Experimental protein-energy deficiency in rats. Ratio of serine+glycine to threonine as an index of deficiency

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1. Rats were given a low-protein (10 g/kg) diet for 16 weeks and the changes occurring in their serum amino acids were studied; during this time a full picture of protein-energy malnutrition was manifested. Groups of rats were killed at intervals of 4 weeks.

2. Food intake decreased gradually from the 4th to the 8th week, then increased slightly from the 9th to the 11th week, and then decreased again.

3. The body-weight of the rats fell progressively from the 1st week on the low-protein diet, remained stationary from the 10th to the 13th week, and then decreased again during the last 4 weeks.

4. The ratio of non-essential to essential amino acid was not correlated with the severity of protein deficiency.

5. The ratios between some individual amino acids were compared with these ratios in control rats during the 4-week periods of protein deficiency. The ratio of serine+glycine to threonine was always significantly higher in the protein-deficient than in the control rats.

During the last 10 years considerable efforts have been made to simulate or produce protein-energy malnutrition, with its clinical manifestations, in experimental animals. The successful production of such an experimental animal model is necessary for studying the time-sequence of the development of the complex biochemical aberrations met with in protein-energy deficiency, since this cannot be achieved in children suffering from protein-energy deficiency diseases. Such models have been produced with monkeys (Gopalan & Ramanathan, 1957; Deo, Sood & Ramalingaswami, 1965) with pigs (McCance & Widdowson, 1966; Heard, Kriegsman & Platt, 1968; Grimble & Whitehead, 1969, 1970) and with rats (Kirsch, Brock & Saunders, 1968; Grimble, Sayer & Whitehead, 1969). Edozien (1968) was able to produce successfully nearly all clinical features of protein-energy deficiency in male rats by feeding them on a diet containing 5 g/kg lactalbumin for 4 months.

One of the complex biochemical changes which takes place in protein-energy deficiency in humans is the distortion in the normal serum amino acid ratio (Arroyave, 1962; Holt, Snyderman, Norton, Roitman & Finch, 1963; Whitehead & Dean, 1964*a*, *b*; Rutishauser & Whitehead, 1969). Similar effects have also been demonstrated in experimental animals when manifestations of protein-energy deficiency were induced in them (Grimble *et al.* 1969; Grimble & Whitehead, 1969).

A study of the amino acid pattern during the various stages of protein-energy deficiency is still lacking and therefore a reliable index is still being sought to enable paediatricians to detect early or even subclinical cases. In the following experiment a systematic study was made of the changes occurring in the serum amino acids of rats fed on a low-protein diet for 16 weeks, during which groups of rats were killed at intervals of 4 weeks. Thus a full picture of protein-energy malnutrition was achieved.

Table 1. *Composition of the experimental diet (g/kg)*

Ingredient	Control diet (NDP:E = 0·096)	Low-protein diet (NPP:E < 0·01)
Casein	160·0	10·0
Sucrose	241·6	291·6
Dextrin	483·4	583·4
B-vitamins mixture*	10·0	10·0
Salt mixture†	5·0	5·0
Maize oil	100·0	100·0

NDP:E, ratio of energy supplied by utilizable protein to total metabolizable energy.

Each rat was given every week 0·1 ml maize oil containing: vitamin A, 2 mg retinol equivalent; vitamin D, 250 μg cholecalciferol equivalent; vitamin E, 34 mg α-tocopherol acetate; and 2 mg menaphthone (Morcos, 1967).

* Thiamin hydrochloride 0·3 g, riboflavin 1·0 g, pyridoxine hydrochloride 0·2 g, calcium pantothenate 6·0 g, nicotinic acid 20·0 g, inositol 20·0 g, *p*-aminobenzoic acid 60·0 g, biotin 0·2 g, folic acid 0·2 g, cyanocobalamin 0·005 g, and choline chloride 60·0 g; made up to 1 kg with maize starch (Miller & Bender, 1955).

† $Ca(PO_4)_2$ 6 g, NaCl 250 g, KCl 150 g, 3 $MgCO_3.Mg(OH)_2.3H_2O$ 65 g, ferrous citrate 25 g, $MnCl_2.4H_2O$ 7 g, $CuCO_3.Cu(OH)_2$ 2 g, $ZnCO_3$ 1 g and NaF 0·1 g (Morcos, 1967).

