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# Influence of methionine availability on glutathione synthesis and delivery by the liver

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*The aim of this study was to define dietary conditions liable to elevate the circulating and tissue levels of glutathione (GSH). For this purpose, GSH synthesis and availability have been compared after supplementation of a low protein diet (10% casein) with 0.6% methionine or after adaptation to a high protein diet (30% casein). the final dietary methionine levels were similar in both cases. To compare the effects of an acute addition of sulfur amino acids to the diet with long-term adaptative changes on GSH status, rats adapted to the 10% casein diet received as a final meal (i) the diet supplemented with 0.6% methionine or (ii) a 30% casein diet.*

*With a 10% casein diet, the plasma and tissue concentrations of methionine and cysteine seem to constitute limiting factors for GSH synthesis. However, in animals adapted to a high protein diet or to a diet supplemented in methionine, the hepatic GSH content was quite enhanced (respectively 1.9 and 2.6 fold that measured in control diet), in keeping with the marked rise of the hepatic cysteine (respectively 2 and 5 fold that measured in the control diet). Acute administration of a final 30% casein meal or 10% casein meal supplemented in methionine induced greater changes in the hepatic GSH content than observed in long-term experiments. Compared with the liver, the muscle and heart GSH content was poorly affected by changes in sulfur amino acids availability or nutritional state.*

*The liver appears as a net site of GSH release, the magnitude of this release was not entirely proportional to the hepatic pool, particularly during the postabsorptive period. Moreover, during this period the hepatic GSH content was markedly depressed compared with the fed state, whatever the dietary conditions.*

*With diets providing a high availability in sulfur amino acids, the activity of the liver γ-glutamyl cysteine synthetase was markedly depressed, even in acute experiments.*

In vitro *experiments confirmed* in vivo *results, and showed that until its extracellular concentration reached 0.4 mM, methionine may constitute a limiting factor for GSH synthesis in isolated hepatocytes.* (J. Nutr. Biochem. 8:246–255, 1997) © *Elsevier Science Inc. 1997*

**Keywords:** rat; glutathione; methionine; high protein diet

## Introduction

Glutathione (GSH), an ubiquitous cellular constituent and the most abundant thiol-reducing agent in mammalian tissues (2 to 10 mmol/L) is implicated in a variety of biological actions. In the reduced form, GSH plays an important role on cellular metabolism and protects against free-radical induced oxidant injury. Moreover GSH, via the GSH-transferase system, reacts with drugs and toxic compounds, and it is also involved in the reduction of disulfide linkages in proteins, in the synthesis of DNA precursors and serves as a reservoir for cysteine.

The cellular level of GSH is regulated by a complex process comprising precursors amino acid transport across

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cell membranes, intracellular synthesizing enzymes, feedback regulation by product formation, and intracellular complexing of GSH.[1] All mammalian cells are capable of glutathione synthesis,[2] but the liver represents the major site of GSH synthesis in humans and animals and is the major source of plasma GSH.[3] This location of GSH synthesis reflects the role of the liver in the metabolism and excretion of ingested toxic compounds. Once synthetized by hepatic cells, GSH is either translocated to plasma or excreted into the bile.[3] The characteristics of carriers mediated GSH transport (efflux) has been largely documented in hepatocytes.[4,5,6] In these cells, GSH efflux into blood and bile is governed by two functionally distinct GSH carriers that have been cloned recently.[7,8] The role of plasma GSH in the control of GSH level in extrasplanchnic tissues is still unclear. However, because of their substantial transpeptidase activity, intestinal and lung epithelial cells are able to use plasma GSH.[9] GSH is cleared by kidneys, as evidenced by the low level of GSH in the renal vein.[10] Thus, there are a significant inter-organ relationships of GSH involving hepatic production, systemic distribution, and renal excretion.

Low-circulating GSH and low-tissue GSH concentrations have been documented in alcoholics, and in patients with hepatic cirrhosis, HIV infection, lung disorders, and Parkinson's disease.[11–15] Whether the GSH depletion observed plays a causative or an accompanying role in the pathogenesis of these diseases, and whether the correction of the deficit might alter their natural history can only be determined by developing strategies for GSH supplementation (pharmacological or nutritional interventions). The daily intake of GSH (approximately 150 mg/day) is too low to significantly influence fasting plasma level of GSH.[16] The increase in plasma and tissue GSH after an oral administration of GSH, is modest in animal models[16] and negligible in human healthy volunteers.[17]

The aim of this work was to study the influence of methionine availability on the efficiency of GSH synthesis and release by the liver. The question arises whether a direct supply of sulfur amino acids constitutes a more efficient strategy than a high protein diet, to favor the replenishement of cellular GSH. Cysteine was not chosen as the source of sulfur amino acids because its administration is associated with significant toxicity[18] and with a reduction in tissue GSH consecutive to the production of hydroxyl radical, generated by the auto-oxidation of cysteine into cystine.[19] Consequently, the experimental diet was supplemented with methionine, which can be easily used by the liver to produce cysteine via the cystathionine pathway.[20] To investigate long-term and acute changes in the systemic and tissular contents of GSH in response to the dietary conditions, two types of experiments have been performed. The first series has been realized on animals adapted for a 3-week period to diets containing 10% or 30% casein, or 10% casein + 0.6% methionine (which mimicks the sulfur amino acid supply of a 30% casein diet). The second series of experiments has been performed on rats adapted to a 10% casein diet and receiving, for their last meal before slaughter a 30% casein diet or a 10% casein diet supplemented with methionine, to assess the adaptation of GSH metabolism to acute changes in sulfur amino acid availability.

**Table 1** Composition of diets

| Ingredients | Diet | | | |
|---|---|---|---|---|
| | 10% casein | 10% casein + L-Met 0.6% | 30% casein | 60% casein |
| | g/kg diet | | | |
| Casein[1] | 100 | 100 | 300 | 600 |
| L-Methionine[2] | — | 6 | — | — |
| Wheat starch[1] | 780 | 774 | 580 | 280 |
| Peanut oil[3] | 50 | 50 | 50 | 50 |
| Mineral mixture[4] | 60 | 60 | 60 | 60 |
| Vitamin mixture[5] | 10 | 10 | 10 | 10 |

[1]Casein and wheat starch was from Louis François (Paris, France).
[2]L-Methionine was from Sigma (L'Isle d'Abeau, France).
[3]Peanut oil was from C.I.O. (Genay, France).
[4]Mineral mixture (per kg of diet): $CaHPO_4$, 15 g; $K_2HPO_4$, 2.5 g; KCl, 5 g; NaCl, 5 g; $MgCl_2$ 2.5 g; $Fe_2O_3$, 2.5 mg; $MnSO_4$, 125 mg; $CuSO_4 \cdot 7H_2O$, 0.2 mg; $ZnSO_4 \cdot 7H_2O$, 100 mg; KI, 0.4 mg. Purchased from UAR (Villemoisson, Epinay-sur-Orge, France).
[5]Vitamin mixture (per kg of diet, except as noted): thiamin, 20 mg; riboflavin, 15 mg; pyridoxin 10 mg; nicotinamide, 100 mg; calcium panthotenate, 70 mg; folic acid, 5 mg; biotin, 0.3 mg; cyanocobalamin, 0.05 mg; retinyl palmitate, 1.5 mg; DL-α-tocopheryl acetate, 125 mg; cholecalciferol, 0.15 mg; menadione, 1.5 mg; ascorbic acid, 50 mg; myo-inositol, 100 mg; choline 1.36 g. Purchased from UAR.

