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Non-essential nitrogen and protein utilization in the growing rat

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Three series of nitrogen-balance experiments were carried out on growing rats fed on purified isonitrogenous diets (16 g N/kg) to study the importance of non-essential N and the essential:total N (E:T) ratio for attaining maximum N balance (NB) and biological value (BV) of protein. Minimum dietary levels of asparagine, proline and glutamic acid required for maximum NB and BV were estimated to be 1·0, 2·0 and 5·0 g/kg respectively. In an essential amino acid-based diet, the levels of individual amino acids were successively reduced to 110% of the requirement. Reducing the level of arginine, lysine or methionine + cystine resulted in a significant increase in NB and BV while the response of rats given the isoleucine-reduced diet significantly decreased. Addition of asparagine, proline and glutamic acid in the estimated minimum amounts to an essential amino acid-based diet resulted in a significant increase in NB and BV. A further significant increase was found when the levels of arginine, lysine and methionine + cystine in the diet were reduced to 110% of the requirement. The performance of rats fed on the latter diet was similar to that of rats given a diet with the optimum E:T ratio. It is concluded that the optimum protein utilization may be influenced by the presence of some non-essential amino acids and by the surplus of some essential amino acids rather than by the E:T ratio *per se*.

Protein utilization: Non-essential nitrogen: Rat

It is generally accepted that, in order to attain maximum growth rate or nitrogen retention, the diet must contain essential amino acids at a particular proportion of dietary protein. Studies with purified diets carried out on rats (Greenstein *et al.* 1957; Stucki & Harper, 1962; Matsuno *et al.* 1976) and chicks (Greene, 1960; Stucki & Harper, 1961; Bedford & Summers, 1985) showed that the animal performance increased as the essential:total N (E:T) ratio increased and reached maximum at E:T ratios between about 0·5 and 0·65. At higher E:T ratios, the response diminished even though the diets contained essential amino acids at levels enabling the synthesis of sufficient quantities of non-essential amino acids. The reason for this decline is not fully understood. It has been suggested that the rate of synthesis of one or more amino acids commonly classified as non-essential may be insufficient to maintain maximal growth (Adkins *et al.* 1966). In our experiments on rats fed on a crystalline amino acid diet, the successive deletion of non-essential amino acids from the diet resulted in a significant decrease in N balance in the case of asparagine and proline while the deletion of glutamic acid caused an insignificant decrease (Heger *et al.* 1987). The importance of asparagine, proline and glutamic acid for the maximal growth of rats has also been demonstrated by other studies (Breuer *et al.* 1964; Hepburn & Bradley, 1964; Adkins *et al.* 1967). Provided the rate of synthesis of these three amino acids limits growth, their supplements in appropriate amounts should increase the utilization of an essential amino acid-based diet to the same level as that found in diets with the optimum E:T ratio. However, the reduced performance at high E:T ratios may also be due to a low efficiency of conversion of some essential amino acids given in excess into non-essential amino acids. Although there is ample evidence indicating that all essential amino acids are converted into non-essential amino acids (Aqvist, 1951), little is known about the efficiency of utilization of individual essential amino acids as sources of non-essential N in quantitative terms.

The objective of the present study was to examine, in balance experiments on growing rats, the effects of supplements of asparagine, proline and glutamic acid and of the excessive *v.* reduced levels of essential amino acids on the utilization of an essential amino acid-based diet.

EXPERIMENTAL

Animals and experimental procedure

Male SPF rats of the Wistar strain, aged 21–23 d and weighing about 65 g, were used. During a 4 d adjustment period, the animals were fed *ad lib.* on a casein-based diet containing 100 g crude protein (N × 6·25)/kg. They were then divided into approximately homogenous groups of five to six rats, placed into individual metabolism cages and fed on experimental diets. The daily amount of diet offered was 11 g. The balance period consisted of a 4 d preliminary period and a 6 d collection period during which faeces and urine were quantitatively collected. N balance (NB) and biological value (BV) of protein were the criteria of response. NB was calculated as the difference between N intake and faecal and urinary N. BV was defined as the proportion of absorbed N that was retained. Details of the balance procedure and the method of calculation have been described previously (Heger *et al.* 1983). Experiments were carried out in an air-conditioned room (12 h light–dark cycle, 23 ± 1°, 50 ± 10% relative humidity).

