Methionine restriction increases blood glutathione and longevity in F344 rats

JOHN P. RICHIE, JR.,*[,1] YVONNE LEUTZINGER,* SAUDHAMINI PARTHASARATHY,† VIRGINIA MALLOY,† NORMAN ORENTREICH,† AND JAY A. ZIMMERMAN[†,‡]

*Division of Nutritional Carcinogenesis, American Health Foundation, Valhalla, New York 10595, USA; †Orentreich Foundation for the Advancement of Science, Inc., Cold Spring-on-Hudson, New York 10516, USA; and ‡Department of Biological Sciences, St. John's University, Jamaica, New York 11439, USA

ABSTRACT **Little is known about the biochemical mechanisms responsible for the biological aging process. Our previous results and those of others suggest that one possible mechanism is based on the loss of glutathione (GSH), a multifunctional tripeptide present in high concentrations in nearly all living cells. The recent finding that life-long dietary restriction of the GSH precursor methionine (Met) resulted in increased longevity in rats led us to hypothesize that adaptive changes in Met and GSH metabolism had occurred, leading to enhanced GSH status. To test this, blood and tissue GSH levels were measured at different ages throughout the life span in F344 rats on control or Met-restricted diets. Met restriction resulted in a 42% increase in mean and 44% increase in maximum life span, and in 43% lower body weight compared to controls ($P < 0.001$). Increases in blood GSH levels of 81% and 164% were observed in mature and old Met-restricted animals, respectively ($P < 0.001$). Liver was apparently the source for this increase as hepatic GSH levels decreased to 40% of controls. Except for a 25% decrease in kidney, GSH was unchanged in other tissues. All changes in GSH occurred as early as 2 months after the start of the diet. Altogether, these results suggest that dramatic adaptations in sulfur amino acid metabolism occur as a result of chronic Met restriction, leading to increases in blood GSH levels and conservation of tissue GSH during aging.—Richie, J. P., Jr., Leutzinger, Y., Parthasarathy, S., Malloy, V., Orentreich, N., Zimmerman, J. A. Methionine restriction increases blood glutathione and longevity in F344 rats. *FASEB J.* 8, 1302–1307 (1994)**

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DESPITE TREMENDOUS INTEREST IN GERONTOLOGICAL research during the last 30 years, the biochemical mechanisms by which the aging process regulates life span remain unclear. Several lines of research have suggested that glutathione (GSH)[2] may play a critical role in the aging process. GSH is an important antioxidant found in high concentrations in nearly all living cells. Its many functional roles include the detoxification of a variety of endogenous and exogenous compounds such as xenobiotics, free radicals, and lipid peroxides; maintenance of protein structure and function; regulation of protein synthesis and degradation; and maintenance of immune function (1, 2). Decreased GSH has been associated with the pathogenesis of specific diseases such as diabetes (3), alcoholic liver disease (4), AIDS (5), and cataracts (6). Depletion of GSH has crucial effects on survival and low GSH levels are associated with cell damage and increased susceptibility to toxic challenge.

Our previous results and those of others have demonstrated that aging is characterized by a loss of GSH. Decreased GSH levels were observed in tissues of senescent organisms including the adult mosquito (7), housefly (8), C57BL/6 mouse (9–13), F344 (14) and Lobund-Wistar (15) rats, and in blood (16–18) and lens tissue (19) from elderly human subjects. Based on the important and wide-ranging functional roles of GSH in health maintenance and survival, we proposed that a GSH deficiency represents a key factor in the aging process and may underlie many changes that occur during senescence (20).

Support for this hypothesis came from the demonstration that nutritional enhancement of GSH levels extended the life span of the mosquito. Biosynthesis of GSH appears to be limited by the availability of cysteine, either from the diet or derived from methionine through the hepatic cystathionine pathway (21). In the aging mosquito model, feeding a specific Cys precursor (thiazolidine carboxylic acid) corrected the aging deficiency of both Cys and GSH and prolonged the life span by 38% (22, 23). This same precursor also increased life span and decreased the accumulation of age pigments in the fruitfly (24). Although the effect of administering GSH precursors on longevity has not been tested in mammalian models, higher GSH levels have been shown to occur during chronic caloric restriction (14, 15), the only well-accepted means of increasing life span potential in mammals (25). Blood GSH levels in calorie-restricted Lobund-Wistar rats were higher than in controls, with increases ranging from 14% in mature adults to as much as 90% in very old animals (15). Likewise, caloric restriction in F344 rats, which enhanced longevity by greater than 40%, also prevented the aging-associated decrease of hepatic GSH (14).

