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Liver DNA and RNA Metabolism in Rats Fed Diets Lacking Methionine plus Cysteine with or without Restricted Energy

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Ponderal parameters, soluble protein content and nucleic acid (DNA and RNA) metabolism were studied in liver of growing male Wistar rats fed with different diets: control diet (Control group) containing 10 % protein (casein 9.8 % plus DL-methionine 0.2 %); diet 2 (Group 2) containing 10 % protein, lacking Met plus Cys; and, diet 3 (Group 3), containing 20 % protein, lacking Met plus Cys and with 50 % of energy restriction (restricted food intake by experimental design). Diets 2 and 3 were compared with the control diet to know the effects produced by the lack of Met plus Cys and the energy restriction, for an experimental period of 14 days, the animals being slaughtered on the 4th, 8th and 14th days. Food intake, body and liver weights, relative liver weight, cellular size and RNA content per organ and per mg protein decreased in groups 2 and 3, group 3 being affected more than group 2. Diet 2 produced a decrease in DNA content, due to lack of Met+Cys. Acid DNAse activity per organ diminished in group 2 on days 8 and 14, and in group 3 on the 8th day. RNA/DNA ratio diminished in group 2 and 3 due to a proportional RNA reduction with respect to the DNA content. Acid RNAse activity per organ diminished in group 2 on the 8th and 14th days and RNAse per mg of protein increased in group 3 at the end of the treatment, therefore the RNA content decreased. The content of DNA in liver is lower than RNA content in rats fed diet 2 and the opposite occurs with diet 3.

Key words: Liver, Nucleic acids, Sulfur amino acid deficiency, Energy, Food intake.

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Nutritional alterations apparently exert modifications in hepatic protein synthesis by acting at transcriptional (22), translational (29) and posttranscriptional level (23). The regulation and control of hepatic synthesis by dietary components, especially amino acids, are complex. DE JONG and SCHREIBER (9) suggest that dietary protein depletion decreases protein synthesis as a result of decreased levels of mRNA.

Low protein diets decrease liver free amino acid concentrations. However, aminoacyl-tRNA-synthetases in liver continue functioning at high level (12). Liver carries out available amino acids for protein synthesis and t-RNAs have high binding capacity to free amino acids in spite of their low concentration, binding with the initiated factors of the 40S ribosomal subunit. This indicate that the low capacity to initiate protein synthesis in this organ is an adaptative mammalian response to an inadequate supply of amino acids.

Liver parenchymal cells grow by cell division in rats, which occurs between 8 and 60 day-old animals, and the total liver DNA increases 12 fold. The growth of tissues are accompanied by a fall in RNA concentration.Altered intakes of protein and amino acids modulate the rates of the major systems (protein synthesis, protein degradation and amino acid oxidation) responsible for the maintenance of organ and whole-body protein and amino acid homeostasis (33).

Methionine is one of the initiators among other amino acids in protein synthesis (32). Therefore, the lack of sulfur amino acids in the diet induces an increase of catabolism in several tissues or a decrease of protein synthesis. Methionine is one of the essential amino acids, but cyst(e)ine is not required for growth in rats and other animals (20). Cyst(e)ine partly diminishes the need of methionine for the growth of animals (30) and is preferentially used for body protein synthesis and its response pattern to the protein synthesis resembles that of the essential amino acids, the incorporation of cysteine into body protein being extensively increased during protein depletion (30).

Therefore, the aim of this work was to find out the effects produced by the lack of S-amino acids and the energy restriction on the nucleic acid turnover and its adaptation, and other related parameters such as food intake, body and liver weights, soluble protein content, number of cells, cellular size (Protein/DNA), RNA/DNA and RNA/protein ratios. Experimental diets lacked methionine plus cysteine, because cystine could replace approximately 70% of the dietary requirement for methionine. The availability of dietary cystine must allow the efficient conservation of a limited methionine pool by means of augmented homocysteine methylation and/or decreased cystathionine synthesis (10), and with energy restriction.