Expt 1. Estimation of minimum levels of proline, asparagine and glutamic acid

The first experiment was designed to estimate the minimum dietary concentrations of proline, asparagine and glutamic acid required for the attainment of maximum N retention. The basal diet in which crystalline L-amino acids were used as the only source of N was formulated to contain 16 g N/kg. The composition of the diet is given in Table 1. The proportion of amino acids in the essential amino acid mixture corresponded to the recommendations of (US) National Research Council (NRC) (1978). The composition of the non-essential amino acid mixture was based on values reported by Itoh *et al.* (1976). The E:T ratio of the basal diet was 0·65; this ratio was found to maximize protein utilization in rats (Heger *et al.* 1987).

To study the effect of proline, asparagine and glutamic acid on NB and BV, the basal diet was modified to provide varying dietary concentrations of one of these amino acids ranging from zero to levels exceeding the requirement as given by NRC (1978). The N content of diets was maintained at a constant level (16 g N/kg) by adjustment of all other non-essential amino acids. The dietary concentrations of amino acids under study are shown in Table 3 (see p. 656).

Expt 2. Influence of essential amino acids given in excess

The aim of this experiment was to study the response of rats to the reduction of dietary level of each essential amino acid. The composition of the basal diet is shown in Table 1. The essential amino acid mixture was used as the principal source of N supplying 15·1 g N/kg. Asparagine, proline and glutamic acid were added to the diet in amounts which had been found to maximize NB in Expt 1. The diet contained 16 g N/kg. In this diet, individual amino acids were successively reduced to a level corresponding to 110% of the optimum requirement (NRC, 1978). The N content was maintained constant by additions of a non-essential amino acid mixture consisting of equal parts of alanine, glutamine, aspartic acid, glycine and serine.

Table 1. *Expts 1 and 2. Composition of basal diets (g/kg)*

	Expt 1	Expt 2
Sucrose	60·0	60·0
Polyethylene	40·0	40·0
Mineral mixture USP XVIII*	50·0	50·0
Vitamin mixture USP XVIII*	10·0	10·0
Soya-bean oil	50·0	50·0
Essential amino acid mixture†	73·6	106·6
Non-essential amino acid mixture‡	33·9	—
Asparagine	—	1·0
Proline	—	2·0
Glutamic acid	—	5·0
Wheat starch	682·5	675·4

* According to the Association of Official Agricultural Chemists (1970).

† Contained (g/kg): arginine hydrochloride 125, histidine 52, isoleucine 86, leucine 129, lysine hydrochloride 151, methionine 69, cystine 35, phenylalanine 86, tyrosine 52, threonine 86, tryptophan 26, valine 103.

‡ Contained (g/kg): alanine 64, asparagine 85, glutamine 242, aspartic acid 66, glutamic acid 22, glycine 265, proline 190, serine 66.

Table 2. *Expt 3. Composition of diets (g/kg)*

Diet	A	B	C	D
Sucrose	60·0	60·0	60·0	60·0
Polyethylene	40·0	40·0	40·0	40·0
Mineral mixture USP XVIII*	50·0	50·0	50·0	50·0
Vitamin mixture USP XVIII*	10·0	10·0	10·0	10·0
Soya-bean oil	50·0	50·0	50·0	50·0
Arginine hydrochloride	14·14	13·32	7·99	9·20
Histidine	5·84	5·51	7·50	3·83
Isoleucine	9·74	9·18	12·50	6·33
Leucine	14·61	13·76	18·75	9·49
Lysine hydrochloride	17·05	16·06	9·63	11·11
Methionine + cystine (1:1 w/w)	11·68	11·02	6·60	7·65‡
Phenylalanine + tyrosine (1:1, w/w)	15·58	14·68	20·00	10·16§
Threonine	9·74	9·18	12·50	6·33
Tryptophan	2·92	2·75	3·75	1·91
Valine	11·69	11·01	15·00	7·58
Asparagine	—	1·00	1·00	—
Proline	—	2·00	2·00	—
Glutamic acid	—	5·00	5·00	—
Non-essential amino acid mixture†	—	—	—	33·90
Wheat starch	677·01	675·57	667·78	682·51

* According to the Association of Official Agricultural Chemists (1970).

† For composition, see Table 1.

‡ Methionine + cystine 2:1, w/w.

§ Phenylalanine + tyrosine 1·6:1, w/w.

