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Biological importance of γ-glutamyl-S-methylcysteine of kidney bean (*Phaseolus vulgaris* L.)

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Abstract

The effects of S-methyl-L-cysteine (SMC) on growth and food intake by rats were investigated and in vivo absorption studies of gamma-glutamyl-S-methyl-L-cysteine and free SMC were performed. The results showed that the peptide is slowly absorbed and only free SMC could be detected in the blood. The diets (10% casein), containing SMC, depressed food intake and animals' growth. Both effects were proportional to the SMC contents in the diets. The results obtained with pair-fed groups led to the conclusion that the depression in growth was caused by the refusal of the SMC diets. The addition of cystine to the SMC diet only partially restored normal growth. Histological examination of livers, kidneys and spleens of rats fed with SMC showed no tissue alterations, except for a significant increase in the kidney weights. The hematological features did not show any significant differences, but there was a slight increase in erythroid precursors and young forms in the bone marrow, which might indicate an increased erythrocyte turnover. These results suggest that the reduction in food intake could be associated with some defence mechanism against toxic substances.

Keywords: Beans; S-methyl-L-cysteine; γ-glutamyl-S-methyl-L-cysteine; Physiological effects; Amino acids absorption

1. Introduction

It has been reported that some legume seeds contain a number of free amino acids and γ-glutamyl peptides, including those with a S atom in their molecules. Free S-methylcysteine and γ-glutamyl-S-methyl-L-cysteine [γ-Glu-Cys(S-Me)] are the most common in *Phaseolus vulgaris* L. seeds, but their occurrence is not limited to *Leguminosae*. They can be found also in the *Alliaceae* and *Cruciferae* families (Kasai & Larsen, 1980). The characteristic flavour of these vegetables is generated partly by the action of enzymatic systems upon disruption of plant tissue giving rise to some volatile sulfur-containing degradation products (Hanum, Sinha, Guyer & Cash, 1995; Lancaster & Shaw, 1989, 1991). Beneficial physiological effects may be attributed to these compounds and their metabolites when these vegetables are ingested by mammals (Krest & Keusgen, 1999; Milner, 1996; Siegers, Steffen, Robke & Pentz, 1999; Sundaram & Milner, 1996). However, the physiological responses differ among the three vegetable families, due to the generation of different patterns of metabolites. The metabolic pathways of the sulfur peptides from *Cruciferae* give rise, mainly, to S-methyl-L-cysteine sulphoxide which can be converted to the highly toxic methanethiol and some disulfides. Ruminants are the most sensitive mammals, developing symptoms of haemolytic anaemia (Greenhalgh, 1969; Griffiths, Mcfarlane-Smith & Boag, 1994). Few studies have been reported about the physiological activity of γ-Glu-Cys(S-Me), found in legume seeds.

The first studies were related to the detection and quantification of the γ-glutamyl peptides in legumes and were performed between 1950 and 1970 (Morris, Thompson & Zacharius, 1963; Rinderknecht, Thomas & Aslin, 1958; Thompson & Morris, 1956; Zacharius, 1970; Zacharius, Morris & Thompson, 1958, 1959). Most of them were focused on investigations of non-protein constituents. Kidney beans (*P. vulgaris*) and *Vigna radiata*

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seeds have been reported to contain considerable amounts of γ-Glu-Cys(S-Me) (Evans & Boulter, 1975; Otoul et al., 1975); *Vigna mungo* has high levels of γ-Glu-Met, but its γ-Glu-Cys(S-Me) content is very low. Evans and Boulter studied the distribution of sulfur-containing peptides in 14 genus and species of legumes and found the highest amount of total SMC in kidney beans (0.87% in protein), which corresponds to approximately 13 μmol/g dry seeds containing 24% protein. Recently, we determined the concentration of this peptide in some varieties of common beans, and we found that its amount in *P. vulgaris* seeds is around 11 μmol/g, which means a proportion of 1:2 in relation to the amount of methionine present in the bean studied (Reis-Giada, Miranda, & Lanfer Marquez, 1998).