Materials and Methods

Animals and experimental design.– Seventy-eight growing male Wistar rats were fed, after weaning, a laboratory stock-diet (type A.04 from Panlab, Barcelona, Spain), and allowed *ad libitum* access up to an average weight of 87 ± 6.9 g (28 days old) had been reached. Then, they were placed into individual metabolism cages in a room with a maintained environmental temperature of 23 °C, suitable humidity, and 12 h light-cycle (07:00-19:00). After three days adapting to the control diet, the experimental procedure was started.

Animals were separated into 3 groups of 26 rats each and fed with different diets (table I): Control diet (Control group), containing 10 % protein (casein 9.8 % +

Table I. *Composition of experimental diets (g/kg).*
Diet 2 formed with a mixture of crystallized amino acids without Met and Cys that were replaced with other non essential amino acids. Diet 3 has double concentration of amino acids, vitamins and minerals. Olive oil, starch and sucrose contents were adjusted to supply half the energy.

Ingredients	Control diet	Diet 2	Diet 3
Casein (delipidate)	98.0	—	—
DL-Methionine	2.0	—	—
Amino acid mixture*	—	105.4	210.80
Cellulose	5.4	50.0	100.00
Starch	200.0	200.0	346.36
Sucrose	534.6	534.6	172.84
Olive oil	50.0	50.0	50.00
Vitamin mixture**	10.0	10.0	20.00
Mineral misture**	50.0	50.0	100.00

*National Research Council (24).
**Association of official agricultural chemist (1).

DL-methionine 0.2 %) (13); Diet 2 (Group 2), containing 10 % protein, a mixture of crystallized amino acids whitout Met and Cys which were replaced with other non essential amino acids; Diet 3 (Group 3), containing 20 % protein (no Met, no Cys), and with an adjustment in the rest of nutrients in order to supply half the food intake of group 2 (by experimental design), and, thus yielding half the energy without altering the other constituents.

Amino acid mixture agreed with the National Research Council (24) and vitamin and mineral mixtures agreed with the Association of Official Agricultural Chemist (2).

During the experiment of 14 days, water and food were administered on demand depending on the need values with the exception of group 3 fed 50 % of the average food intake of group 2.

Food intake of group 2 was daily measured in each rat and 50 % of the average food intake of this group was used as food intake in group 3, but supplied one day after.

In group fed diet 3 (restricted intake), the experiment was limited to 10 days because of the physical impairment that they showed which caused the death of almost all the animals of group 3 from the 12th day on.

On 4th, 8th and 14th day, six rats of each group were anaesthetized with sodium pentobarbital, i.p. (30 mg/kg). Animals were killed by decapitation between 09:00 and 12:00 am, and livers were removed, frozen in liquid nitrogen, weighed and kept at -20 °C before analysis.

Analytical procedures.– Aliquots (1 g) of liver were homogenized with a cold buffer (NaCl 0.1 mol/L, $NaCO_3H$ 0.05 mol/L, pH = 7.4) with a g/L ratio of 200 g/1000 ml. Afterwards, the sample was centrifuged at 100,000 g for 10 min at a temperature of 0-4 °C, and aliquots of supernatant were used to evaluate soluble proteins and hydrolising DNA and RNA enzyme activities. Protein was measured using the method of LOWRY *et al.* (17). DNAse (Deoxyribonuclease I EC 3.1.21.1) was measured by the method of MC DONALD (19), results were expressed in I.U. RNAse (Ribonuclease EC 3.1.27.5) was measured by the method of

KALNITSKY *et al.* (16). DNA was determined by the technique of BURTON (6) and RNA by the technique of SCHMIT and THANNHAUSER (27) with a modification from MUNRO and FLECK (21).

Statistical analysis.– Results were analyzed by two independent analysis (by one way ANOVA in each case) (15), ANOVA for each day to compare different diets and ANOVA for each diet to compare different days.

Test of TUKEY (31) between days or between diets was performed on account of the impossibility to estimate each term from the two way ANOVA to calculate Diet x Time, as the table results were incomplete due to the death of the animals of group 3 on the 14th day. Tukey Test was used due to this defect, so that temporal correlation was not used. Tukey-Cramer adjustment was used, when there was different size of cell and there was no continuity on the time. The significant degree between the diet and the days was 5 %, if $P > 0.05$, values are indicated as non significant.