MATERIALS AND METHODS

Animals and diets

One hundred albino rats of both sexes of the Sprague–Dawley strain, weighing 100–110 g, were used. They were housed individually in cages with galvanized-screen floors. Room temperature during the whole experiment ranged from 25–28°. Seventy of the rats were fed on a low-protein diet with a ratio of energy supplied by utilizable protein to total metabolizable energy (NDP:E) of < 0·01. The other thirty rats were given a control diet of NDP:E of 0·096 (Table 1). Water and food were freely available. The weights of rats, as well as their food intakes were recorded daily. At the end of each 4-week interval animals were killed by decapitation and blood was collected; the serum was separated and stored in a deep-freeze at −18° until used for analysis. Only sixteen samples of the control and thirty of the low-protein groups were analysed. The animals were not allowed any food during the night before they were killed. During the last 4 weeks of the experiment (last interval), food was withheld from the rats for only 6 h before decapitation. At this stage the rats were weak and could not tolerate overnight fasting.

Methods of analysis

Serum total proteins were determined by the biuret method (King & Wootton, 1959). For the determination of serum amino acids, a known volume of serum was deproteinized with 95 % (v/v) ethanol according to the method described by Whitehead & Dean (1964*a*) and the precipitate was washed twice with small portions of ethanol. The filtrate and washings were dried under reduced pressure, then redissolved in ethanol. The amino acids were determined by the buffered method of Levy & Chung (1953) with the two-dimensional ascending paper-chromatography technique on Whatman No. 1 filter paper. Normal butanol–acetic acid–water-mixture (12:3:5, by

Table 2. *Dietary energy intake and weight change of control rats (C) and rats given the low-protein diet (LP)*

Period	No. of weeks	No. of survivors		Food intake (g/rat per d)		Energy intake (J/g body-weight per d)		Body-weight (g)	
		C	LP	C	LP	C	LP	C	LP
1	0	30	70	—	—	—	—	104·0	103·4
	1	30	70	11·00	9·89	1883	1694	117·00	91·16
	2	30	70	13·70	9·76	2085	1907	130·00	85·00
	3	30	70	13·42	9·18	1867	1915	149·50	81·57
	4	30	70	13·51	7·74	1615	1658	166·00	78·58
2	5	23	60	12·80	6·18	1389	1401	168·00	74·50
	6	23	59	12·68	5·62	1344	1359	179·00	70·77
	7	23	58	12·45	5·52	1259	1389	185·00	67·14
	8	23	56	12·33	5·21	1199	1379	189·00	64·81
3	9	16	45	11·82	4·54	1092	1266	192·50	62·44
	10	16	43	11·80	4·72	1080	1372	194·00	60·00
	11	16	39	11·42	4·92	1112	1507	196·00	59·57
	12	16	36	11·38	4·70	1016	1448	198·50	60·00
4	13	8	22	11·75	4·48	1030	1401	208·00	59·00
	14	8	20	11·55	4·00	1014	1321	213·00	57·00
	15	8	19	10·88	4·05	955	1485	214·00	52·50
	16	8	18	10·76	3·90	944	1516	214·00	46·00

The difference in the number of rats between the end of each period and the beginning of the next are the rats killed for analysis. The changes in the number of rats during one and the same period is due to mortality of the rats.

volume) was used for 24 h as the first solvent and the buffered *m*-cresol–phenol mixture (2:1, by volume) for 24 h as the second solvent (pH 8·3). The ratio of non-essential to essential amino acids was calculated as described by Whitehead & Dean (1964*a*).

RESULTS

During the first 3 weeks the food intakes of the control animals and the animals given the low-protein diet were about the same. At the end of the 3rd week the appetite of the animals receiving the low-protein diet was reduced. Compared with the controls, their food intake fell markedly during the 4th week and continued to fall till the end of the 9th week: during the third period of the experiment (10–12 weeks), it increased by 15%, after which it decreased again (Table 2).

The calculated energy intake per g body-weight per d of the rats fed on the low-protein diet was constant from the 5th week onwards; that of the control rats decreased continuously as their weight increased (Table 2).