Early metabolic studies, made on S-methylcysteine, showed that, when replacing dietary methionine and cysteine, this amino acid failed to induce a growth response in animals, such as rabbits, chicks and rats. In addition, antinutritional effects of free S-methyl-L-cysteine (SMC) on laboratory animals have been reported (Benevenga, 1974; Benevenga, Yen & Lalich, 1976, Case & Benevenga, 1976), despite of some results showing no significant adverse effects (Evans & Bandemer, 1976; Eyre, Phillips, Evans & Thompson, 1983). Mice growth depression has been observed when these animals received a diet composed of a mixture of synthetic amino acids supplemented with 75% SMC and 25% methionine in molar proportions. Compared to a control diet containing 100% methionine, the experimental group presented a weight gain of only 13% (Friedman, 1994; Friedman & Gumbman, 1984). Benevenga et al. reported that the consumption of a 10% casein diet, containing 2.4% of SMC, caused an 85% depression in growth and a 65% reduction in food intake. The incorporation of equimolar quantities of methionine depressed the growth to approximately the same extent and excessive consumption of both amino acids resulted in an increase in red blood cells in the splenic sinusoids and deposition of iron in the spleen. The most widely accepted explanation is that SMC and methionine, when at high concentrations, compete in a common metabolic pathway, producing some intermediate toxic products, such as methanethiol (Finkelstein & Benevenga, 1986; Mitchell & Benevenga, 1978). Because of the presence of SMC in the structure of the peptide, the same kind of toxic effect might also be expected for the dipeptide γ-Glu-Cys(S-Me).

Based on these findings and on the elevated consumption of *P. vulgaris* by humans, we carried out some experiments on rats in order to clarify the conflicting biological effects reported and to determine the extent and the reasons for growth depression when the SMC was added in increasing levels to casein diets for young rats. Intestinal absorption rates and physiological effects of γ-Glu-Cys(S-Me) and free SMC on rats were also studied.

2. Materials and methods

2.1. Chemicals

All chemicals, including the amino acids and ion-exchange resins, were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Samples

The *P. vulgaris* seeds, Carioca variety, were obtained from producers of Itapetininga, São Paulo, Brazil. The grains were stored at 4°C and powdered in an analytical mill to 60 mesh size for analysis.

2.3. Animals

Male Wistar albino rats of our colony were used throughout. All rats were housed individually in wire-bottomed cages in a room with controlled light cycle (12/12 h light/dark). They had free access to food and water right up to the time of experiment.

2.4. Preparation of crude extracts

The whole bean flour samples were extracted with 70% EtOH during 96 h, ensuring the inactivation of enzymes and allowing the extraction of amino acids and γ-glutamyl peptides according to the procedure described by Reis-Giada et al. (1998). The crude extract was submitted to purification to remove the neutral compounds (mainly carbohydrates) on an Amberlite IR 120 column (15×30 cm, 32.5 meq H^+; Scheme 1).

2.5. Absorption tests

Animals, weighing 150–200 g, were divided into three groups (four rats each); they were housed individually in wire-mesh cages and submitted to an 18 h fasting period. They received equimolar quantities (66 μmol) of methionine (9.83 mg), SMC (8.91 mg) and the peptide (17.42 mg) from the crude and partially purified extracts. Blood samples (100 μl) were collected from tail vein several times and the quantifications of the compounds were carried out as depicted in Scheme 2.

2.6. Nutritional experiments

Rat feeding trials were conducted to evaluate the in vivo nutritional quality of a reference protein supplemented with protein amino acids and also with SMC.

In the first two experiments, the animals were divided into six groups (10 rats each). The animals weighing 50–60 g were housed individually in suspended wire-mesh cages at room temperature with a controlled 12 h light-dark cycle. They were fed six different diets, prepared in

accordance with the recommendation of AIN-93 (Reeves, Nielsen & Fahey, 1993), containing 10% casein and supplemented with the sulfur amino acid to be investigated. The groups were fed one of the six diets over a 14-day period. Food and water were available ad libitum, and food intake and body weight gains were measured daily.