## Methods and materials

### *Animals and diets*

Male Wistar rats were fed a commercial nonpurified diet (A03 pellets, U.A.R., Villemoisson/Orge, France) until body weight reached ≈170 g. They were fed for 21 days semi-purified diets (distributed as a moistened powder) containing various proportions of dietary proteins (10, 30, and 60% casein, 10% casein + 0.6% methionine) as described in *Table 1*. Three additional experimental groups of rats, adapted for 21 days to the 10% casein diet, were used: two groups received for their last meal before slaughter a 10% casein diet supplemented either with 0.6% methionine and the third group received a 30% casein diet as a final meal. The animals were housed two per cage and were maintained in temperature-controlled rooms (22°C). They had free access to food for a 8 hr period from 0900 hr to 1700 hr each day, the lighting schedule being 12 hr dark (0900 to 2100 hr): 12 hr light (2100 to 0900). The rats were sampled either 8 hr (fed state) or 24 hr (postabsorptive state) after the onset of food intake.

### *Sampling procedures*

The rats were anaesthetized with sodium pentobarbital and maintained at 37°C. Blood (≈0.8 mL) was withdrawn into heparinized syringes from the hepatic vein, portal vein then abdominal aorta. The efferent blood from the liver was sampled in a branch of the hepatic vein, inside the left hepatic lobe. Portal and hepatic blood flows were determined by an indicator-dilution method, with *p*-aminohippurate as indicator.[21] The indicator (200 mg/L in salin) was infused in mesenteric vein using a 10/4 needle at a rate of 0.1 mL/min during 10 min, after administration of a priming dose of 0.1 mg. After 10 min infusion, blood was sampled either from the hepatic vein then aorta (hepatic blood flow) or from the portal vein (portal blood flow). It was assessed that the relatively short-term

infusion used in the present experiments was sufficient to obtain a stable concentration of the indicator in the studied blood vessels; about 1 min elapsed between blood sampling from hepatic or portal vein or from the aorta. Blood from each animal was placed in a plastic tube containing heparin and centrifuged at 20,000 g for 2 min, then 0.5 mL of plasma was removed and used immediately for GSH determination or kept at −20°C for amino acids analysis. The absence of hemolysis in the samples was checked by measuring the absorbance at 546 nm. The heart, portions of liver tissue (≈1 g) and of quadriceps femoris (≈0.4 g) were removed, quickly frozen in liquid nitrogen, and stored at −80°C. Another portion of liver (≈500 mg) was used immediately to measure the cystathionase activity and the remainder of the liver was excised and weighed to determine the total liver weight.

### *Preparation and incubation of isolated hepatocytes*

Hepatocytes were isolated from fed rats by the collagenase perfusion method, essentially as described by Krebs et al.[22] Cell viability was estimated by cell-membrane refractoriness in phase-contrast microscopy and ranged over 95 to 98%. Isolated liver cells (20 mg wet weight/mL) were finally resuspended in a pregassed ($O_2/CO_2$, 95/5, v/v) Krebs-Henseleit bicarbonate buffer pH 7.40, containing 2% bovine serum albumin and 10 mM glucose. Aliquots of cells were incubated at 37°C in stoppered vials with shaking for 30 min in the presence of glutamine 2 mM, glycine 1 mM and various concentrations of methionine or *N*-acetylcysteine.

### *Determination of the amino acids concentrations in plasma and tissues*

The frozen tissues were crushed in 5 vol 0.6 M $HClO_4$ and then neutralized with $K_2CO_3$. The plasma samples were deproteinized with 5% sulfosalicylic acid (1 vol:1 vol) and all extracts were adjusted to pH 2.2 with a lithium citrate buffer 0.2 N before analysis. Amino acids were determined on a Chromakon 500 autoanalyzer (Kontron, Zürich, Switzerland), using lithium citrate buffers (Pharmacia, LKB Biochrom, Cambridge, UK) and post-column ninhydrin detection.

### *GSH and cysteine determinations*

**Sample preparations.** The tissues were weighed and homogenized (1:10, w/v) with cold homogenization buffer containing 0.5 M perchloric acid and supplemented with EDTA (1 mM) to reduce oxidation. Immediately after its obtention, plasma (9 vol) was deproteinized with (1 vol) $HClO_4$ 5M/EDTA 1 mM. Aliquots of isolated liver cells (2 mL) were removed and quickly centrifuged at 20,000 g, the supernatant was withdrawn and the cell pellet was vigourously resuspended and deproteinized with 200 µL of an ice-cold solution containing 3 M $HClO_4$ and 6 mM EDTA. After homogenization samples were immediately centrifuged (4°C, 20,000 g, 2 min). At this step, plasma-treated samples must be immediately used for GSH determination, whereas centrifuged samples from tissue or from cells can be stored for 2 days at −20°C without noticeable changes of the GSH content. Before injection in the HPLC system, all perchloric supernatants were diluted in sodium dihydrogenphosphate buffer (0.1 M, pH 2.5) containing EDTA (0.5 mM).

**HPLC/electrochemical detection conditions.** The HPLC system consisted of a Dionex pump, model GPM2 (Dionex, Sunnyvale, CA, USA), a Gilson 234 refrigerated autosampler, and fitted with a 5 µm C-18 Spherisorb 5 ODS-2 analytical column (250 × 4.6 mm ID) (Interchim, Montluçon, France). The mobile phase was 100 mM sodium dihydrogenphosphate, 5% methanol and 0,1 mM EDTA, the pH was set to 2.5 with orthophosphoric acid. It was filtered through a 0.2 µm filter before use and degassed with a helium sparging system. Elution was isocratic at a flow rate of 0.5 mL/min at ambient temperature. The detection was performed on an electrochemical detector (Dionex, model PED), using a gold working electrode, with detector voltage at +0.5 V. The current product was monitored using an integrator (Enica 31, Delsi, France).

