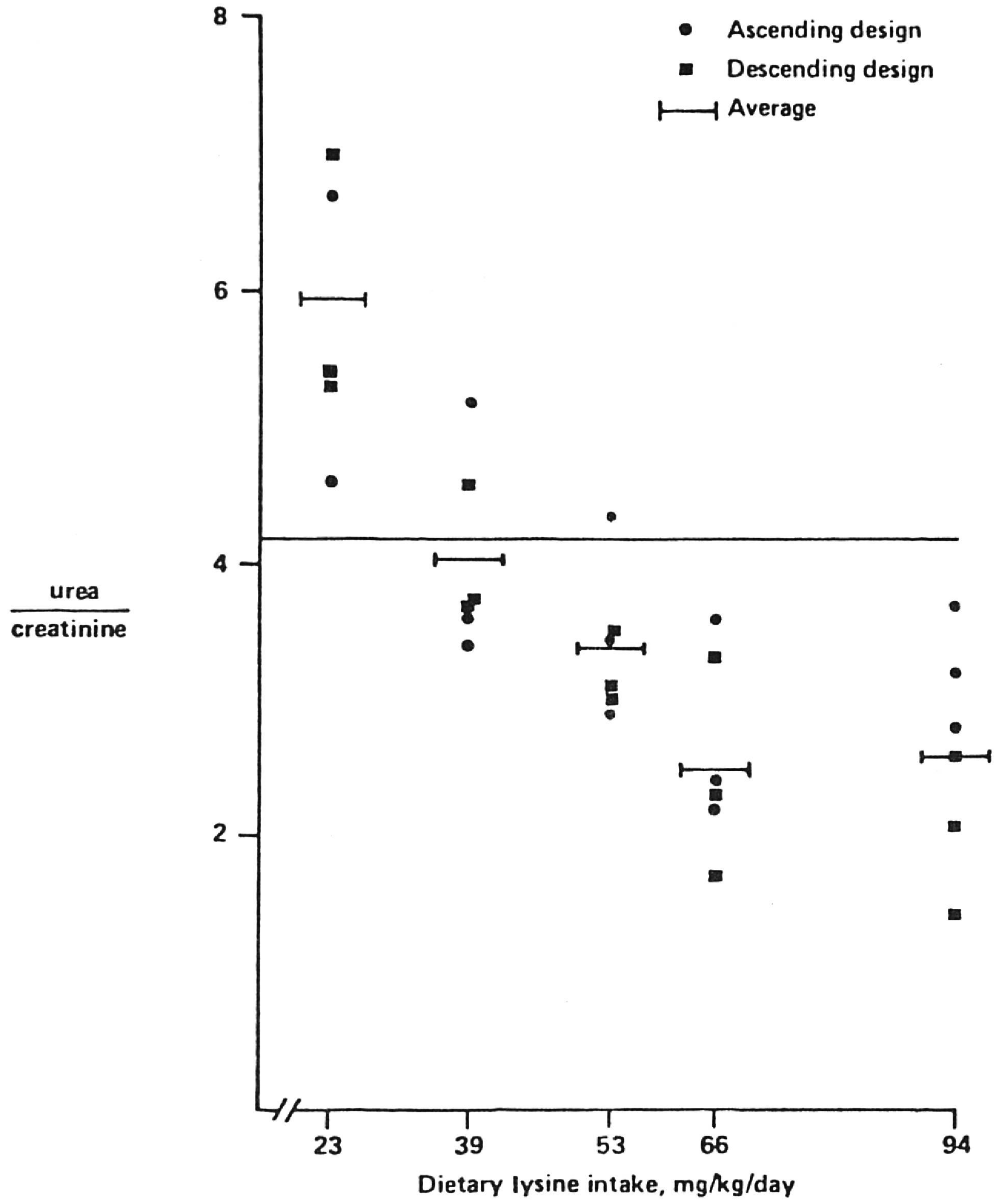

FIG. 3.3. UREA/CREATININE RATIO VALUES CALCULATED FROM ANALYSES OF 96-HOUR URINE COLLECTIONS (4-DAY BALANCE PERIODS) FROM CHILDREN FED DIFFERENT LEVELS OF LYSINE

of protein intake determined with cow's milk and a soybean protein isolate. They are similar to the lower limit of the threonine range. Therefore, it was decided to use 38 and 14.4 mg/kg/day, respectively, as the recommended levels of intake. These figures must be corrected for the "true" digestibility of the dietary protein sources, because if it is not the limiting amino acid in either milk or soy protein, the recommended level might be closer to 12 mg/kg/day.