In the first experiment one group received the non-protein diet for the net protein ratio (NPR) determination; the second group was fed the 10% casein diet, which is known to be limited in sulfur amino acids, and the remaining four groups were fed the amino acid-supplemented diets.

In the second experiment, conducted subsequently, SMC was added to the casein diets in increased levels from 0.6 to 2.4%. Two groups of rats were pair-fed to 100% of the consumption level of the corresponding groups fed casein diet supplemented with 0.6 and 1.2% SMC.

On the 14th day, the rats were killed by ether; liver, spleen, and kidneys were removed, blotted and weighed, and samples of each were fixed in buffered formalin and histologically analysed by light microscopy.

Rat groups for the first and second experiments had the following diets:

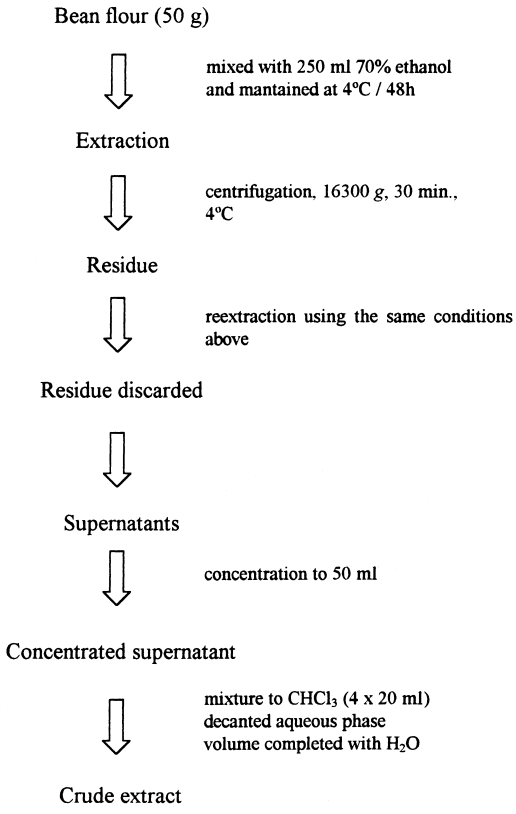
First Experiment

Group 1 — Free protein diet
Group 2 — 10% casein
Group 3 — 10% casein + 0.3% cystine
Group 4 — 10% casein + 0.3% cystine + 0.37% methionine
Group 5 — 10% casein + 0.3% cystine + 0.34% S-methylcysteine
Group 6 — 10% casein + 0.34% S-methylcysteine

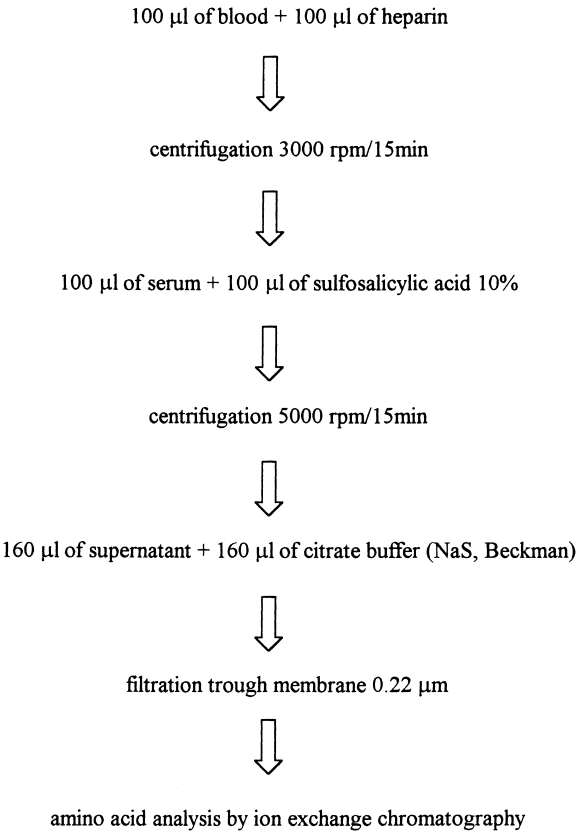
Second Experiment

Group 1 — 10% casein + 0.3% cystine (control diet)
Group 2 — 10% casein + 0.6% S-methylcysteine
Group 3 — 10% casein + 1.2% S-methylcysteine
Group 4 — 10% casein + 2.4% S-methylcysteine
Group 5 — Pair fed group 2
Group 6 — Pair fed group 3