**Standards.** Standards solution (GSH: 10 mM, Cys: 1 mM) were prepared in sodium dihydrogenphosphate buffer (0.1 M, pH 2.5) containing EDTA (0.5 mM) and aliquoted before storage at −80°C. Before use, they were diluted as necessary with the same buffer, to give final concentrations covering those found in extracts to be analyzed. The external standard method was used for quantification.

### *Measurement of enzyme activities*

**Cystathionase activity.** Immediately after animal sacrifice, a portion of liver was homogenized in 9 vol of an ice-cold potassium phosphate buffer 100 mM, pH 7.0 containing 1 mM EDTA and 20% glycerol. The homogenate was centrifuged at 28,000 g for 30 min at 4°C and the supernatant fluid could be stored 1 week at −80°C before the determination of enzyme activity. Cystathionase assay was performed as described by Heinonen.[23] Briefly, the reaction mixture contained the following, in a total volume of 0.5 mL: Tris HCl buffer (50 mM, pH 8.4); pyridoxal 5′-phosphate, (3 mM); L-cystathionine (5 mM); and liver extract diluted in 0.1 mL of 100 mM potassium phosphate buffer, pH 7.0. The incubation was performed at 37°C for 30 min and the reaction was stopped by cooling the tubes in an ice bath. Blank assays, in which cystathionine was omitted in the reaction mixture, were performed in parallel. Dithiothreitol (final concentration: 50 mM) was added to bring all cysteine to the reduced form and the amount of cysteine was determined spectrophotometrically by the method of Gaitonde.[24]

**γ-glutamyl cysteine synthetase activity.** A portion of freezed liver (1 g) was crushed in 4 vol of an ice-cold sodium dihydrogenphosphate buffer 10 mM, pH 7.4 containing 250 mM sucrose. The homogenate was centrifuged at 100,000 g for 60 min at 4°C. The enzyme activity was determined on the supernatant according to the procedure described by Richman and Meister.[25] The standard enzyme assay reaction mixture contained Tris-HCl buffer (100 mM, pH 8.2), sodium L-glutamate (10 mM), L α-aminobutyrate (10 mM), $MgCl_2$ (20 mM), ATP (5 mM), EDTA (2 mM), BSA (40 µg/mL), and enzyme in a final volume of 0.5 mL. Blanks assays were performed in the absence of L α-aminobutyrate. After an incubation at 37°C for 30 min, the reaction was stopped by adding 0.5 mL of 25% (w/v) trichloracetic acid. The inorganic phosphate released was determined using a kit obtained from Sigma (L'Isle d'Abeau, France).

Enzyme activities were expressed in µmol of product formed per 30 min per mg protein. Protein was assayed using the Pierce B.C.A. protein reagent kit (Interchim, Montluçon, France).

### *Statistics*

Values are means ± SEM and significance of the differences between mean values was determined by one-way analysis of variance (ANOVA) coupled with the Student-Newman-Keuls multiple comparison test. Values of $P < 0.05$ were considered to be significant.

**Table 2** Effect of the dietary protein level and of a supplementation in methionine on body and liver weights, food intake and on hepatic blood flow

| | Casein in the diet | | |
|---|---|---|---|
| | 10% | 30% | 10% + Met 0.6% |
| Body weight, g | 265 ± 11[a] | 332 ± 7[b] | 312 ± 6[c] |
| Food intake, g/day | 23.5 ± 0.6 | 24.0 ± 0.6 | 25.1 ± 0.3 |
| Weight gain, g/day | 4.5 ± 0.2[a] | 7.0 ± 0.3[b] | 6.5 ± 0.2[b] |
| Liver wt, g | | | |
| Postprandial | 9.5 ± 0.4[a] | 13.3 ± 0.5[b] | 14.7 ± 0.6[c] |
| Postabsorptive | 8.7 ± 0.5[a] | 11.2 ± 0.3[b] | 12.5 ± 0.4[c] |
| Hepatic blood flow, mL/min | | | |
| Postprandial | 22.1 ± 1.9[a] | 31.9 ± 2.1[b] | 31.0 ± 2.4[b] |
| Postabsorptive | 20.2 ± 2.2[a] | 25.1 ± 1.8[b] | 25.0 ± 1.9[b] |
| Sulfur amino acid concentration derived from diet, % | | | |
| Methionine | 0.3 | 0.9 | 0.9 |
| Cystine | 0.03 | 0.09 | 0.03 |
| Total sulfur amino acid | 0.33 | 0.99 | 0.93 |

Values are means ± SEM; $n$ = 12 rats.
Values in a line not sharing a common superscript letter are significantly different at $P < 0.05$.

## Results

### *Influence of the dietary casein level and of a supplementation in methionine on body and liver weights and on hepatic blood flow*

As shown in *Table 2*, increasing the dietary casein level from 10 to 30% had little effect on food intake whereas the daily body weight gain was significantly increased (+56%). Supplementation of a 10% casein diet with 0.6% methionine improved (+45%) the body weight gain, compared with the 10% casein diet. The liver weight was dependent on the diet conditions, it represented 3.6%, 4.0%, or 4.7% of the body weight in rats fed the 10% casein diet, the 30% casein diet, or the 10% casein diet supplemented with methionine, respectively. There was a noticeable increase in the liver weight after feeding, except with the 10% casein diet. Hepatic blood flow changed in parallel to the liver weight, but it was significantly lower in rats fed the 10% casein diet than in those fed the other diets. Food deprivation slightly reduced hepatic blood flow in all dietary groups (−10% to −20%). It could be noted that in rats fed the 10% casein diet supplemented with 0.6% methionine and in those fed the 30% casein diet, the final methionine levels were similar (0.9%) and the sulfur amino acid concentration derived from diet was of the same magnitude (0.93 to 0.99%).

### *Plasma and tissue concentrations of amino acids involved in GSH metabolism*

In rats fed the 10% casein diet, the concentration of methionine in plasma was relatively low (≈60 μM, *Table 3*). The plasma concentration of methionine was poorly affected in rats fed a 30% casein diet, whereas the supplementation of the diet with 0.6% methionine markedly increased this parameter (+170%). When the diet was supplemented with methionine, the concentrations of methionine were markedly enhanced in liver (×3), muscle (×2) and heart (×2.8) compared with values observed with the 10% casein diet (*Table 3*). In rats adapted to a 30% casein diet, the tissular concentrations of methionine were also enhanced, but the changes were of lesser magnitude.