Results

Food intake was significantly lower in groups 2 and above all, 3 when compared with the control group. The food intake decreased by 50 %, 42 % and 37 % on the 4th, 8th and 14th days respectively, in group 2 and 74 % and 72 % on the 4th and 8th days in group 3.

The decrease of food intake produced a parallel decrease in body weight by 17 %, 37 % and 53 % in group 2 (days 4th, 8th and 14th, respectively) and 29 % and 41 % in group 3 (4th and 8th days).

Liver weight was significantly greater in control group from the 4th day up to 8th day by 26 % and did not vary between the 8th and 14th days. In group 2 liver weight diminished by 34 %, 54 % and 65 % on the 4th, 8th and 14th days compared with the control group, and in group 3 it was lower by 50 % and 67 % on the 4th and 8th days, respectively.

Relative liver weight decreased by 27 % and 26 % on the 8th and 14th days in group 2, and by 30 % and 45 % on the 4th and 8th days in group 3.

Soluble protein content (table II) did not vary during the experimental period, in control and 3 groups, although it decreased by 71 % on the 14th day with respect to the 4th day and by 69 % on the 14th day with respect to the 8th day in group 2. Both experimental diets did not modify the liver soluble protein content on the 4th day, but it decreased in group 2 on the 8th and 14th day by 32 % and 81 %; and by 54 % on the 8th day in group 3.

Liver DNA content (table III) increased through the experimental period by 30 % and 40 % on the 8th and 14th days, respectively, compared to the 4th day in control group. The lack of Met+Cys in the diet reduced the DNA content by 69 %, 68 % and 65 % on the 4th, 8th and 14th days respectively. The energy restriction did not modify the DNA content.

Acid DNAse activity per organ (table III) diminished by 57 % and 70 % on the 8th and 14th days in group 2 compared with the control group, and this activity per mg of protein decreased by 33 % and 17 % on 4th and 8th days. The energy restriction did not modify either parameter.

The number of liver nuclei (table III) increased in groups control and 2 through the experimental period. The lack of Met+Cys in the diet reduced the nucleus number by 69 %, 68 % and 65 % on the 4th, 8th and 14th days, respectively, and the diet with energy restriction produced

Table II. *Food intake, body and liver weights, relative liver weight, liver soluble proteins in growing male Wistar rats fed different diets. Control diet: 10 % protein (casein + DL-Methionine), Diet 2, lacking Met+Cys, and Diet 3, lacking Met+Cys and 50 % energy restriction.* Data are means + SEM of 6 rats. Significant differences ($p < 0.05$): a, versus control; b, versus diet 2; *, between days.

Treatment	Day	Food intake (g)	Body weight (g)	Liver weight (g)	Rel. Liver weight (mg)	Liver sol. prot. (mg)
Control	4	13.3 ± 0.60	109.6 ± 4.7	5.86 ± 0.22	5.33 ± 0.47	337.47 ± 3.98
	8	13.9 ± 0.60	125.4 ± 3.1	7.41 ± 0.46*	5.90 ± 0.15	431.52 ± 7.29
	14	14.4 ± 0.70	146.8 ± 5.6*	7.40 ± 0.28*	5.07 ± 0.16	463.06 ± 0.17
Diet 2	4	6.6 ± 0.30[a]	90.7 ± 2.3[a]	3.88 ± 0.29[a]	4.27 ± 0.12	313.55 ± 46.83
	8	8 0 ± 0.50[a*]	79.3 ± 5.9[a*]	3.44 ± 0.23[a]	4.33 ± 0.40[a]	292.03 ± 29.71[a]
	14	9.0 ± 0.60[a]	68.9 ± 3.3[a*]	2.58 ± 0.10[a*]	3.74 ± 0.30[a*]	90.16 ± 14.17[a*]
Diet 3	4	3 .4 ± 0.20[ab]	77.6 ± 4.6[a]	2.91 ± 0.15[ab]	3.75 ± 0.33[a]	250.46 ± 27.97
	8	3.9 ± 0.02[ab]	74.5 ± 2.1[a]	2.41 ± 0.24[a]	3.23 ± 0.11[a]	213.20 ± 19.32[a]

Table III. *DNA, DNAse/liver, DNAse/soluble proteins, number of nucleus and cellular size in liver of growing male rats fed different diets. Control diet: 10 % protein (casein + DL-Methionine), Diet 2, lacking Met+Cys, and Diet 3, lacking Met+Cys and 50 % energy restriction* Data are means + SEM of 6 rats. Significant differences ($p < 0.05$): a, versus control; b, versus diet 2; *, between days.