A marked loss in weight of the animals was recorded as early as the 1st week of feeding on the low-protein diet (Table 2). This was followed by a gradual reduction in weight, which ceased during the 10th–12th week. During the latter period food intake increased slightly.

By the end of the experiment the rats fed on the control diet had increased their body-weight by 106%, whereas those in the deficient group had lost 56% of their original body-weight.

Table 3. *Percentage of rats showing gross changes due to protein deficiency during the 16-week experiment*

Criterion	♂	♀
Diarrhoea	50	57
Oedema	54	51
Loss of hair	33	38
Mortality rate	30·5	17·6

From the 10th week of feeding on the low-protein diet 29 % of the female and 38 % of the male rats began to lose their hair; 54 % of the females and 51 % of the males developed oedema (Table 3). The loss of hair always began before the appearance of oedema. The loss of hair and oedema were mostly localized in the region between the fore-limbs. The weight of oedematous rats was constant during the 10 d during which oedema was manifested. After the disappearance of oedema the weight of the rats fell and the animals died or were killed.

Diarrhoea accompanied the onset of oedema and loss of hair—that is the period in which protein deficiency was established (Table 3).

The mortality rate was higher among male rats (30·5 %), and deaths occurred at an earlier period (after 5 weeks) than among females. Female rats had a mortality rate of 17·6 % and deaths occurred during the third period onward.

Biochemical changes in serum

A sharp fall (40 %) in total serum proteins was recorded after the first 4 weeks of feeding on the low-protein diet; after that the decrease was gradual (Table 4). Serum total proteins of the control rats remained unchanged throughout the experiment.

During the first 4 weeks of the experiment the non-essential: essential amino acid ratio in the serum of rats given the low-protein diet was 147 % of that found in the controls. This value had increased to 261 % by the end of the second 4 weeks. During the third 4 weeks the ratio was equal to that of normal animals, and then increased abruptly to 607 % during the last 4 weeks. Only in the last period did these ratios differ significantly from the control values.

When the changes that occurred in the concentration of each amino acid in the serum of rats given the low-protein diet were studied, it was shown that threonine was the main amino acid that was significantly reduced from the first period till the end of the experiment (Table 5); other essential amino acids – valine, leucine, isoleucine, and methionine – were also reduced during the first and second periods. The non-essential amino acids (aspartic acid, glutamic acid, serine, glycine, alanine and histidine) were almost normal, except for glutamine which was markedly reduced.

During the third period there was a marked increase in the essential amino acids (tyrosine, valine, leucine and isoleucine and methionine) and the non-essential amino acids (aspartic acid, glutamic acid, alanine and glutamine). During the 4th period a significant reduction occurred in both essential and non-essential amino acids.

The ratios between individual amino acids were calculated during the four periods

Table 4. *Serum total proteins and non-essential:essential amino acid ratios in control rats (C) and rats given a low-protein diet (LP)*

(Mean values with their standard errors: numbers of rats in parentheses)

	Total proteins (g/l)				Amino acid ratios			
	C		LP		C		LP	
Period no.	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1 (0–4 weeks)	71·2 (4)	0·6	42·0 (4)	1·9	1·15 (4)	0·14	1·69 (6)	0·23
2 (5–8 weeks)	66·7 (4)	1·8	41·3 (6)	3†	1·19 (4)	0·12	3·05 (6)	1·60NS
3 (9–12 weeks)	67·6 (4)	0·5	33·0 (4)	1·6†	1·47 (4)	0·11	1·43 (7)	0·42 NS
4 (13–16 weeks)	67·5 (4)	0·3	28·8 (8)	1·6†	1·27 (4)	0·07	7·71 (6)	2·08**

NS, not significant.
Values significantly different from those of control groups: $**P < 0.01$; † $P < 0.005$.

(Table 6). The ratios alanine:threonine, and serine+glycine:threonine were always significantly higher than normal during the four periods. The latter ratio showed a negative, though not significant correlation coefficient (−0·14) with body-weight of the animals given the low-protein diet.