Expt 3. Optimization of an essential amino acid-based diet

In this experiment, an attempt was made to increase the utilization of an essential amino acid-based diet with the E:T ratio approaching 1·0 by supplements of asparagine, proline and glutamic acid and by reducing the levels of arginine, lysine and methionine + cystine. The composition of diets is given in Table 2. Diet A contained essential amino acids as the only source of N in proportions corresponding to the requirements given by NRC (1978).

To this diet, supplements of asparagine, proline and glutamic acid were added in the same amounts as in Expt 2, thus forming diet B. The composition of diet C was similar, but the levels of arginine, lysine and methionine + cystine were reduced to 110% of the requirement. All diets were kept isonitrogenous (16 g N/kg) by adjusting the levels of essential amino acids. For comparison, a diet with the E:T ratio 0·65 was also included (diet D). The composition of this diet was identical with the basal diet used in Expt 1.

Statistical analysis

The data were subjected to analysis of variance and differences between means were assessed using Duncan's multiple-range test. Where applicable, the results were expressed as means with their standard errors. The minimum levels of proline, asparagine and glutamic acid required for attaining maximum NB were estimated by a broken-line regression model relating NB to the dietary concentration of the respective amino acid. The minimum 'requirement' was estimated as the amino acid level equivalent to the breakpoint in the response (Heger *et al.* 1987).

RESULTS

With a few exceptions, the rats consumed all the food offered and gained approximately 3·3 g/d. No diet exhibited any adverse effect on food intake.

Expt 1

The results of Expt 1 are summarized in Table 3. There was no significant response of rats to graded supplements of asparagine. Both NB and BV slightly increased as the level of dietary asparagine increased from 0 to 1 g/kg. Thereafter, they remained practically unchanged.

Increasing the dietary concentration of proline from 0 to 2 g/kg produced a significant increase in NB and BV. Further increases up to 10 g/kg were without any effect. Since there were no values below the level of 2 g proline/kg to identify the breakpoint in the response, this part of the experiment was repeated using lower levels of proline and narrower intervals between the supplements. The results showed a gradual increase in NB and BV up to the highest proline level (2 g/kg) which produced a similar response as the same level used in the first part of the experiment.

Increasing the glutamic acid concentration from 0 to 5 g/kg resulted in a non-significant increase in NB and BV. A further increase exceeding 10 g glutamic acid/kg was associated with a significant decrease in the response.

Expt 2

Mean values for NB and BV of diets containing the reduced levels of individual essential amino acids are presented in Table 4. Reducing the dietary level of arginine, lysine and methionine + cystine to 110% of their requirements increased both variables. Protein utilization in rats fed on the isoleucine-reduced diet decreased. There was no significant response to changes in dietary concentrations of other essential amino acids.

Expt 3

The results of the comparison of the diets differing in E:T ratios and in amino acid composition are summarized in Table 5. Protein utilization of the essential amino acid-based diet supplemented with asparagine, proline and glutamic acid (diet B) was significantly higher than that of the diet containing essential amino acids as the only source

Table 3. *Expt 1. Effects of graded additions of asparagine, proline or glutamic acid on nitrogen balance (mg/g N intake) and biological value of protein in growing rats*

(Mean values with their standard errors)

Asparagine (g/kg)		0	0·5	1	2	4	8
N balance:	Mean	746^{a}	753^{a}	777^{a}	777^{a}	778^{a}	783^{a}
	SE	16	15	9	7	10	3
Biological value:	Mean	919^{a}	926^{ab}	953^{ab}	961^{b}	955^{ab}	957^{b}
	SE	18	15	11	6	9	5
Proline (g/kg)		0	2	4	6	8	10
N balance:	Mean	645^{a}	747^{b}	746^{b}	732^{b}	746^{b}	750^{b}
	SE	17	9	7	10	6	4
Biological value:	Mean	808^{a}	910^{b}	915^{b}	904^{b}	913^{b}	919^{b}
	SE	17	8	4	7	7	2
Proline (g/kg)		0	0·2	0·6	1·2	2·0	
N balance:	Mean	682^{a}	686^{ab}	709^{abc}	739^{bc}	760^{c}	
	SE	19	16	15	16	17	
Biological value:	Mean	851^{a}	856^{a}	876^{ab}	909^{bc}	927^{c}	
	SE	17	14	13	14	14	
Glutamic acid (g/kg)		0	1	5	10	25	50
N balance:	Mean	737^{a}	748^{a}	759^{a}	754^{a}	729^{a}	668^{b}
	SE	12	5	5	5	17	11
Biological value:	Mean	913^{ab}	932^{ab}	934^{a}	932^{a}	899^{b}	837^{c}
	SE	13	4	5	6	16	12

$^{a, b, c}$ Means within rows with unlike superscript letters were significantly different ($P < 0·05$).