A recent report (26) described another dietary intervention that enhanced life span in mammals: increases in both median and maximum life span were observed in F344 rats fed a diet severely restricted in the essential amino acid methionine (Met). Met restriction appeared to have a similar effect on growth and life span as caloric restriction (25), but the mechanisms involved are apparently different. Met restriction resulted in impaired growth, even though food was provided ad lib and consumption was only 10% below that of controls. Indeed, the caloric intake of Met-restricted

[1]To whom correspondence and reprint requests should be sent, at: Division of Nutritional Carcinogenesis, American Health Foundation, One Dana Road, Valhalla, NY 10595, USA.

[2]Abbreviations: GSH, glutathione; Met, methionine; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); MPA, metaphosphoric acid.

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animals expressed on a per gram of body weight basis was actually 62–193% greater than that of controls. Also, pair-feeding the control diet to match the intake of the Met-restricted animals did not inhibit growth and Met-restricted animals did not resume growth when their diet was calorically enriched to match the intake of control rats.

Because Met is a precursor for Cys and GSH biosynthesis, a Met-restricted diet might be expected to deplete GSH. Indeed, this has been observed in short-term feeding studies in young animals (27, 28). However, based on their increased longevity, we hypothesized that animals on a chronic Met-restricted diet would demonstrate adaptive changes in sulfur amino acid metabolism resulting in elevated blood GSH levels. To test this hypothesis, we examined blood and tissue GSH and Cys levels in adult animals of different ages on control and Met-restricted diets.

MATERIALS AND METHODS

Animals and diets

Male F344 rats were obtained from Taconic Farms (Germantown, N.Y.) at 4 wk of age. The rats were fed Purina Rat Chow until 6–8 wk of age when they were randomized into control and experimental diet groups. Both groups were fed a chemically defined diet (26) based on the AIN-76 diet with the protein replaced by an essential amino acid mixture (**Table 1**). As a control, Met was fed at the level of 0.86% (w/w) of the diet to mimic the sulfur amino acid content in the standard AIN-76 formulation; in the restricted diet Met was provided at 0.17% (w/w), a level below which the animals could not survive. To compensate for the lower Met, glutamic acid was raised from 2.70% in the control diet to 3.39% in the experimental diet. Animals were housed in a conventional animal facility in groups of five in solid-bottomed cages lined with wood chips. All animals were given free access to food and acidified water and maintained on a 12 h light/dark cycle throughout the study. A separate cohort consisting of 16 animals per diet group was used to assess life span characteristics, and survival was measured by assessing mortality daily.

Tissue collection and processing

All tissues were routinely collected between 9:00 and 10:00 AM to avoid the confounding effects of circadian fluctuations. From some rats, whole blood samples were collected at 20–25 months of age from the suborbital vein into heparinized tubes. Other animals were killed at either 3–4 months or 29–32 months of age by CO_2 exposure after overnight fasting. Before death, blood was obtained by cardiac puncture, and immediately afterward tissues including liver, kidneys, lungs, brain, and spleen were removed and rinsed in ice-cold saline. After collection, blood and tissues were maintained at 0–4°C and processed within an hour, with blood and kidney treated within the first 15 min after removal. Blood was processed by the addition of 4 volumes of 5% (w/v) metaphosphoric acid (MPA) (Mallinckrodt, Paris, Ky.). After centrifugation at 14,000 × *g* for 2 min, the acid-soluble fraction was removed and stored at −70°C until analysis. Tissue samples were homogenized (10%, w/v) in 5% MPA, centrifuged, and stored as acid-soluble fractions at −70°C.

Measurement of glutathione and cysteine

Unless otherwise stated, total glutathione (GSH and GSSG) was measured by the method of Tietze (29) modified for use with 96-well plates: 50 μl of extract diluted 10-fold in 100 mM $NaHPO_4$/5 mM EDTA buffer at pH 7.5 was added to 50 μl of glutathione oxidoreductase (5 units/50 μl) and 50 μl of 2.5 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The reaction was initiated by the addition of 50 μl of 1.2 mM NADPH in buffer. The rate of color change at 410 nm, which is proportional to the amount of total glutathione in the samples, was monitored in a Dynatech MR700 ELISA plate reader. On selected samples, both the levels of GSH and GSSG were quantitated using our HPLC with electrochemical detection method (30). All samples were analyzed in duplicate. An aliquot of blood was hemolyzed in distilled H_2O and analyzed spectrophotometrically for hemoglobin content using Drabkin's reagent (31). Cysteine and cystine were determined by the method of Gaitonde (32). Briefly, samples were reduced in acidic medium with 10 mM dithiothreitol and conjugates of ninhydrin were assayed spectrophotometrically at 560 nm.