DISCUSSION

From the beginning of the experiment the animals given the low-protein diet consumed less food than the controls, and whereas the latter gained weight normally the former lost weight progressively. The calculated daily energy intake per g body-weight of animals given the low-protein diet was found to be constant from the 5th week of the experiment. The reduction in appetite, constant daily energy intake per g body-weight and loss of weight of the animals show that the dietary amino acids were not sufficient to meet the demands for tissue synthesis.

The weight of the rats given the low-protein diet decreased continuously till the end of the 9th week and then remained constant during the next 4 weeks. A slight increase in food intake was observed. During that period, loss of hair, oedema and diarrhoea began to appear—signs similar to those reported in children with kwashiorkor. The slight increase in food intake suggests an adaptation of the animal to compensate for the loss of body constituents through diarrhoea.

This experiment demonstrated the possibility of using the rat as an experimental model for induction of protein-energy malnutrition with its known clinical manifestations similar to those of kwashiorkor in children. To achieve this, rats had to be given a low-protein diet (10 g/kg) for at least 10 weeks. Grimble *et al.* (1969) were unable to produce such changes in rats because the diet they gave was not low enough in its protein concentration (60 g/kg).

Early biochemical changes were indicated by the 41% fall in serum protein

Table 5. *Concentration (mg/l) of some amino acids in the serum of control rats (C) and rats given a low-protein diet (LP)*

Amino acid		Period 1 (0–4 weeks) Mean	SE	Period 2 (5–8 weeks) Mean	SE	Period 3 (9–12 weeks) Mean	SE	Period 4 (13–16 weeks) Mean	SE
Cystine	C	19·9	3·7	19·7	3·7	44·2	14·5	17·7	0·4
	LP	14·8	2·0	23·8	7·1	27·8	6·8	13·6	4·7
Aspartic acid	C	8·1	1·7	5·1	0·1	28·6	12·6	6·2	0·2
	LP	10·1	4·2	7·8	1·7	18·6	5·2	1·7	0·8***
Glutamic acid	C	7·8	1·8	4·4	0·6	11·8	3·1	4·4	0·7
	LP	7·0	2·9	7·0	1·5	18·2	6·0	1·9	0·4*
Serine	C	11·0	1·4	17·0	4·7	11·8	2·7	7·0	1·2
	LP	15·9	2·9	9·2	2·5	18·9	4·0	7·2	0·8
Glycine	C	13·7	0·9	10·9	2·6	9·7	2·6	5·9	0·6
	LP	13·0	3·7	9·4	2·4	15·2	5·2	3·0	0·7**
Alanine	C	16·6	2·6	10·2	2·8	13·0	1·9	10·1	1·8
	LP	14·8	3·3	13·3	2·1	23·7	7·2	6·9	1·7
Glutamine	C	21·2	2·6	46·2	15·2	35·0	3·8	28·1	3·2
	LP	17·0	1·7	15·1	2·5*	31·0	6·2	14·8	2·7***
Tyrosine	C	18·6	3·6	22·3	3·6	20·2	5·5	14·6	3·9
	LP	13·0	3·0	21·4	7·8	33·2	7·1	5·3	1·5***
Threonine	C	13·7	1·1	11·9	2·4	14·1	1·8	14·8	3·8
	LP	6·6	1·5***	5·8	0·8*	11·5	2·8	2·8	0·8***
Valine	C	9·3	0·9	7·7	0·3	8·9	1·7	7·0	1·5
	LP	7·0	1·1	4·6	1·4*	14·1	4·1	2·5	0·5***
Methionine	C	6·6	1·2	8·2	0·8	11·3	0·7	—	—
	LP	4·6	0·2	5·8	0·2	21·0	5·5	—	—
Leucine and isoleucine	C	11·7	0·8	11·5	0·7	9·2	2·0	6·8	1·7
	LP	8·0	1·7	7·2	2·9	20·2	6·8	1·7	0·5***
Phenylalanine	C	5·4	0·6	7·4	0·05	8·5	0·2	8·6	3·2
	LP	5·9	0·7	5·4	2·9	11·8	3·7	6·8	2·3
Lysine	C	8·6	1·4	13·5	0·1	13·6	3·8	3·9	0·1
	LP	8·6	1·8	15·5	4·1	12·4	2·1	3·8	1·5
Histidine	C	11·5	4·2	39·0	0·5	23·6	0·5	8·3	3·0
	LP	13·1	5·6	35·3	9·2	32·4	6·8	8·0	3·0

Values significantly lower than for control group: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

concentration during the first 4-week period, the reduction afterwards being gradual. Clinical and pathological changes that occurred in the livers of rats given the low-protein diet will be described in another report.