Treatment	Day	DNA (mg)	DNAse/liver (UI)	DNAse/sol. prot (UI/mg)	Nucleus number (millions)	Cellul. size (ng)
Control diet	4	16.22 ± 0.97	206.1 ± 10.7	0.64 ± 0.07	2617 ± 156	2.25 ± 0.06
	8	21.08 ± 0.74*	306.1 ± 71.0	0.69 ± 0.14	3401 ± 119*	2.20 ± 0.17
	14	22.74 ± 0.79*	326.6 ± 41.3	0.72 ± 0.06	3668 ± 127*	2.03 ± 0.14
Diet 2	4	5.09 ± 0.70[a]	139.1 ± 40.1	0.43 ± 0.13[a]	821 ± 113[a]	5.24 ± 1.04[a]
	8	6.67 ± 0.32[a*]	133.0 ± 16.5[a]	0.50 ± 0.07[a]	1076 ± 52[a*]	3.26 ± 0.33[a*]
	14	7.93 ± 0.96[a*]	97.9 ± 11.0[a]	1.19 ± 0.20*	1279 ± 155[a*]	2.12 ± 0.21*
Diet 3	4	17.63 ± 0.67[b]	275.1 ± 11.3	1.21 ± 0.58[b]	2844 ± 108[b]	1.02 ± 0.03[ab]
	8	14.94 ± 1.40[b]	168.7 ± 16.6[ab]	0.84 ± 0.16[b]	2409 ± 226[ab]	1.03 ± 0.13[ab]

Table IV. *RNA, acid RNAse, RNAse/soluble proteins, RNA/DNA and RNA/soluble proteins in liver of growing male rats fed different diets.* Control diet: 10 % protein (casein + DL-Methionine), Diet 2, lacking Met+Cys, and Diet 3, lacking Met+Cys and 50 % energy restriction. Data are means + SEM of 6 rats. Significant differences ($p < 0.05$): a, versus control; b, versus diet 2; *, between days.

Treatment	Day	RNA (mg)	Acid RNAse (UI liver)	RNAse/sol. prot. (mUI/mg)	RNA/DNA (mg/mg)	RNA/sol. prot. (mg/mg)
Control diet	4	159.3 ± 1.93	2.03 ± 0.51	6.42 ± 1.85	9.98 ± 0.69	0.49 ± 0.54
	8	167.8 ± 8.29	2.67 ± 0.76	6.53 ± 1.89	7.94 ± 0.49	0.40 ± 0.04
	14	191.7 ± 15.1	3.88 ± 0.13	8.99 ± 1.09	8.52 ± 0.82	0.44 ± 0.06
Diet 2	4	19.76 ± 1.93^{a}	1.77 ± 0.12	6.19 ± 0.10	3.99 ± 0.28^{a}	0.07 ± 0.01^{a}
	8	25.15 ± 1.16^{a}	1.50 ± 0.16^{a}	5.56 ± 1.20	3.78 ± 0.07^{a}	0.09 ± 0.01^{a}
	14	26.70 ± 3.50^{a}	1.26 ± 0.79^{a}	12.85 ± 0.83*	3.35 ± 0.06^{a}	0.36 ± 0.11*
Diet 3	4	6.63 ± 0.60^{ab}	2.76 ± 0.48	10.72 ± 0.95	0.38 ± 0.03^{ab}	0.03 ± 0.003^{a}
	8	5.76 ± 1.04^{ab}	3.46 ± 0.35^{b}	16.21 ± 0.82^{ab}*	0.37 ± 0.04^{ab}	0.03 ± 0.003^{a}

a decrease of this parameter on the 8th day (29 %) compared to control group.

Cellular size (table III) did not vary in control and 3 groups and decreased in group 2 through the experimental period. The lack of Met+Cys in the diet produced a hypertrophy (133 % and 48 % on the 4th and 8th day) and the energy restriction produced an atrophy (55 % and 49 % on the 4th and 8th day) compared with the control group, with respect to soluble protein content.