Statistical analysis

Data are expressed as means ± SEM. Differences between groups were considered significant at $P < 0.05$ by Student's *t* test or by ANOVA with Scheffe's posthoc test.

RESULTS

Met restriction resulted in a dramatic increase in life span over control-fed rats (**Fig. 1**). Mean life span was increased 42% from 103 ± 5.85 wk in controls to 146 ± 9.86 wk in Met-restricted animals ($P < 0.0005$), and median life span was also increased from 108 wk in controls to 154 wk in Met-restricted animals. A 44% increase in maximum life span was also observed, from 133 wk in controls to 191 wk in Met-restricted animals.

Body weight was monitored throughout the life span and the data are provided in **Fig. 2.** Control-fed animals grew throughout the first 52 wk, reaching an average adult weight of about 450 g, typical for this strain of rat. On the other hand, growth was greatly reduced in the Met-restricted animals, with adults achieving a maximum body weight of about 200 g.

Total glutathione (GSH and GSSG) levels were determined in retro-orbital blood samples from 20- to 25-month-old rats from the same cohort (**Table 2**). A striking 81% increase in concentration was observed in Met-restricted animals compared to controls. When samples were analyzed using HPLC with electrochemical detection (30), results demonstrated that the increase was due primarily to enhanced GSH levels as GSSG levels were low and unchanged in Met-restricted animals. Finally, the increase was not due to changes in hematologic parameters such as erythrocyte

TABLE 1. *Composition of the methionine-restricted diet*

Ingredient	Concentration in diet, %	Ingredient	Concentration in diet, %
L-Arginine	1.12	L-Phenylalanine	1.16
L-Lysine	1.44	Glycine	2.33
L-Histidine	0.33	Dextrin	5.00
L-Leucine	1.11	Corn starch	43.61
L-Isoleucine	0.82	Sucrose	20.00
L-Valine	0.82	Solca floc	5.00
L-Threonine	0.82	Choline bitartrate	0.20
L-Tryptophan	0.18	Vitamin mix-AIN	1.00
L-Methionine	0.17 (0.86)[a]	Mineral mix-AIN	3.50
Glutamic acid	3.39 (2.70)[a]	Corn oil	8.00

[a] Amino acid concentrations of control diet are given in parentheses.

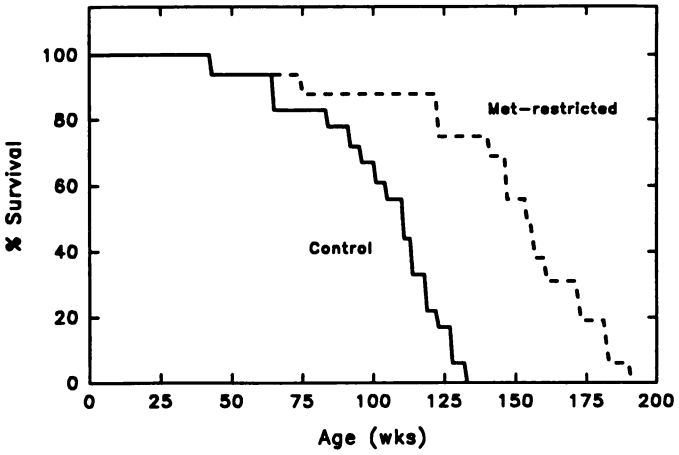

Figure 1. Increased longevity in F344 rats fed a Met-restricted diet. Thirty-two 6-wk-old animals were randomized into a control and a diet group of 16 animals each. Protein in the AIN-76-based diet was replaced by an essential amino acid mixture containing Met at 0.86% (w/w) or 0.17% (Met-restricted).