In rats receiving the 30% casein diet, as well as in those adapted to the diet supplemented with methionine, the plasma taurine level was twice that measured in rats fed the 10% casein diet (*Table 3*). Whatever the diet, the levels of taurine in muscle and heart were higher than those measured in liver; however in this last tissue, the concentration of taurine was more sensitive to nutritional changes (*Table 3*). The diet supplemented with methionine and that containing 30% casein induced a 9 fold and a 16 fold increase, respectively, in the liver concentration of taurine, compared to the values obtained with the 10% casein diet (*Table 3*).

As shown in *Figure 1*, the concentration of cysteine was particularly low (≈28 μM) in the liver of rats adapted to the 10% casein diet, this level was increased in rats fed a high

**Table 3** Arterial, hepatic, muscle, and heart concentrations of amino acids in rats fed various casein levels or a diet supplemented in methionine

| | Casein in the diet | | |
|---|---|---|---|
| | 10% | 30% | 10% + Met 0.6% |
| | Artery, mM | | |
| Methionine | 0.06 ± 0.01[a] | 0.10 ± 0.01[b] | 0.17 ± 0.01[c] |
| Taurine | 0.02 ± 0.01[a] | 0.05 ± 0.01[b] | 0.05 ± 0.01[b] |
| Serine | 0.42 ± 0.03[a] | 0.28 ± 0.01[b] | 0.23 ± 0.01[c] |
| Glycine | 0.31 ± 0.01[a] | 0.17 ± 0.02[b] | 0.25 ± 0.01[c] |
| Glutamine | 0.64 ± 0.02[a] | 0.54 ± 0.01[b] | 0.68 ± 0.04[a] |
| Alanine | 0.64 ± 0.03[a] | 0.59 ± 0.03[b] | 0.72 ± 0.01[b] |
| | Liver, μmol/g | | |
| Methionine | 0.08 ± 0.01[a] | 0.14 ± 0.01[b] | 0.25 ± 0.01[c] |
| Taurine | 0.46 ± 0.01[a] | 7.54 ± 0.15[b] | 4.18 ± 0.05[c] |
| Serine | 1.93 ± 0.06[a] | 0.57 ± 0.08[b] | 0.74 ± 0.08[c] |
| Glycine | 3.82 ± 0.17[a] | 1.89 ± 0.09[b] | 2.37 ± 0.11[c] |
| Glutamate | 1.85 ± 0.06[a] | 2.40 ± 0.09[b] | 2.41 ± 0.04[b] |
| Glutamine | 5.40 ± 0.31[a] | 4.15 ± 0.30[b] | 5.39 ± 0.35[a] |
| Alanine | 3.84 ± 0.12[a] | 3.15 ± 0.23[b] | 3.75 ± 0.12[a] |
| | Muscle, μmol/g | | |
| Methionine | 0.09 ± 0.01[a] | 0.12 ± 0.01[a] | 0.18 ± 0.02[c] |
| Taurine | 4.90 ± 0.28[a] | 11.2 ± 0.42[b] | 10.3 ± 0.61[b] |
| Serine | 2.71 ± 0.10[a] | 0.85 ± 0.03[b] | 1.34 ± 0.03[c] |
| Glycine | 6.01 ± 0.45[a] | 2.25 ± 0.09[b] | 4.06 ± 0.12[c] |
| Glutamate | 1.58 ± 0.10[a] | 1.92 ± 0.04[b] | 1.78 ± 0.12[a] |
| Glutamine | 5.81 ± 0.12[a] | 4.42 ± 0.16[b] | 4.65 ± 0.16[b] |
| Alanine | 5.02 ± 0.30[a] | 3.56 ± 0.06[b] | 4.04 ± 0.15[c] |
| | Heart, μmol/g | | |
| Methionine | 0.06 ± 0.01[a] | 0.23 ± 0.01[b] | 0.17 ± 0.04[c] |
| Taurine | 9.94 ± 0.71[a] | 17.3 ± 0.80[b] | 14.3 ± 1.45[c] |
| Serine | 1.29 ± 0.10[a] | 0.42 ± 0.01[b] | 0.35 ± 0.03[c] |
| Glycine | 0.80 ± 0.03[a] | 0.52 ± 0.01[b] | 0.48 ± 0.01[c] |
| Glutamate | 8.10 ± 0.48[a] | 9.44 ± 0.12[b] | 6.88 ± 0.35[c] |
| Glutamine | 6.28 ± 0.36[a] | 6.09 ± 0.73[a] | 6.02 ± 0.56[a] |
| Alanine | 2.01 ± 0.09[a] | 1.89 ± 0.08[a] | 1.64 ± 0.12[b] |

Values are means ± SEM; $n$ = 12 rats.
Values in a line not sharing a common superscript letter are significantly different at $P < 0.05$.

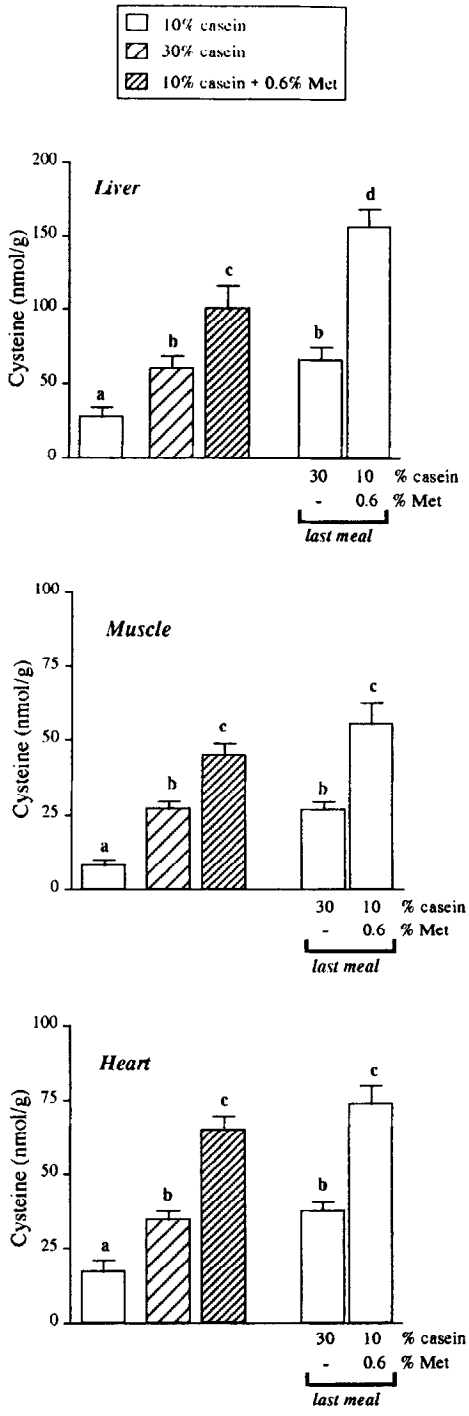


**Figure 1** Changes in tissue concentrations of cysteine in response to dietary conditions. In a first series of experiments, rats were adapted for 3 weeks to differents diets containing: 10% casein, 30% casein, or 10% casein + 0.6% methionine. In the second series of experiments, rats adapted to a 10% casein diet received for their last meal before slaughter, a 30% casein diet or a 10% casein diet supplemented with 0.6% methionine. The concentration of cysteine was measured on tissue extracts by a HPLC method, according to the procedure described in the Materials and methods section. Samples were withdrawn during the fed period. Values are means ± SEM for 12 rats in each diet. Different subscript letters indicate significant differences.