Liver RNA content and RNA/DNA (RNA content in each cell) (table IV) did not vary in the three groups during the experimental period; the lack of Met+Cys provoked a decrease in RNA content, RNA/DNA ratio on the 4th, 8th and 14th days compared with the control group (88 %, 85 % and 86 % on RNA content; and 60 %, 52 % and 61 % on RNA/DNA ratio). RNA/soluble proteins ratio decreased in group 2 on the 4th and 8th days (86 % and 78 %, respectively). The energy restriction imposed to animals (group 3) provoked a decrease on RNA content (96 % and 97 %), on RNA/DNA ratio (96 % and 95 %) and on RNA/protein (94 % and 93 %) on days 4th and 8th, respectively.

Acid RNAse activity per organ (table IV) diminished by 56 % and 32 % on 8th and 14th day in group 2 and per mg of protein did not vary compared with the control group. The diet with energy restriction imposed on animals in group 3 did not provoke any variation in the acid RNAse activity per organ, however it was higher per mg of protein in this group on the 8th day (148 %) than in the control group.

Discussion

Amino acids play an important role in the regulation of food intake by the synthesis of neurotransmitters. BEVERLY *et al.* (5) observed that the lack of essential amino acids in the diet influences the protein synthesis which is necessary for improving the anorexia and adversion to imbalanced amino acid diets.

The food intake reduction decreased body weight in both experimental groups, which agrees with JABLONSKI and RAFALSKI (14). Changes were not observed among either experimental group, which could be due to the use of amino acids from the diet and to the increase of fat mobilization from adipose tissue, the rate of myofibrillar protein breakdown being reduced in growing rats (26).

Liver weight increased in control group due to the fact that animals were in growth period. The decreased liver weight in groups 2 and 3 can be due to the lack of methionine, one of the amino acid initiators in the protein synthesis.

The decrease in relative liver weight on the 8th day in group 2 and on the 4th day in group 3 is related to a diminution of protein synthesis as the study of soluble proteins and RNA content on those days indicates.

Protein breakdown in liver may well increase initially (33) when the availability of amino acids is restricted, but with a continued low intake of amino acids, the breakdown of liver proteins declines (11).

The lack of sulfur amino acids has been reported to be related to a great decrease of protein synthesis, especially when the diet lacks methionine (18). In this paper a reduction of soluble protein content was observed on days 8 and 14 in group fed diet 2 and on day 8 in group fed diet 3, although protein synthesis was being controlled in liver by different mechanisms from that in muscle. The lack of methionine plus cysteine could reduce the content of soluble protein content as a consequence of the reduction of RNA synthesis, which has the same intensity on the 8th day in both experimental diets.

Omission of methionine and cysteine from a nutritionally adequate diet produces the same nucleolar changes as if they were removed from all amino acids (3). Deficiencies in some of the essential amino acids cause either little result (methionine, tryptophan, threonine, lysine) or none at all (3). AOI *et al.* (1) suggests that a close relationship exists between amino acid metabolism and the regulation of nuclear DNA synthesis in liver. Inducing hepatic DNA synthesis in protein-deficient rats required at least six amino acids: valine, threonine, isoleucine, tryptophan, methionine and lysine.

Liver DNA content increased in control group as it was constituted by young rats with the liver in hyperplasia phase. Protein malnutrition lessened the nucleic acid synthesis, so that the lack of Met+Cys reduced the DNA content compared to control group. The energy restriction did not paradoxically modify this parameter due to a decrease in RNA synthesis as a consequence of a diminution of RNA polymerase A, which synthesizes ribosomal RNA (7) and RNA polymerase B, which synthesizes mRNA. It was also observed that a major RNAse acid activity per mg of protein with the energy restriction diet produced a liberation of polyribonucleotides that could improve the DNA synthesis. This hypothesis was confirmed with the experiment with starved animals (28). The activity of acid DNAse per mg of protein decreased in group 2, while the DNA content was higher in group 3, which could mean a clear reduction in the synthesis rather than in DNA degradation in group 2.