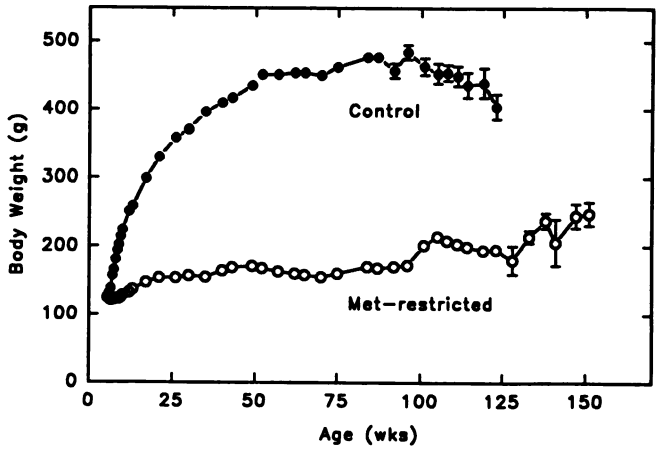

Figure 2. Body weight of animals on Met-restricted and control diets.

number or size, as a similar increase due to Met-restriction was observed when GSH levels were expressed on a per gram of hemoglobin basis.

The effect of life-long Met restriction was also assessed in old animals, age 29–32 months. Mean body weight was reduced from 349 ± 32.3 g for the control-fed animals to 208 ± 24.3 g for Met-restricted animals (P = 0.0013) (**Table 3**). Liver, lung, and kidney weights were correspondingly lower in Met-restricted rats and brain size was unchanged. Thus, organ to body weight ratios remained the same for all tissues except brain, where it increased 69% in the Met-restricted group (P = 0.01).

Blood GSH levels of these old animals were increased with Met restriction to 267% of control levels ($P < 0.002$) (**Table 4**). In contrast, liver GSH was reduced by more than 50% ($P < 0.002$) and kidney levels were decreased by 25% ($P < 0.03$). No differences were observed in all other tissues examined. A 41% decrease in cyst(e)ine levels was observed due to Met restriction in plasma ($P < 0.04$). A decrease of 20% was also observed in whole blood, but the difference was not statistically significant. Liver and kidney cyst(e)ine levels were unchanged in Met-restricted animals.

To establish how soon after the introduction of the experimental diet the changes in sulfur amino acid metabolism had occurred, animals were examined 2 months after the start of the diet. Results obtained with these young animals were similar to those observed with the old rats. As presented in **Table 5,** blood GSH levels increased to 175% of controls, and liver and kidney levels were reduced to 51% and 70% of controls, respectively ($P < 0.002$). The levels of GSH in other tissues were unchanged except for a 21% decrease in spleen. Thus, the same changes in GSH observed in old animals are evident as early as 8 wk after the start of the diet. In this same period, cysteine concentration was similar in both dietary groups for all tissues except the liver, which was 32% lower in Met-restricted animals (Table 5).

To assess the effects of Met restriction on the previously observed developmental and aging-related changes in GSH levels, rats were separated into age groups as follows based on the survival characteristics of this strain: young (3–4 months), mature (20–25 months), and old (29–32 months). Although an aging decrease of 18% was observed in blood of control animals ($P < 0.05$), the levels of GSH in old (29–32 months) Met-restricted rats were 22% greater than in mature Met-restricted adults ($P < 0.05$) as well as being 167% greater than in control-fed animals ($P < 0.001$) (**Fig. 3**). In control rats, a 24% decrease was observed between young and mature rats representing the growth and maturation period. The elevation of GSH by Met restriction occurred in all age groups. Indeed, despite the 42% drop in GSH between young and old control-fed rats, GSH levels in Met-restricted animals of both ages were elevated to approximately the same level.

DISCUSSION

In the present study, Met restriction resulted in dramatic increases in both mean and maximum life span, as previously observed (26). Although Met restriction inhibited growth, all animals appeared otherwise healthy, based on gross inspection of coat, internal organs, and behavior. These data suggest that life-long Met restriction results in an actual delay of the biological aging process in F344 rats. In addition, this effect on survival and body weight is not due to caloric restriction (26).

TABLE 2. *Increased blood glutathione in adult F344 rats fed a Met-restricted diet*

	GSH + GSSG		(GSH + GSSG)/hemoglobin	
Diet[a]	(μeq. GSH/ml)[b]	% of control	(mg/g)[b]	% of control
0.86%	0.945 ± 0.0316	(100)	1.06 ± 0.114	(100)
0.17%	1.71 ± 0.103*	181	1.74 ± 0.132*	164

[a]Diets are 0.86% Met (control) and 0.17% Met (Met-restricted). [b]Results are expressed as means ± SEM, n = 6. *Values are significantly different from control values, $P < 0.001$.