protein diet (≈60 μM with the 30% casein diet) and reached 100 μM in rats receiving a 10% casein diet supplemented with methionine. The cysteine concentrations in muscle and heart underwent changes parallel to liver concentrations (*Figure 1*). When rats adapted to a 10% casein diet were supplemented with methionine (0.6%) for their last meal, the hepatic concentration of cysteine was markedly enhanced (≈150 μM). On the other hand, short-term and long-term effects of a 30% casein diet on liver cysteine concentration were of the same magnitude.

Adaptation to a high protein diet markedly depressed tissue serine and glycine (*Table 3*); this effect was particularly marked in the liver, but it was also observed in muscle and heart. Moreover, the supplementation of the 10% casein diet with methionine also significantly decreased the plasma and tissue levels of serine and glycine.

As shown in *Table 3*, the increase of the dietary casein level from 10 to 30% induced (i) a significant decrease of the hepatic concentrations of alanine and glutamine, in response to the acceleration of their catabolism and (ii) a net increase in the liver concentrations of glutamate (+30%). By contrast, the supplementation of a 10% casein diet with 0.6% methionine poorly affected the tissue concentrations of glutamate, glutamine, and alanine.

### *Liver concentrations in GSH*

This parameter has been measured in the liver of rats sampled during the fed and the postabsorptive periods (*Figure 2*). In the liver of fed rats, the concentration of GSH (1.81 mM with the 10% casein diet) was greater in animals adapted to a diet supplemented with methionine (4.7 mM) than in those receiving a high protein diet (3.4 mM). The hepatic GSH content markedly increased and reached values close to 6.5 mM in rats submitted to an acute supplementation of methionine (0.6%) in their last meal. It could be noted that similar concentrations in liver GSH were found when cysteine was administered instead of methionine for the last meal (*data not shown*). Moreover, when animals adapted to a 10% casein diet received a 30% casein diet as a final meal, the liver concentration of GSH was significantly increased (5.2 mM), but was lesser than with a direct methionine supplementation.

During the postabsorptive period (*Figure 2*), there was a drastic decrease in hepatic GSH in all the nutritional conditions studied and this phenomenon was particularly marked with the 30% casein diet.

### *GSH content in muscle and heart*

Whatever the diet, the concentrations of GSH in the muscle and in the heart of fed rats (*Figure 3*) were markedly lower than those found in the liver. The GSH content in muscle was slightly enhanced in rats receiving a high protein diet as well as in those supplemented with methionine: +60% compared with values obtained with the 10% casein diet. When animals adapted to a 10% casein diet received for their last meal, an acute administration of 0.6% methionine or a 30% casein diet, changes in the GSH content in muscle were moderate when compared to control value (+10%). In the heart, the GSH level was poorly affected by nutritional changes, except in rats fed the high protein diet. During the

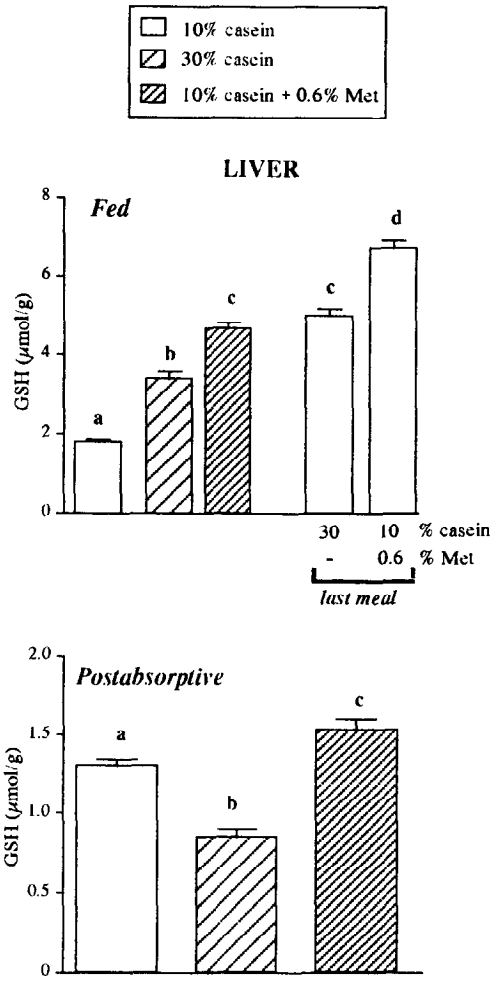


**Figure 2** Changes in the liver GSH concentration during the fed and postabsorptive periods in rats adapted to different nutritional conditions as detailed in the legend of the *Figure 1*. The liver GSH concentration was determined by HPLC, according to the procedure described in the Materials and methods section. Values are means ± SEM for 12 rats in each diet. Different subscript letters indicate significant differences.

postabsorptive period, changes in the GSH concentration of muscle and heart were quite similar to those measured in the fed state.

### *Plasma concentrations of GSH*

Compared with the levels of glutathione found in tissues, the concentrations of GSH in the plasma were quite low (10 to 30 μM, *Table 4*). As in the liver, the GSH level in plasma was responsive to an increase in dietary protein or to a supplementation of the diet with methionine (0.6%). The highest plasma concentrations of GSH were reached in rats receiving for their last meal an acute addition of methionine or a 30% casein diet. Whatever the diet, the plasma GSH levels were depleted during the post absorptive period.