Liver RNA content, RNA/DNA and RNA/protein ratios decreased in group 2 due to a minor RNA synthesis and not to a major acid RNAse activity, while in group 3 the decrease of liver RNA content, RNA/DNA ratio and RNA/soluble proteins were due to a minor RNA synthesis and major acid RNAse activity per mg of protein on 8th day.

The lack of Met+Cys reduced the RNA/DNA ratio indicating a diminution in the protein synthesis per unit of DNA, and they were accompanied by a diminution of cellular ribosome content, which was most affected when animals were fed the energy restricted diet.

The energy restriction produced a loss of RNA/protein ratio in spite of having a greater DNA content than in group 2; RNA content diminished more with energy restriction by a higher acid RNAse activity by mg of protein as discused before. The energy restriction affected more the RNA/protein ratio than the lack of Met+Cys, probably due to fall of polymerase activity and reduced rates of rRNA synthesis, in agreement with COWARD *et al.* (8).

Liver DNA content in group 3 was similar to control group, but the machinery activity of protein synthesis was reduced at a lower level than in group 2 on days 4th and 8th with respect to the soluble protein content. The cellular size was greater in group 2 than in control and group 3, with a major RNA content per cell in group 2 than in group 3, due to a diminution in plasma insulin content studied by us (25). The insulin reduction can affect phosphorylation/dephosphorylation of transcription factors, and it may be a common mechanism by which insulin regulates the rate of transcription of differents genes (4), diminishing both the RNA content in each cell and the soluble protein synthesis.

In conclusion, the results of this study showed that in comparison to control casein-fed rats, those fed a lack of Met+Cys diet exhibited a diminution of DNA synthesis and acid DNAse activity, and a major RNA content and lower acid RNAse activity than the rats fed the energy restricted diet.

J. L. REY DE VIÑAS, M. GONZÁLEZ-FERNÁNDEZ y M. C. SANDOVAL. *Metabolismo del DNA y del RNA en hígado de ratas sometidas a dietas carentes de metionina y de cisteína, con y sin restricción energética.* Rev. esp. Fisiol. (J. Physiol. Biochem.), 51 (3), 129-138, 1995.

Se estudian parámetros ponderales, proteínas solubles y el metabolismo de los ácidos nucleicos en hígado de ratas machos Wistar en período de crecimiento, alimentadas durante 14 días con diferentes dietas: del 10 % de proteína, Dieta control (caseína 9,8 % + DL-Metionina 0,2 %) y Dieta 2, (aminoácidos cristalinos carentes de Met y Cys); del 20 % de proteína, Dieta 3 (sin Met+Cys) y con el 50 % de restricción energética por ingesta restringida de alimento. Se comparan las dietas 2 y 3 con la control, para conocer los efectos provocados por la carencia de Met+Cys y la restricción energética. Los animales se sacrifican a los 4, 8 y 14 días. La ingesta, el peso corporal, el total y el relativo del hígado, el tamaño celular y el contenido de RNA por órgano y por mg de proteína disminuyen por efecto de las dietas 2 y 3, siendo mayor la disminución debida a la restricción energética. La dieta 2 disminuye el contenido de DNA debido a la carencia de Met+Cys. La actividad enzimática DNAsa por órgano disminuye en el grupo 2 en los días 8 y 14 y en el grupo 3 el día 8. La relación RNA/DNA disminuye en los grupos 2 y 3 ya que hay una reducción proporcional de RNA respecto al contenido de DNA. La actividad RNAsa ácida por órgano disminuye en el grupo 2 en los días 8 y 14, y por mg de proteína aumenta en el grupo 3 al final del tratamiento respecto al control, por lo que disminuye el contenido de RNA. El contenido de DNA y el de RNA es menor en las ratas alimentadas con la dieta 2, y en las ratas de la 3, el contenido de RNA es menor que el de DNA.

Palabras clave: Hígado, Acidos nucleicos, Aminoácidos azufrados, Energía, Ingesta.