TABLE 3. *Body and organ weights in old Met-restricted rats*

	Weight, g[a]		Relative weight, g/100 g body wt[a]	
	Control	Met-restricted	Control	Met-restricted
Whole body	349 ± 32.3	208 ± 24.3*	–	–
Liver	11.1 ± 0.681	6.32 ± 0.464*	3.21 ± 0.297	3.11 ± 0.170
Kidney	3.57 ± 0.400	1.97 ± 0.108*	1.06 ± 0.040	0.977 ± 0.076
Lung	1.83 ± 0.052	1.08 ± 0.041*	0.535 ± 0.061	0.616 ± 0.106
Brain	2.02 ± 0.118	2.02 ± 0.067	0.597 ± 0.088	1.01 ± 0.088†

[a]Values are mean ± SEM; $n = 3$ for the control group and $n = 5$ for the Met-restricted group. *Significantly different from control values, $P < 0.002$. †Significantly different from control values, $P < 0.01$.

TABLE 4. *Blood and tissue glutathione and cyst(e)ine levels in old rats on a Met-restricted diet*

	GSH + GSSG, μeq. GSH/ml or g[a]		Cys + cystine, neq. Cys/ml or g[a]	
Tissue	Control	Met-restricted	Control	Met-restricted
Whole blood	0.780 ± 0.083	2.08 ± 0.102*	80.1 ± 21.4	64.2 ± 9.44
Plasma	–	–	106 ± 14.3	62.9 ± 14.0†
Liver	3.05 ± 0.332	1.43 ± 0.212*	274 ± 60.8	242 ± 44.7
Kidney	1.69 ± 0.224	1.20 ± 0.076†	895 ± 94.3	859 ± 50.8
Brain	1.04 ± 0.110	1.16 ± 0.071	–	–
Lung	0.821 ± 0.318	0.660 ± 0.226	–	–

[a]Results are expressed as means ± SEM; $n = 3$–5 rats per group. *Values are significantly different from control values, $P < 0.002$. †Values are significantly different from control values, $P < 0.05$.

TABLE 5. *Blood and tissue glutathione and cyst(e)ine levels in young rats after 2 months on a Met-restricted diet*

	GSH + GSSG, μeq. GSH/ml or g[a]		Cys and cysteine, neq. Cys/ml or g[a]	
Tissue	Control	Met-restricted	Control	Met-restricted
Whole blood	1.34 ± 0.035	2.35 ± 0.046*	–	–
Plasma	–	–	141 ± 9.32	131 ± 18.1
Liver	2.92 ± 0.212	1.50 ± 0.122*	304 ± 14.5	207 ± 22.5*
Kidney	1.41 ± 0.111	0.997 ± 0.056*	914 ± 78.6	789 ± 38.0
Brain	1.36 ± 0.037	1.24 ± 0.067	116 ± 31.8	131 ± 21.5
Spleen	1.08 ± 0.054	0.852 ± 0.112†	279 ± 43.7	274 ± 34.3
Stomach	1.52 ± 0.060	1.37 ± 0.089	206 ± 27.1	154 ± 19.3
Lung	1.35 ± 0.072	1.44 ± 0.081	235 ± 31.9	203 ± 31.7
Testis	2.89 ± 0.045	2.59 ± 0.104	283 ± 57.5	327 ± 28.2

[a]Results are expressed as means ± SEM; $n = 10$. *Values are significantly different from control values, $P < 0.004$. †Values are significantly different from control values, $P < 0.05$.

Of major importance is our finding that despite severely restricting the dietary intake of Met, the only available precursor for GSH or Cys, substantial increases in blood GSH levels were observed. Indeed, to our knowledge the extent of GSH enhancement in blood that we report here is greater in magnitude than that observed in adult animals by any dietary means previously reported. It is particularly remarkable because GSH levels are tightly regulated by feedback inhibition of the GSH biosynthetic pathway (33). In addition, with the exception of decreases in liver and kidney GSH, GSH and cyst(e)ine levels were both unchanged in other tissues. These unusual findings clearly suggest that major metabolic adaptations have occurred, as early as 2 months into the diet, that allow for the conservation of GSH and the efficient utilization of available precursors to synthesize GSH.

Although earlier studies had examined the effect of Met restriction on GSH and Cys levels, they all used young animals and much shorter (1 wk) feeding periods (27, 28). In one study where young Sprague-Dawley rats were fed a Met-restricted diet for 15 days, decreased GSH levels were observed in liver and other tissues, but no GSH changes were observed in erythrocytes or brain (34). Thus, the metabolic changes apparent in the present study are likely to have occurred between 2 and 8 wk of feeding. Indeed, Mortensen (35) has shown an increase in blood GSH of weanling rats after 4 wk on a Met-restricted diet.