Twenty rats weighing 150–200 g were used for the third experiment. They were divided into two groups and kept in boxes (five animals each) maintained at room temperature in a room with a 12 h light-dark cycle. Water and a



Scheme 1. Extraction of γ-glutamyl peptides from bean flour.



Scheme 2. Flow diagram for the preparation of serum samples for amino acid analysis.

commercial lab chow were offered ad libitum. One group was given, by gavage, 180 mg of SMC, solubilized in 1.5 ml of water, every other day, over a 14-day period. After this time, liver, spleen, and kidneys were removed, blotted and weighed, and samples were fixed in buffered formalin and histologically analysed; blood and bone marrow samples were collected for hematological studies. The other group did not receive anything except water and it was considered as a control.

2.7. Histological assessment

Samples of tissues from each organ removed were fixed in buffered 10% formalin solution, during 48 h. Following subsequent dehydration in multiple alcoholic solutions, the tissues were embedded in paraffin. Sections of about 4–6 μm were stained with hematoxilin and eosin and observed under a standard light microscope at magnifications of ×400. Additional sections were cut and treated with potassium ferrocyanide (Perls' test) for the identification of hemosiderin pigment (Michalany, 1980).

2.8. Hematological analysis

Heparinized blood samples were collected concomitantly with the samples for histological studies. Total and differential counts of leucocytes and erythrocytes were done in a hemocytometer measuring hemoglobin and hematocrit (Lecoq, 1972).

2.9. Tissue analysis

Bone marrow cells were obtained by flushing the femoral cavity with a calcium-magnesium-free phosphate-buffered saline solution, pH 7.4. The cells were quantified in a hemocytometer, and cytocentrifuge smears were stained by the standard May-Grunwald and Giemsa solution, and observed under a light microscope (10×40).

Spleen cells were obtained by cell dissociation equipment with the calcium-magnesium-free-phosphate buffered saline solution, and analyzed in the same way as the bone marrow cells.

2.10. Statistical analysis

The data were analyzed by parametric (Tukey's test) and non-parametric (Mann–Whitney) variance analysis with a level of significance of 95%.

3. Results and discussion

3.1. Absorption rates

Preliminary studies showed that the dipeptide γ-glutamyl-S-methyl-L-cysteine is completely stable when incubated for 6-h under acidic conditions and in the presence of pepsin, imitating the stomach environment, although it has been reported previously that the gamma bond is more labile than the bonds in the alpha position (Kasai & Larsen, 1980).

The following experiments were carried out, administrating equimolar quantities of SMC, methionine, and peptide from crude and partially purified extracts.

The in vivo intestinal absorption tests showed that the ingestion of the peptide results in the appearance, in the peripheral blood, of free SMC only, confirming the hypothesis that the molecule undergoes hydrolysis, probably by a specific peptidase located in the brush border membrane or in the cytoplasm of the enterocyte, before entering the blood. In this study, a group of rats received 66 μmol of the partially purified peptide by gavage in a single dose but the intact peptide was not detected in the blood. Values for SMC during the 6-h study are presented in Fig. 1. The mean concentration of SMC reached a maximum 2 h after the start of the experiment and decreased slowly thereafter.

