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A Decrease in Cysteine Levels Causes the Glutathione Deficiency of Aging in the Mosquito (42660)

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Abstract. Our previous results indicated that a glutathione (GSH) deficiency is a determinant of the aging process in many tissues and organisms. Correction of this deficiency in the aging mosquito by feeding the cysteine (Cys) precursor magnesium thiazolidine carboxylic acid (MgTc) suggested that the cause could be a lack of Cys. Adult mosquitoes (*Aedes aegypti*) were fed either a control diet or a diet supplemented with MgTC and then were analyzed for their Cys, cystine, GSH, and glutathione disulfide contents with our HPLC method. The life span profile of Cys levels paralleled that of GSH in the control group with high levels in the young that decreased during maturity and aging. Cystine and glutathione disulfide were undetectable. The causal relationship between the Cys and the GSH deficiencies was shown in the MgTC-supplemented group with an 83% increase in Cys and a 39% increase in GSH relative to control values. Further the conversion steps of MgTC to Cys and then to GSH were verified by use of buthionine sulfoximine. These results demonstrate that a Cys deficiency occurs in the aging mosquito and is the cause of the GSH deficiency.

A deficiency of glutathione (GSH), a universally found antioxidant with a wide variety of important metabolic functions, appears to be a general phenomenon of aging tissues. This decrease, which occurred in a variety of tissues from different organisms such as mosquito (1, 2), mouse (3), and man (4, 5), supports our earlier findings of aging decreases in reducing capacity, such as lower levels of several $NADP^+$ -linked enzyme activities (6, 7), $NADPH/NADP^+$ ratios (8), protein biosynthesis (9), and DNA replication *in vivo* (10).

In the mosquito the deficiency of GSH was due to a decrease in its biosynthesis (11). However, the specific impairment was unknown and could be due to a lack of precursor amino acids or cofactors or to decreased enzyme activities.

Recent results led us to hypothesize that a lack of cysteine (Cys), a GSH precursor, is responsible for the decreased biosynthesis (12) and thus the GSH deficiency of aging. We found that both GSH levels and life span increased concomitantly in adult mosquitoes fed magnesium thiazolidine-4-carboxylic acid (MgTC) (12), a putative Cys precursor (13, 14). Further, old mosquitoes retained the same capacity for this GSH enhancement as younger adults.

Our objectives were to test this hypothesis by determining the levels of Cys and GSH in the aging mosquito and to investigate the role of Cys in the MgTC enhancement of GSH. This approach was possible because of our recent development of an HPLC method for the simultaneous determination of Cys, cystine, GSH, and glutathione disulfide (GSSG) levels (15).

For these experiments the yellow fever mosquito (*Aedes aegypti*) was selected because of its unique advantages. It is a multicellular, eukaryotic organism that resembles mammals in its biochemical composition and nutritional requirements for growth and has been validated as a model of aging (16–18).

Materials and Methods. *Experimental organism and its culture.* The experimental organism was the yellow fever mosquito, *A. aegypti* (Louisville), which has been colonized in this laboratory for over 20 years, amounting to about 300 generations. The chronological and biological ages of adult mosquitoes were as follows: 0–6 days, end of metamorphosis; 7–22 days, mature; 23–35 days, old; 35+ days, very old. These ages were characterized from survival curves and from DNA and protein profiles (19) of cohort populations that were cultured and aged under our

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standard conditions. The median survival time for these mosquitoes was 29 ± 2 days, a value which has been reproducible for several decades.

Our standardized culture procedure with defined conditions was used to produce mosquitoes of all ages throughout the life cycle (17). The procedure is outlined as follows: Eggs stored at 22°C for less than 1 month were synchronously hatched by agitating them for several minutes in distilled water on a vortex mixer. Larvae which emerged within 15 min were transferred to culture pans containing distilled water with a mineral supplement corresponding to that described by Lang *et al.* (16). An aqueous 10% (w/v) suspension of dessicated hog liver (Teklad, Madison, WI) was added daily to maintain a bacterial infusion, which is the principal source of nutrients for the growing larvae.

Pupae were transferred to distilled water for adult emergence. Adult mosquitoes were fed from cotton pads moistened with 10% (w/v) sucrose solution, which simulates the normal diet of plant and fruit nectars for adult mosquitoes. Blood meals, which are required only for ovarian development in females (20), were withheld to avoid this complication. Indeed, results comparing blood-fed vs sucrose-fed adults indicated a much shorter life span for the blood-fed mosquitoes (21).