Several metabolic pathways are affected by feeding a Met-restricted diet and could result in the observed GSH changes. A lack of Cys in the diet necessitates a shift in the hepatic cystathionine pathway toward Cys synthesis (21). An increase in GSH biosynthesis in the liver is likely, as lower

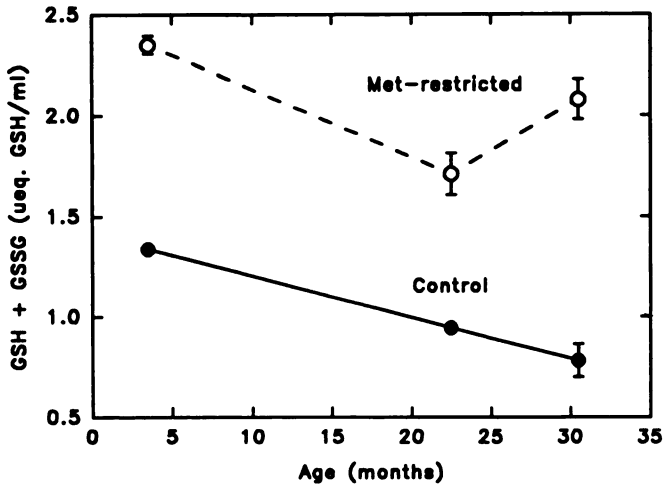

Figure 3. Effect of age on blood GSH in Met-restricted rats. Blood GSH was determined in young (3-4 month), mature (20-25 month), and old (29-32 month) rats by the method of Tietze (29). Each symbol and bar represent the mean ± of 3-10 rats. Bars are omitted if SEM was less than the size of the symbol.

liver GSH levels would decrease the feedback inhibition by GSH on the GSH biosynthetic pathway (33). In addition, Cys can be incorporated into GSH more efficiently because the competing demand for Cys by protein synthesis (36) is reduced, as evidenced by the absence of growth.

Increased efflux of GSH from the liver into the blood (as reflected by the large increase in blood GSH) may be responsible for the maintenance of GSH in extrahepatic tissues. Indeed, greater than 90% of the turnover of GSH in the liver has been attributed to efflux (37). Increases in hepatic GSH synthesis and efflux also occur in starvation (37) as well as during strenuous exercise (38). Thus, the liver GSH reserve is sacrificed in order to deliver GSH to other critical tissues. If, as has been suggested recently, the kidneys contribute significantly to the RBC GSH (39), our finding of a 25% reduction in kidney GSH may point to an increased efflux from the kidney. We note that the average level of liver GSH does not appear to be an important factor in longevity as Met-restricted rats have an extended life span despite having less than half the GSH of controls.

The effect of Met restriction is not limited to young animals. Indeed, in old Met-restricted rats, at an age where a GSH decline is evident in control-fed animals, blood GSH levels remain high: at the same level as young Met-restricted animals and 167% greater than controls.

The differential response of blood and liver GSH to Met restriction provides further evidence that different metabolic mechanisms are responsible for the effects of caloric restriction and Met restriction. In previous reports, both liver and blood GSH levels were increased in response to caloric restriction (14, 15). There may be, however, a relationship between Met restriction and overall protein restriction. Protein restriction is believed by some researchers to extend life span, although few studies have distinguished protein restriction from caloric restriction in the same study design (40-43). Protein restriction has been shown to enhance various antioxidant defense systems: in Swiss mice, increased activity of superoxide dismutase and decreased lipid peroxidation in liver have been demonstrated together with decreased accumulation of lipofuscin pigments in brain, liver, and intestine (44); decreased liver protein oxidation was found in F344 rats (45). We have not yet established whether Met restriction has similar effects on antioxidants other than GSH.

Dietary Met restriction represents an important tool for exploring the biochemical mechanisms responsible for the biological aging process. We propose that metabolic adaptations have occurred that optimize the synthesis of GSH by the liver and its delivery via blood to critical extrahepatic tissues. The importance of maintaining optimal GSH levels is indicated by the many essential functions of GSH. The role of GSH in Met restriction, together with previous evidence on the involvement of GSH in aging, strongly support the hypothesis that GSH deficiency is an important factor in the regulation of the aging process. In addition, these new findings indicate that the GSH content of blood is an important index of longevity. FJ

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