### *Hepatic pool and release of GSH*

The release of GSH by the liver and the hepatic pool of GSH (GSH conc × liver wt.) have been represented in *Table 5*. In fed rats, the GSH release seems dependent on the size of the hepatic GSH pool, however the GSH release was not directly proportional to the hepatic pool value: with the 10% casein diet, the GSH release was quite high (232 nmol/min) for a 17 μmol/liver pool of GSH. In rats fed the high protein diet, the hepatic GSH release was about 324 nmol/min, whereas the hepatic pool was of 45 μmol/liver. The highest changes in the hepatic GSH pool and release were observed with the diet supplemented with methionine: +300% for the GSH pool and +115% for the GSH release, compared with data obtained in rats fed the 10% casein diet. Similar changes in the GSH pool were observed after an acute administration of methionine for the last meal (*Table 5*). Moreover, long-term and acute effects of a 30% casein diet on the hepatic pool of GSH were of the same magnitude.

During the absorptive period, the hepatic GSH pool and release were drastically depressed in comparison with those observed in the fed state (*Table 5*). However, as in fed rats, the hepatic GSH release was still noticeable (100 nmol/min), whereas the pool of GSH was quite low (≈11 μmol/liver).

### *Effect of the diet on enzyme activities involved in GSH metabolism*

As shown in *Table 6*, the activity of cystathionase was not affected by changes in dietary conditions. By opposite, the activity of γ-glutamyl cysteine synthetase was markedly depressed in rats fed the 30% casein diet or the methionine supplemented diet (−50 to −60%), when compared with that measured in the liver of rats fed the 10% casein diet. A similar repression of γ-glutamyl cysteine synthetase was observed in rats submitted to an acute supplementation of methionine in their last meal before slaughter. On the other hand, the lowering of this enzyme activity was lesser (−35%) in rats submitted to an acute administration of a 30% casein diet for their last meal. Such a result is in agreement with the differences between the liver GSH concentrations measured in rats adapted for 3 weeks to a 30% casein diet or in rats receiving a 30% casein diet only for their last meal.

### *Effect of methionine on cellular glutathione content*

Isolated hepatocytes obtained from rats adapted to diets containing 10, 30, or 60% casein or to a 10% casein diet supplemented with 0.6% methionine, have been incubated for 30 min. in the presence of increasing concentrations of methionine (0 to 0.8 mM), then the cellular GSH content has been determined (*Figure 4*). It could be noted that, whatever the diet, the cellular GSH content was largely depleted during the perfusion and washing procedures. *Figure 4* shows that, in the absence of methionine in the incubation medium, the GSH content in hepatocytes from rats adapted to the 10% casein diet (0.40 μmol/g) was half of that measured in cells from rats adapted to other diets. In hepatocytes from rats fed a 60% casein diet, the addition of methionine was ineffective to markedly change the cell GSH content (*Figure 4*). The plot of hepatocytes GSH content as a function of methionine additions showed no significant differences between adaptation to a 10% casein

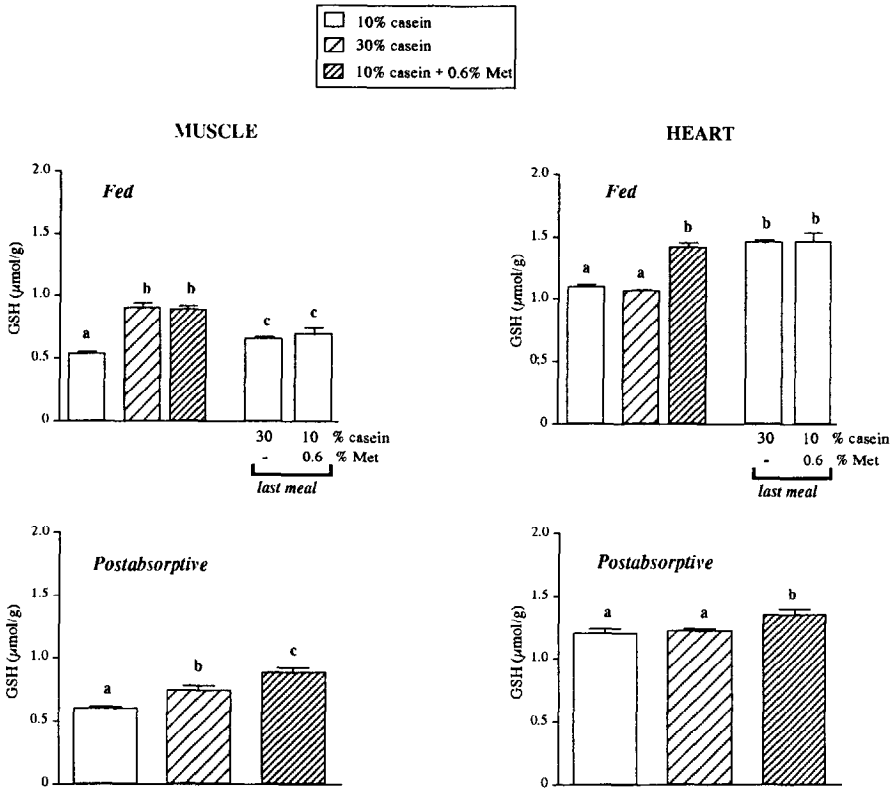


**Figure 3** Changes in muscle and heart GSH concentrations during the fed and postabsorptive periods in rats adapted to different dietary conditions as detailed in the legend of the *Figure 1*. Values are means ± SEM for 12 rats in each diet. Different subscript letters indicate significant differences.

diet supplemented with 0.6% methionine or to a 30% casein diet.

When the extracellular concentration of methionine varied from 0 to 0.4 mM, the slope of the changes in GSH content was markedly higher in hepatocytes adapted to the 10% casein diet (×3) than in those from rats adapted to the other diets. For concentrations of methionine greater than 0.4 mM, changes in cell GSH were moderate in all dietary conditions: the concentrations of GSH reached about 1.7 mM in hepatocytes from rats fed the 10% casein diet, supplemented or not with 0.6% methionine, as well as in those from rats adapted to the 30% casein diet.

**Table 4** Influence of the diet and of the nutritional state on plasma GSH concentrations

| | Plasma GSH | | |
|---|---|---|---|
| | Artery | Portal vein | Hepatic vein |
| | (μM) | | |
| Fed | | | |
| Rats adapted to: | | | |
| casein 10% | 13.1 ± 0.5[a] | 11.7 ± 0.4[a] | 22.7 ± 1.0[a] |
| casein 30% | 17.5 ± 1.6[b] | 17.3 ± 1.7[b] | 27.6 ± 2.3[b] |
| casein 10% + Met 0.6% | 17.5 ± 1.5[b] | 15.2 ± 1.0[b] | 32.0 ± 2.5[b] |
| Rats adapted to a 10% casein diet and receiving for their last meal: | | | |
| casein 10% + Met 0.6% | 25.7 ± 0.8[c] | 24.5 ± 1.6[c] | 43.1 ± 2.2[c] |
| casein 30% | 26.0 ± 1.7[c] | 22.8 ± 1.4[c] | 38.9 ± 2.0[c] |
| Postabsorptive | | | |
| Rats adapted to: | | | |
| casein 10% | 8.4 ± 0.8[d] | 8.0 ± 0.5[d] | 13.1 ± 0.7[d] |
| casein 30% | 9.9 ± 0.6[d] | 9.1 ± 0.9[d] | 15.1 ± 0.8[e] |
| casein 10% + Met 0.6% | 7.5 ± 0.2[d] | 6.4 ± 0.2[e] | 12.3 ± 0.7[d] |

Values are means ± SEM for six rats in each nutritional condition. Values in a column not sharing a common superscript are significantly different at $P < 0.05$.