TABLE 3.5. COMPARISON OF RECOMMENDED AMINO ACID INTAKES (mg/kg/day) WITH THE CONTENTS OF SAFE LEVELS OF PROTEIN INTAKES FOR 2-YEAR OLD CHILDREN

Amino acid	Amino acids provided by		Recommended amino acid intakes
	0.79 g of milk protein/kg/day	0.98 g of soy protein isolate/kg/day	
Isoleucine	46	51	32
Lysine	66	61	66
Methionine + Cystine	30	26	28
Threonine	35	36	38
Tryptophan	13.8	16.3	13
Valine	52	52	39

The recommended levels of intake of the six essential amino acids investigated in this study and supported by the studies on protein requirements are 21−35% lower than the currently accepted recommendations of isoleucine, sulfur amino acids, threonine, and valine for 2-year old children, which are based on the FAO/WHO (1973) amino acid scoring pattern and a safe intake level of egg or milk protein of 2 g/kg/day (Table 3.6). The recommendation for tryptophan, on the other hand, is 8% higher although this may be an overestimation. These differences are important in terms of protein quality assessment since they may lead to changes in the amino acid scoring pattern for proteins, as discussed in detail in another section of this Conference (Torún *et al.*, Chapter 20). Some proteins which are considered to be limited by their methionine or threonine contents may, in fact, have a better nutritive quality than present assessments suggest, provided that other essential amino acids do not limit their quality to the same extent as the currently accepted methionine (or threonine) related limitations. In contrast, many vegetable protein sources will still be considered of a low quality since lysine or tryptophan may be their limiting amino acid. The results of the present study does not indicate that the FAO/WHO estimates for these amino acids are excessive. Furthermore, the nutritional value of such proteins is impaired by relatively low digestibilities which implies that larger amounts of these proteins must be ingested to secure adequate levels of total nitrogen absorption, which is independent of their essential amino acid composition.

TABLE 3.6. COMPARISON OF ESSENTIAL AMINO ACID INTAKES (mg/kg/day) RECOMMENDED BY FAO/WHO (1973) AND BY THIS STUDY FOR 2 YEAR-OLD CHILDREN

Amino acid	(A) FAO/WHO[1]	(B) This Study	(B) as % of (A)
Isoleucine	48	32	67
Lysine	66	66	100
Methionine + Cystine	42	28	67
Threonine	48	38	79
Tryptophan	12	13	108
Valine	60	39	65

[1] Based on the FAO/WHO (1973) amino acid scoring pattern and a safe level of protein intake equivalent to 1.2 g of milk or egg protein/kg day.

SUMMARY

The FAO/WHO essential amino acid scoring pattern was based on the requirements of preschool children. Those requirements, however, were theoretical estimates derived from information on amino acid requirements of infants under six months of age, 10–12-year old children and non-pregnant adults. That information was based on nitrogen balance studies and on weight gains of infants. The present study investigated the requirements of children 21–27 months-old for isoleucine, lysine, methionine + cystine, threonine, tryptophan, and valine. Each amino acid was studied with six children who ate a mixture of cow's milk and synthetic amino acids which provided increasing (3 children) or decreasing (3 children) amounts of the specific amino acid under study at 9-day intervals. Among the various indicators explored, only the plasma concentration of the free amino acid under study after an overnight fast, the nitrogen balance and the urinary urea/creatinine ratio changed in relation to the amino acid intake. Daily requirements of all the children studied were satisfied by the absorption of 31 mg isoleucine, 64 mg lysine, 27 mg methionine + cystine, 36–52 mg threonine, 12.5 mg tryptophan and 38 mg valine/kg body weight. The corresponding requirements suggested by FAO/WHO in 1973 were 48, 66, 42, 48, 12 and 60 mg amino acid/kg. Further studies (Torún *et al.*, Chapter 20) suggested 37 mg/kg/day as the requirement for threonine absorption, and confirmed that the FAO/WHO requirements for isoleucine, methionine + cystine, threonine, and valine are too high. Those studies also supported the conclusion that the values established in our investigations are similar to "recommended" or "safe levels" of intake rather than mean requirements. These new recommendations may lead to changes in amino acid scoring patterns and in the protein quality assessment of some protein sources.

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