Table 4. *Expt 2. Effect of reducing dietary levels of essential amino acids to 110% of the requirement on nitrogen balance and biological value of protein in growing rats*

(Mean values with their standard errors)

	N balance (mg/g N intake)		Biological value	
Reduced amino acid	Mean	SE	Mean	SE
None (basal diet)	662	3	83·9	0·2
Arginine	693**	3	87·1**	0·3
Histidine	670	6	84·7	0·4
Isoleucine	628**	6	79·8**	0·4
Leucine	666	6	83·9	0·6
Lysine	710**	5	88·4**	0·6
Methionine + cystine	690*	7	86·5*	0·9
Phenylalanine + tyrosine	656	13	82·8	1·6
Threonine	647	9	81·5	0·9
Tryptophan	661	4	83·0	0·6
Valine	664	6	83·7	0·5

Mean values were significantly different from those for the basal diet: $^{*}P < 0·05$, $^{**}P < 0·01$.

of protein (diet A). A further increase in NB and BV was found in the diet containing reduced levels of arginine, lysine and methionine + cystine (diet C). The performance of rats fed on this diet and on the diet with the optimum E:T ratio (diet D) was practically the same.

Table 5. *Expt 3. Nitrogen balance and biological value of protein in growing rats fed on diets with various proportions of essential and non-essential amino acids*

(Mean values with their standard errors)

	N balance (mg/g N intake)		Biological value	
Diet*	Mean	SE	Mean	SE
A	614[a]	12	80·7[a]	1·4
B	666[b]	7	84·8[b]	0·7
C	737[c]	10	91·4[c]	1·0
D	725[c]	3	90·7[c]	0·2

* For details, see Table 2.

[a, b, c] Means within columns with unlike superscript letters were significantly different ($P < 0·05$).

DISCUSSION

A significant response to proline supplements indicates that this amino acid must be included in the diet to obtain maximum protein utilization. The essential nature of proline has been demonstrated in several other experiments on growing rats (Breuer *et al.* 1964; Adkins *et al.* 1966), chicks (Sugahara & Ariyoshi, 1967) and young pigs (Ball *et al.* 1986).

The response to additions of asparagine or glutamic acid was less pronounced. Contrary to our previous study in which similar diets were used (Heger *et al.* 1987), only a non-significant increase in NB was observed as the level of asparagine in the diet increased. Similarly, Ranhotra & Johnson (1965) reported a non-significant increase in N retention due to asparagine supplementation. Breuer *et al.* (1966) and Crosby & Cline (1973) demonstrated the importance of asparagine for maximum growth of rats. However, they found that after several days of deprivation, the rats seemed to adapt to an asparagine-free diet irrespective of the presence or absence of three metabolically related amino acids. The opinion on the requirement for glutamic acid is also controversial. Hepburn *et al.* (1960) and Breuer *et al.* (1964) found that glutamic acid was essential for growth of the rat. They showed that even though the need for glutamic acid could be met partly by arginine, both amino acids had to be present in the diet to maximize growth rate. On the other hand, there are several reports suggesting no specific need of the rat for glutamic acid (Adkins *et al.* 1966; Womack, 1969; Newburg *et al.* 1975).

A significant decrease in protein utilization found in rats consuming higher amounts of glutamic acid is rather surprising. Glutamic acid is known to be one of the most abundant amino acids serving as the main medium in amino-N exchange and being readily converted to other non-essential amino acids. No adverse effects of higher levels of glutamic acid have been observed in rats (Adkins *et al.* 1967) or chicks (Sugahara & Ariyoshi, 1968). It seems that the decline in N retention found in the present experiment may be attributed to the deficiency of proline and asparagine. To keep the diets isonitrogenous, the amount of the non-essential amino acid mixture was decreased as the level of glutamic acid in the diet increased. Thus, the dietary concentrations of proline and asparagine decreased from 7·1 and 3·2 g/kg to 1·1 and 0·5 g/kg respectively. The latter levels were considerably lower than those which were found to maximize N retention.