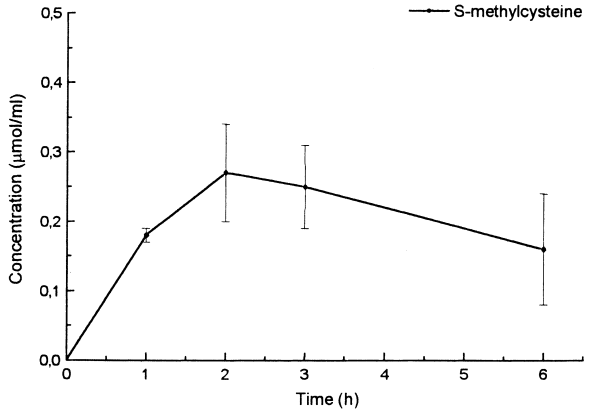


Fig. 1. Changes in peripheral blood S-methylcysteine concentration (μmol/ml) of rats after administration by gavage of 66 μmol (17.4 mg) of γ-glutamyl-S-methyl-L-cysteine from the purified extract.

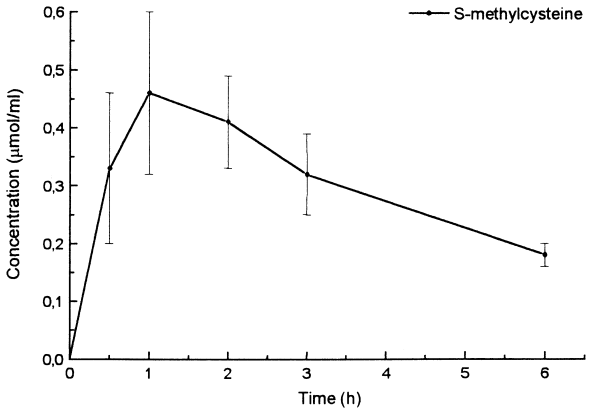


Fig. 2. Changes in peripheral blood S-methylcysteine concentration (μmol/ml) of rats after administration by gavage of 66 μmol (8.9 mg) of the amino acid.

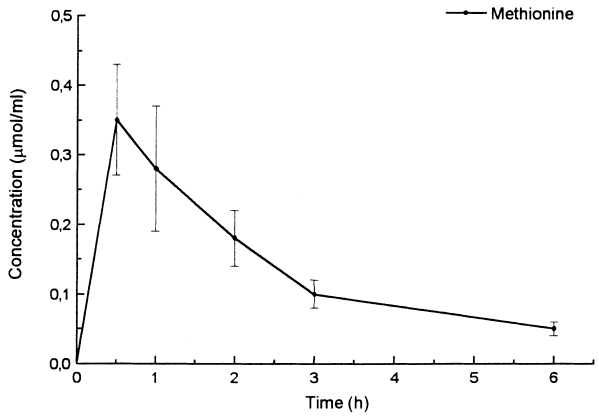


Fig. 3. Changes in peripheral blood methionine concentration (µmol/ml) of rats after administration by gavage of 66 µmol (9.8 mg) of the amino acid.

There was a delay in assimilation of the peptide, in comparison to free SMC, as shown in Fig. 2. This delay can probably be explained by free amino acid absorption into the entherocyte across the brush-border membrane via group-specific amino acid transport systems. Small peptides are transported intact across the intestinal brush border via specific peptide transport systems and are then hydrolyzed, first by highly active cytosolic peptidases, generating free amino acids. The basolateral membranes of the entherocytes possess a number of group-specific amino acid transport systems, which are responsible for the exit of the amino acids produced, from the cell into the blood circulation (Matthews, 1991).

The presence of high levels of carbohydrates, in the crude extract, causes a slowing of the absorption rate, reaching the maximum level at 3 h, 1 h after the administration of the purified peptide, but the carbohydrates do not interfere with the total amount of the absorbed peptide (results not shown). These findings are possibly due to interference with the transport system.

Changes in methionine concentrations in peripheral blood after a single dose of methionine can be observed in Fig. 3. The appearance and the decay of methionine in blood is fast and the highest concentration of methionine is at 30 min after ingestion. SMC showed a different absorption/metabolism pattern from methionine; it remains longer in the blood and seems to be assimilated more slowly than methionine. A significant difference was found between the serum concentrations of these amino acids: 6 h after gavage, 0.2 µmol/ml of SMC were still observed while the concentration of methione was only 0.05 µmol/ml. These results are consistent with a fast assimilation process and an even faster metabolization of methionine by hepatic enzymes.