Distortion of the serum non-essential:essential amino acid ratio occurred simultaneously with the reduction in the concentration of serum proteins, with very large changes during the first and second periods. However, during the third period, the ratio returned to normal, suggesting that great biochemical changes preceded the clinical manifestations. This distortion in the ratio was due to the abrupt increase in the essential amino acids threonine, valine, leucine and isoleucine (Table 5) to values even higher than normal.

Similar findings were previously reported by Holt *et al.* (1963). In their study some

Table 6. *Ratios between individual amino acid in the serum of control rats (C) and rats given a low-protein diet (LP)*

(Mean values with their standard errors; numbers of rats in parentheses)

	Ratio, (serine + glycine): (valine + leucine)				Ratio, alanine: threonine				Ratio, (serine + glycine): threonine			
	C		LP		C		LP		C		LP	
Period no.	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1 (0–4 weeks)	1·11 (4)	0·14	2·00 (6)	0·17†	1·19 (4)	0·39	2·20 (6)	0·26*	1·45 (4)	0·24	4·61 (6)	0·58†
2 (5–8 weeks)	0·94 (4)	0·22	4·18 (6)	1·63*	0·91 (4)	0·14	1·56 (6)	0·22*	1·48 (4)	0·09	3·95 (6)	0·68†
3 (9–12 weeks)	1·20 (4)	0·17	1·48 (8)	0·43 NS	0·91 (4)	0·14	2·82 (6)	0·97*	1·35 (4)	0·20	3·31 (8)	0·51†
4 13–16 weeks)	1·29 (4)	0·36	4·07 (10)	0·82**	0·92 (4)	0·17	4·01 (10)	1·14†	1·36 (4)	0·54	6·03 (10)	0·84†

NS, not significant.
Values significantly lower than for control group: * $P < 0.05$; ** $P < 0.01$; † $P < 0.005$.

of the children suffering from protein-energy deficiency showed normal non-essential: essential amino acids ratios. Such observations could be explained by the increase in the circulating essential amino acids valine, leucine and isoleucine due to muscle breakdown. This suggestion was supported by the findings of Sidransky & Verney (1970) and Waterlow (1968), who demonstrated in rats that protein catabolism of skeletal muscles increased when the protein content of the diet was reduced.

The possibility that the non-essential: essential amino acid ratio might be affected by malabsorption which developed in these rats and manifested by diarrhoea has been refuted by Kirsch, Saunders & Brock (1968). They demonstrated normal amino acid absorption from the gut in protein-deficient rats in spite of the presence of shortening and blunting of the villi in the intestine of these animals.

The significant increase in the amino acid ratio during the fourth period of our study is surprising. Sidransky & Verney (1970) reported that during protein deficiency skeletal muscles respond by decreasing protein synthesis and increasing protein catabolism, therefore diverting the amino acids into the circulation and maintaining normal or increased liver protein synthesis. Thus, during the fourth period, liver protein synthesis must have been increased and the circulating amino acids diverted towards this synthesis. So changes in the serum amino acid ratio are not an indication of the progressive changes that occur in the different stages of protein-energy malnutrition.

A better approach is by calculating the ratio of one or two individual amino acids to each other (Table 6). Our experiment showed that the ratio of alanine to threonine and that of serine + glycine to threonine were always significantly higher than normal, the latter being higher in all stages of protein deficiency. Besides, there was a tendency of the ratio to increase with body-weight deficit.

This method is also simple since only two or three amino acids need to be determined, even by using the one-dimensional paper-chromatography technique. The rats fasted overnight as well as those fasted for a few hours showed almost similar ratios. The use of this ratio in applied nutrition programmes would help to indicate the subclinical as well as the different stages of protein-energy malnutrition among children.

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