References

1. Aoi, S., Wen, L-T., Kuwahata, T. and Tsukada, K. (1987): *J. Nutr.*, 117, 673-667.
2. Association of official agricultural chemists (1970): Official methods of analysis. Washington, D. C. p. 800-801.
3. Bailey, R. P., Wooman, M. J., Sawai, Y., Tsukada, K. and Lieberman, I. (1976): *Proc. Natl. Acad. Sci. USA*, 73, 3201-3205.
4. Blackshear, P. J. (1992): *Biochem. Soc. Trans.*, 20, 682-685.
5. Beverly, J. L., Gietzen, D. W. and Rogers, Q. S. (1991): *J. Nutr.*, 121, 754-761.
6. Burton, K. (1956): *Biochem. J.*, 62, 315-323.
7. Coupar, B. E. H., Davies, J. A. and Chesterton, C. J. (1978): *Eur. J. Biochem.*, 84, 611-623.
8. Coward, W. A., Whitehead, R. G. and Lunn, P. G. (1977): *Br. J. Nutr.*, 38, 115-126.
9. De Jong, F. A. and Schreiber, G. (1987): *J. Nutr.*, 117, 1795-1800.
10. Finkelstein, J. D., Martin, J. J. and Harris, B. J. (1986): *J. Nutr.*, 116, 985-990.
11. Garlick, P. J., Millward, D. J., James, W. P. T. and Waterlow, J. C. (1975): *Biochim. Biophys. Acta*, 414, 71-84.
12. Harper, A. E. (1974): In "The control of Metabolism" (Sink J. D., ed). Pennsylvania State University Press, University Park, PA, pp. 49-74.
13. Heger, J. and Frydrych, Z. (1985): *Br. J. Nutr.*, 54, 499-508.
14. Jablonski, E. and Rafalski, H. (1984): *Br. J. Nutr.*, 51, 235-243.
15. Johnson, R. and Bhattacharyya, G. (1987). Statistics: principles and methods. John Wiley.
16 Kalnitsky, G., Hummel, J. P., Resminick, H., Carter, J. P., Barnett, L. B. and Diersks, C. (1959): *Ann. N. Y. Acad. Sci.*, 81, 542-566.
17. Lowry, D. A., Rosebrough, N. J., Fair, A. L. and Randall, R. J. (1951): *J. Biol. Chem.*, 193, 265-275.
18. Martínez, J. A., Goena, M., Santidrián, S. and Larralde, J. (1987): *Ann. Rev. Metab.*, 31, 146-153.
19. Mc. Donald, M. R. (1955): In "Methods in enzymology" (S. P. Colowick and N. O. Kaplan, eds.). Academic Press. New York, Vol. II, pp. 437-447.
20. Meister, A. (1965). Biochemistry of the amino acids. Vols. I and II, Academic Press, New York.
21. Munro, H. N. and Fleck, A. (1966): *Analyst*, 91, 78-88.
22. Murty, C. N. and Sidransky, H. (1972): *Biochim. Biophys. Acta*, 262, 328-335.
23. Murty, C. N., Verney, E. and Sidransky, H. (1976): *Lab. Invest.*, 34, 77-85.
24. National Research Council (1978): In "Nutrient requeriments of the laboratory rat". Natural Academy of Science. Washington, pp. 13-26.
25. Rey de Viñas, J. L., Sánchez-Barco, J. L., González-Fernández, M., Rubio, J., de Arribas,

B., Osuna, C. and Cañellas, S. (1992): In "Branched-Chain Amino Acids: Biochemistry, Physiopathology, and Clinical Science (Schauder, P. *et al.*, eds.), pp.245-251. Raven Press, New York.
26. Santidrián, S. (1981): *Rev. esp. Fisiol.*, 37, 23-30.
27. Schmidt, G. and Thannhauser, S. J. (1945): *J. Biol. Chem.*, 161, 83-89.
28. Shyamala, V. (1986): *Biochem. Int.*, 13, 33-40.
29. Sidransky, H., Sarma, D. S. R., Bongiorno, M. and Verney, E. (1986): *J. Biol. Chem.*, 243, 1123-1132.
30. Tanaka, H., Nakatomi, Y., Mori, M. and Ogura M. (1990): *Agric. Biol. Chem.*, 54, 2093-2099.
31. Tukey, J. W. (1987): Exploratory data analysis. Addison Wesley. Reading Mass.
32. Yokogoshi, H. and Yoshida, A. (1980): *J. Nutr.*, 110, 375-382.
33. Young, V. R. and Marchini, J. S. (1990): *Am. J. Clin. Nutr.*, 51, 270-289.