Table 1
Effect of S-methyl-L-cysteine (SMC) supplementation on growth, food intake and net protein ratio (NPR) values[a]

Diet	Weight gain (g)[b]	Food intake (g)[b]	NPR
Control (10% casein + 0.3% Cys)	76.9±11.8a	176.0±18.9a	4.66±0.27a
Control + 0.34% SMC	47.9±7.8b	132.9±12.1b	4.09±0.30b
10% casein	56.4±10.8b	164.5±19.1a	3.95±0.30bc
10% casein + 0.34% SMC	37.6±10.3c	128.7±15.8b	3.58±0.42c

[a] Different letters indicate significant differences ($P < 0.05$).
[b] Weight gain and food intake during a 14-day period.

3.2. Nutritional studies

To clarify the effect of added SMC on weight gain and net protein ratio, rats were fed diets with and without cystine, S-methylcysteine, or both amino acids.

As shown in Table 1, the consumption of the 10% casein diet containing 0.34% of SMC caused a 51% depression in growth and a 27% reduction in food intake in relation to the diet supplemented with 0.3% of cystine, confirming that SMC does not replace cysteine in its physiological function. The weight gain was even lower than the one caused by the casein diet without any sulfur amino acid supplementation. The non-supplemented casein diet caused a 27% depression in growth when compared to the control diet (10% casein + 0.3% Cys). The concomitant addition of cystine partially restored the normal growth, although the weight gain was 38% lower than that of the control group (Table 1). The data shows that NPR values relative to weight changes seem to be parallel. Therefore, the weight gains indicate protein synthesis and consequently the nutritional value of the diets. The weight gain of the rats fed for a 14-day period with the different diets is shown in Fig. 4.

The addition of SMC caused a marked reduction in weight gain and food intake and did not contribute any beneficial effect; the NPR values for the SMC diet were significantly lower than the control diet, and even lower

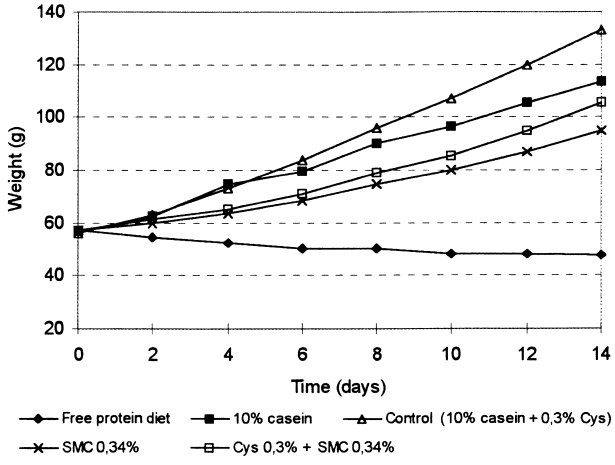


Fig. 4. Growth curves of male rats on control diets enriched with S-methyl-L-cysteine and/or other sulfur amino acids.

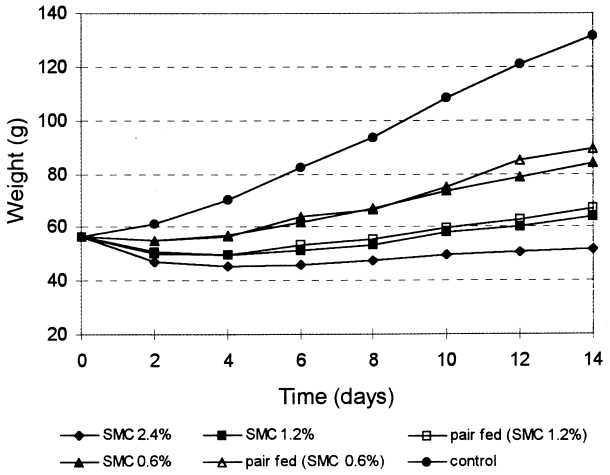


Fig. 5. Effect of increasing amounts of S-methyl-L-cysteine on the weight gain of rats compared to control and pair feeding diets.

than the diet without any supplementation with sulfur amino acid (Table 1).