Based on the analysis of the dose-response relationship, the minimum dietary levels of asparagine, proline and glutamic acid were estimated to be 1·0, 2·0 and 5·0 g/kg respectively. It is evident that these values cannot be taken as requirements since the optimum dietary concentration of any non-essential amino acid depends on the quantity of one or more other amino acids (Rogers *et al.* 1970). However, the sum of estimated minimum amounts of asparagine, proline and glutamic acid was considered to meet all the specific needs for non-essential N provided the diet contained essential amino acids in excess. The supplements of the three amino acids were, therefore, included into essential amino acid-based diets used in Expts 2 and 3, thus reducing the E:T ratio of these diets from 1·0 to about 0·95.

The results of Expt 2 show that not only a lack of non-essential amino acids, but also an excess of some essential amino acids may impair protein utilization. The increase in N balance due to the reduction of dietary levels of arginine, lysine or methionine+cystine suggests that these amino acids are not used efficiently as sources of non-essential N. Allen & Baker (1974) compared the efficacy of various sources of non-essential N in growing chicks using a slope-ratio technique. Of the amino acids tested, the lowest efficacy was found in arginine and lysine. Methionine was not included in the study.

The low utilization of arginine as a source of non-essential N is not surprising since this amino acid is closely associated with the synthesis of urea. Urea is known to be a poor source of N for the synthesis of non-essential amino aids (Birnbaum *et al.* 1957; Allen & Baker, 1974). Stein *et al.* (1986) measured the excretion of ^{15}N in adult males after the administration of ^{15}N-labelled amino acids. They found that about 35% of ^{15}N from arginine was excreted in urine. The percentage of ^{15}N excreted in all other amino acids was significantly lower.

The reasons for the low conversion efficiency of lysine and sulphur-containing amino acids are less clear. Although lysine catabolism yields glutamate via both the pipecolic acid and saccharopine pathways, little is known of the rate of these reactions. It has been shown that some steps in lysine catabolism, such as the conversion of lysine to α-aminoadipic acid or the conversion of α-aminoadipic acid to α-ketoadipic acid, are extremely slow (Berg & Kolenbrander, 1970).

The N of S-containing amino acids is incorporated into urea after it is released as ammonia or is used for the synthesis of polyamines and taurine (Stipanuk, 1986). Polyamine degradation leads to the formation of many derivatives, but there is no evidence for their participation in amino acid synthesis (Pegg & McCain, 1982). Taurine serves as a constituent of bile acids or is excreted in urine. Chesney *et al.* (1985) have shown that the dietary level of S-containing amino acids and renal clearance of taurine are positively correlated. Alternative pathways of methionine and cystine catabolism that involve transamination do not appear to be quantitatively important in mammals (Stipanuk, 1986). It seems, therefore, that only a part of N of the catabolized methionine+cystine is incorporated into non-essential amino acids.

Taking into consideration the amino acid patterns of diets used in Expt 2, an imbalance could be expected. Nevertheless, with the exception of leucine, no adverse effects of imbalance were found. This might be due to relatively high levels of limiting amino acids with respect to the requirement. As has been shown by Harper (1964), the negative effects of amino acid imbalance diminish as the level of the amino acid in shortest supply increases. The decreased N retention in rats consuming the leucine-reduced diet might be a result of an antagonism between the branched-chain amino acids. Benton *et al.* (1956) observed a depression of food intake and growth rate in rats given excessive amounts of isoleucine and valine which was prevented by additional leucine. Similar effects were found in diets low in isoleucine when leucine was added in excess (Harper *et al.* 1955). In the present experiment, however, no signs of antagonism were apparent in the isoleucine-reduced diet, although the degree of isoleucine reduction and the surplus of other branched-chain amino acids were similar to those of the leucine-reduced diet.

The results of Expt 3 suggest that the concept of optimum E:T ratio as a prerequisite for attaining the maximum protein utilization may not be fully justified. The N retention in rats fed on diet D with the E:T ratio 0·65 which had been considered to be optimal was essentially the same as that of rats receiving diet C with the E:T ratio 0·94. It seems that the optimum utilization of dietary N may be influenced by the presence of some non-essential amino acids such as proline, asparagine and glutamic acid and by the surplus of some essential amino acids such as arginine, lysine and methionine + cystine rather than by the E:T ratio *per se*.

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