**Table 5** Effect of the dietary conditions on the total hepatic GSH content and on the hepatic GSH release in rats during the fed and postabsorptive periods

| | Hepatic GSH Pool | Hepatic GSH Production |
|---|---|---|
| | μmol/liver | nmol/min |
| Fed | | |
| Rats adapted to: | | |
| casein 10% | 17.3 ± 1.5[a] | 232 ± 26[a] |
| casein 30% | 45.4 ± 6.2[b] | 324 ± 20[b] |
| casein 10% + Met 0.6% | 68.6 ± 9.1[c] | 480 ± 37[c] |
| Rats adapted to a 10% casein diet and receiving for their last meal: | | |
| casein 10% + Met 0.6% | 61.3 ± 2.9[c] | 363 ± 31[b] |
| casein 30% | 64.1 ± 7.8[c] | 410 ± 43[b] |
| Postabsorptive | | |
| Rats adapted to: | | |
| casein 10% | 11.3 ± 0.7[d] | 99 ± 12[d] |
| casein 30% | 25.0 ± 3.4[e] | 112 ± 13[d] |
| casein 10% + Met 0.6% | 19.1 ± 2.1[ae] | 140 ± 11[e] |

Values are means ± SEM; $n = 12$ rats in each nutritional conditions. Values in a column not sharing a common superscript are significantly different at $P < 0.05$.

**Table 6** Changes in cystathionase and γ-glutamyl cysteine synthase activities in the liver of fed rats adapted to different dietary protein levels or to diets supplemented in methionine

| | Cystathionase | γ-Glutamyl Cysteine Synthase |
|---|---|---|
| | μmol Cys.mg prot$^{-1}$.30 min$^{-1}$ | nmol Pi.mg prot$^{-1}$.30 min$^{-1}$ |
| Rats adapted to: | | |
| casein 10% | 0.43 ± 0.02[a] | 13.10 ± 0.66[a] |
| casein 30% | 0.48 ± 0.03[a] | 6.75 ± 0.67[b] |
| casein 10% + Met 0.6% | 0.42 ± 0.02[a] | 5.55 ± 0.27[c] |
| Rats adapted to a 10% casein diet and receiving for their last meal: | | |
| casein 10% + Met 0.6% | 0.42 ± 0.01[a] | 5.73 ± 0.27[bc] |
| casein 30% | 0.43 ± 0.03[a] | 8.34 ± 0.45[d] |

Values are means ± SEM; $n$ = 6 rats.
Values in a column not sharing a common superscript are significantly different at $P < 0.05$.

Additional experiments have been performed on isolated hepatocytes from rats adapted to the four types of diets to compare the effects of an addition of 0.4 mM *N*-acetylcysteine to those of 0.4 mM methionine on the cell GSH content. For a given diet, data obtained showed no significant differences in the cell GSH level in response to methionine and to *N*-acetylcysteine (result not shown).

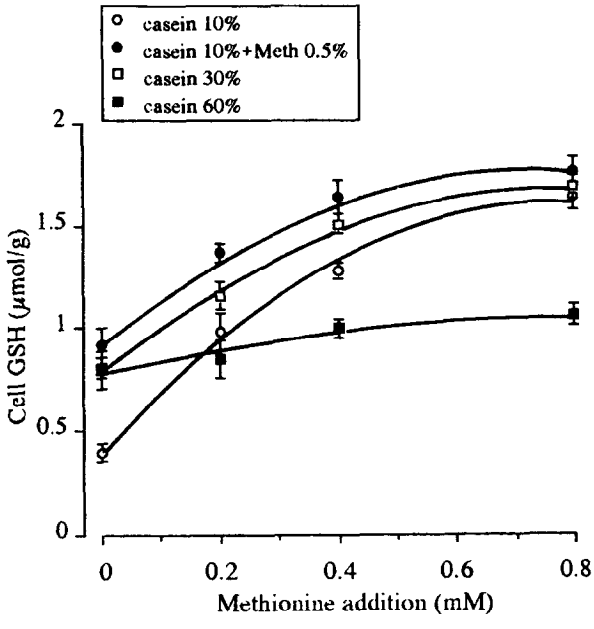


**Figure 4** Changes in the concentration of GSH in response to the addition of increasing concentrations of methionine in hepatocytes isolated from rats adapted to diets containing different casein levels. Isolated liver cells were incubated for 30 min in the presence of 2 mM glutamine, 1 mM glycine, and various concentrations of methionine. Then an aliquot of cellular suspension was withdrawn, centrifuged and the GSH concentration in the pellet was determined according to the procedure described in the Materials and methods section. Results shown are the means ± SEM of four cell preparations for each dietary conditions and in which GSH determinations were performed in triplicate.

## Discussion

Previous works have shown that the dietary protein supply strongly influenced GSH synthesis[26–28] and it has been shown that the liver and plasma contents of GSH are responsive to the dietary casein level, up to about 24%.[27] GSH available for peripheral tissues was not markedly elevated in rats fed excess protein (>30%).[28] When the dietary protein level is increased, the sulfur amino acids availability is strongly enhanced,[28] but there is also a general induction of amino acids catabolism. Thus, it seemed interesting to study the specific effects of a supplementation of the diet with sulfur amino acids on GSH synthesis. In this view, methionine appears as an effective source of sulfur amino acid for GSH synthesis; first, because its circulating level is greater than that of cysteine; second, because its hepatic uptake may be very efficient[29]; and third, it constitutes a substrate for the cystathionine pathway in the liver, thus providing cysteine.[30]

This study shows that a direct supplementation of the diet with methionine is slightly more efficient to increase the liver GSH content than does a high protein diet, considering that the final methionine supply was similar in both cases. This could be related to the higher concentration of cysteine found in the liver of rats supplemented with methionine than in those adapted to a 30% casein diet. Methionine appears quite efficient to increase, in parallel, the body weight gain, the hepatic mass, and the GSH status. Nevertheless, the enhancement of the hepatic GSH pool consecutive to a supplementation of the diet with methionine could be ascribed to the rise in liver GSH content, rather than in hepatic mass. Hum et al.[28] have reported that, in rats fed a 40% casein diet, the total liver GSH content was unchanged compared with that measured in rats receiving a 20% casein diet. On the other hand, low protein diets induce a sparing effect of thiol groups that favors cysteine salvage and GSH synthesis. Thus, the present data indicate that a high protein diet is less effective to supply cysteine for liver GSH synthesis than does a direct supplementation of the diet with sulfur amino acids.