In another experiment, the depression in growth and reduction in food intake were proportional to the amount of SMC in the diet. Nevertheless, the pair feeding groups showed similar results, leading to the conclusion that the depression in growth is caused by the refusal of the SMC diet (Fig. 5).

The physiological role of SMC is not clear and the literature is conflicting. Benevenga and co-workers (1976) reported an 85% depression in growth and a 65% reduction in food intake of rats fed a basal diet (10% casein + 0.3% methionine) supplemented with an excess of SMC (2.4%). However, it is important to note that a SMC dosage almost 8 times lower, utilised in our experiment, was able to produce a reduction of 51% in growth.

Friedman and Gumbmann (1984) studied the physiological role of several methionine analogues in mice and observed that the experimental group, which received a diet supplemented with 0.21–0.52% SMC, presented a weight gain of 13% compared to the control group. On the other hand, Eyre et al. (1983) showed that the supplementation of Maris Bead bean, a microbial protein product and egg albumin protein with SMC did not significantly alter their biological values, and concluded that this amino acid appears to have a largely passive nutritional role.

At SMC concentrations of 0.34, 0.6, 1.2, and 2.4% in diets used in our experiments, histological examination of hematoxilin- and eosin-stained sections of liver, spleen, and kidneys of rats did not show tissue alterations. These results differ from those published by Benevenga et al. (1976), who demonstrated that the consumption of diets containing high levels (2.4%) of SMC, during a 13-day period, resulted in an increase in red blood cells in the splenic sinusoids and increased deposition of iron in the spleen. In the same study, they

Table 2
Effect of increasing levels of S-methyl-L-cysteine (SMC) in diets on liver, kidney and spleen weights[a]

Diet	Liver	Spleen	Kidney
Control (10% casein + 0.3% Cystine)	5.68±0.61a	0.41±0.11a	0.84±0.06a
Control + 0.6% SMC	5.43±0.77a	0.31±0.07a	1.17±0.06b
Control + 1.2% SMC	5.86±1.62a	0.31±0.06a	1.22±0.13b
Control + 2.4% SMC	5.43±0.50a	0.28±0.04a	1.49±0.08c

[a] Different letters indicate significant differences ($P < 0.05$).

Table 3
Effect of S-methyl-L-cysteine (SMC) given by gavage on liver, kidney and spleen weights[a]

Treatment	Liver	Spleen	Kidney
Control	3.76±0.37a	0.27±0.03a	0.83±0.07a
SMC	3.98±0.36a	0.30±0.08a	0.97±0.10b

[a] Different letters indicate significant differences ($P < 0.05$).

reported an increase in the weight of liver, spleen and kidney. Our results show that only the kidneys underwent a significant increase in weight, which was proportional to the amount of SMC in the diet (Table 2). When SMC was administered for 14 days by gavage (180 mg/animal/2 days), which corresponds to 3× the amounts that the rats ate per day with the SMC-supplemented diet, the same enhancement in kidney weight was observed, probably due to a highly functional demand; but no histologic alterations were evident (Table 3).

The hematological features did not show any significant difference between SMC diet- and control diet-fed rats (results not shown). However, an increase in erythroid precursors and a slight rise in the young forms in the bone marrow, and consequently a leucocytosis in peripheral blood, could indicate an increased erythrocyte turnover and the initiation of an inflammatory process.

The mechanism by which SMC and methionine, in excess, cause adverse effects is not at hand. Methanethiol, a metabolic product of these amino acids, via transamination pathway, has been held responsible for these effects (Benevenga et al., 1976; Finkelstein & Benevenga, 1986). On the other hand, recent studies with humans could not explain methionine toxicity through its degradation into methanethiol (Blom, Boers, Elzen, Gahl & Tangerman, 1989; Gahl et al., 1987).

The significant reduction in food intake seems to be associated with some defence mechanism against toxic substances derived from SMC metabolism. We believe that the slow metabolization of SMC, producing methanethiol and its metabolites through the transamination pathway, prevents high concentrations of these compounds causing more pronounced effects.

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