Moreover, additional experiments have shown that the effects of an acute administration of a 10% casein meal supplemented with 0.6% methionine, or of a 30% casein meal, were more effective on the liver GSH content than long-term adaptation to the corresponding diets. Moreover, the cysteine availability seems to constitute a key component to favor GSH synthesis with diets supplemented with sulfur amino acids. On the other hand, the higher concentrations of GSH found in the liver of rats receiving a 30% casein diet for their last meal, compared with that measured in adapted rats, were not accompanied by any significant change in hepatic cysteine concentrations. However in these nutritional conditions, the differences in GSH concentrations could be related to changes in the activity of γ-glutamyl-cysteine synthetase.

Except in premature infants, requiring a dietary cysteine supply because of their low capacity for cysteine synthesis by the transsulfuration pathway,[31,32] the synthesis of cys-

teine from methionine should not represent a limiting step for GSH synthesis. Besides, the activity of cystathionase in rat liver was unaffected by drastic changes in amino acids availability. In contrast, the activity of γ-glutamyl-cysteine synthetase was markedly reduced with a high protein diet as well as with a dietary methionine supply. This effect could not be explained by a direct inhibition of this enzyme by GSH, because hepatic GSH was highly diluted in the assay medium used to determine the enzyme activity. Although altered γ-glutamyl cysteine synthetase activity could result from posttranslational modifications, accumulating evidence suggests a prominent role for a regulation at the transcriptional level.[33] In this way, it could be speculated that changes in γ-glutamyl cysteine synthetase activities observed in some of the dietary conditions tested could result from transcriptional or posttranscriptional modifications.

Changes in methionine availability markedly depressed the hepatic content in serine and glycine and mimicked the effects of high protein diets. Serine is implicated in the conversion of methionine to cysteine, whereas glycine constitutes a direct precursor for GSH synthesis. However, the question arises whether the fall of these amino acids concentrations could affect GSH synthesis. By maintaining sufficient levels of glutamate in the liver, glutamine could play a role in GSH synthesis; in this view, hepatocytes cultured in glutamine-free medium are rapidly depleted in glutamate, resulting in depressed intracellular GSH level.[34] With high protein diets, the stimulation of gluconeogenesis and glutaminase activities depressed hepatic glutamine; however, the levels of glutamate were not affected.[35] Moreover, it has been reported that a glutamine-supplemented nutrition preserves hepatic GSH during acetominophen toxicity.[36]

In the liver, GSH can act directly as a major antioxidant and as a cofactor for many enzymes: GSH peroxydase, GSH reductase, and GSH transferase.[26] In rats adapted to a high protein diet as well as in those receiving a supplementation in methionine, the GSH release in the blood was greater than that measured with a low protein diet, but the magnitude of this release was not directly proportional to the total hepatic GSH content. The involvement of a carrier mechanism in the concentration-driven efflux of sinusoidal and biliary GSH is now well documented.[4–6] When the hepatic pool of GSH is high, it is conceivable that the carrier system reaches its maximal activity, hence a possible plateauing of GSH release. Furthermore, it could be proposed that, when the total hepatic GSH content is relatively low, the carrier-mediated GSH transport is not rate limiting for GSH efflux and, accordingly, any stimulation of the efflux would contribute to deplete the liver GSH content. This interpretation is in accordance with previous reports describing a sigmoidal relationship between liver GSH content and hepatic sinusoidal GSH efflux.[4,37] Whatever the dietary casein level, the hepatic pool and the release of GSH were markedly depressed during the post-absorptive period, however the liver remained a net site of GSH release. Moreover, when the diet was supplemented with methionine, the hepatic GSH was less depleted during the post-absorptive period.

The procedure of hepatocytes isolation appears to induce a marked depletion of GSH concentration, particularly in cells from rats fed a 10% casein diet; this suggests a relationship between the initial level of GSH and GSH preservation during cell preparation. In vitro, the cell GSH content markedly increased until the concentration of methionine in the extracellular medium reached 0.4 mM. As far as the plasma concentration of methionine in vivo is low (≈0.1 mM), this amino acid could constitute a limiting factor for GSH synthesis. Moreover, in vitro, the effects of methionine and *N*-acetylcysteine on GSH synthesis were similar, which supports the hypothesis for a key role of methionine to favour GSH synthesis in vivo. In hepatocytes isolated from rats adapted to a very high protein diet (60% casein), no stimulatory effect of methionine on GSH synthesis could be observed. This could be explained by an accelerated catabolism of sulfur amino acids[29] and/or by the enhanced glucagon secretion observed with this diet.[38] It has been established that glucagon is liable to stimulate the phosphorylation of γ glutamyl-cysteine synthetase, leading to its inhibition.[39]

In contrast with the liver, the muscle GSH content underwent a similar rise with a high protein diet or a low protein diet supplemented with methionine and this GSH concentration was maintained practically unchanged during the post-absorptive period. On the other hand, only a supplementation of the diet with methionine or an acute administration of a 30% casein meal was effective to significantly increase the heart concentration of GSH in fed rats.

In some diseases, a severe loss of GSH has been demonstrated.[40] From a prophylactic or therapeutic standpoint of view it has to be emphasized that a compensation of the GSH loss is required before a critical lower threshold is reached. Therefore, it appears of great interest to develop strategies for GSH supplementation. The direct administration of GSH to animals or to humans has not given convincing results.[16,17] On the other hand, it has been demonstrated that a significant rise of plasma and tissue levels of GSH can be achieved in animals treated with GSH esters;[41] however, caution is advised because high levels of GSH esters were associated with evidence of toxicity.[42] Given that the tolerance of the organism toward an amino acid supply is limited, it would be more advisable to recommend the intake of foods containing proteins rich in sulfur amino acids to favor the rapid recovery of GSH stores when these last are depleted. Nevertheless, in some cases of enteral nutrition, the supplementation in methionine could constitute an interesting mean to ensure an efficient GSH synthesis.

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