Materials. DL-Buthionine-(*S,R*)-sulphoximine (BSO) was obtained from Chemical Dynamics Corp. (South Plainfield, NJ) MgTC was a generous gift from Dr. Jaime Miquel (Farmacia Diana, Denia, Spain). GSH, GSSG, CYS, and cystine were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade, and the water used was double glass-distilled.

Experimental feeding regimen. From 25 to 30 female 12-day-old adult mosquitoes of different ages were placed in cages consisting of glass lantern chimneys fitted with nylon net tops and screened petri dish bottoms. Control groups were fed a normal diet of 10% (w/v) sucrose solution. The experimental groups were fed 10% sucrose solution supplemented with either 0.05% (w/v) MgTC or 0.1% (w/v) BSO or with a combination of 0.05% MgTC and 0.1% BSO. This concentration of MgTC was previously shown to increase both GSH levels and longevity (12), and preliminary results demonstrated that this level of BSO decreased GSH levels by about 50%. All solutions were provided on cotton pads placed on the tops of the cages and were replaced every other day. An environmental temperature of 29 ± 1°C and a 12-hr light cycle was maintained throughout the investigation. After 8 days of treatment, the mosquitoes were processed for HPLC analysis.

Preparation of samples. Samples of 8–15 mosquitoes were cold inactivated, and homogenates (10% (w/v)) were prepared in ice-cold 5.0% (w/v) metaphosphoric acid using an all-glass Ten Broeck homogenizer. After centrifugation at 14,000*g* for 15 min, the supernatants were collected and analyzed by HPLC.

Analysis of glutathione and cyst(e)ine. GSH, GSSG, Cys, and cystine were separated and quantified simultaneously by HPLC with dual electrochemical detection using a novel method (15). In brief, portions of acid supernatants were diluted 1/20 with HPLC solvent, and 20-μl aliquot was injected onto the reverse-phase column and eluted isocratically using a solvent of 96% (v/v) 0.1 *M* monochloroacetic acid, pH 3.0, and 4% (v/v) methanol and an ion-pairing reagent of 2.0 m*M* heptane sulfonic acid with a flow rate of 1 ml/min. The compounds were detected in the eluant with a dual electrochemical detector of two Au/Hg electrodes in series. The resultant profiles (nA vs sec) were quantified on the basis of peak area and the use of authentic external standards. The standards contained Cys (0.5–5 μ*M*), cystine (1–10 μ*M*), GSH (8–80 μ*M*), and GSSG (1–20 μ*M*) and were prepared daily.

Statistical methods. Student's *t* test was used for statistical comparisons, and a test for independence was used to demonstrate whether slopes were different from zero (22).

Results. *Validation of the method.* The key feature of this investigation was the use of an analytical method specific for Cys, cystine, GSH, and GSSG. This method was validated in mosquito samples by adding known amounts of authentic compounds to mosquito samples before homogenization and determining their recovery (Table I). Several

TABLE I. RECOVERY OF CYSTEINE, CYSTINE, GSH, AND GSSG ADDED TO ADULT MOSQUITO SAMPLES

Analyte	Sample	Analyte added	Sample + addition	% Recovery
Cysteine	6.98 ± 0.226[a]	7.50[a]	14.9 ± 0.422[a]	103
Cystine	<0.05	5.00	4.73 ± 0.430	95
GSH	82.3 ± 6.23	79.5	157 ± 4.46	97
GSSG	<0.05	5.00	5.22 ± 0.231	104

Note. Known amounts of cysteine, cystine, GSH, and GSSG were added to different samples of 8–12 adult (12-day-old) mosquitoes before tissue processing. The results were expressed as the means ± SEM for three samples, and recovery was given as percentages of added analyte recovered compared to calculated amounts.

[a] nmole/sample. The sample size was 1 ml for each analyte and contained 91 mg of tissue.

concentrations of each sample were assayed to ensure proportionality between chromatographic peak area and sample size. Recoveries of the added compounds ranged from 95 to 104%, indicating that the processing and analytical methods were valid for the quantitative determination of cyst(e)ine and glutathione in mosquito tissues.

HPLC profiles. Typical chromatographic profiles of a standard solution of authentic compounds and a mosquito sample are shown in Fig. 1. Only GSH and Cys were found in the samples since cystine and GSSG were below the detection limits of 1 pmole/mg. All peak areas of samples corresponded to 300 to 600 pmole of GSH and 10 to 40 pmole of Cys.

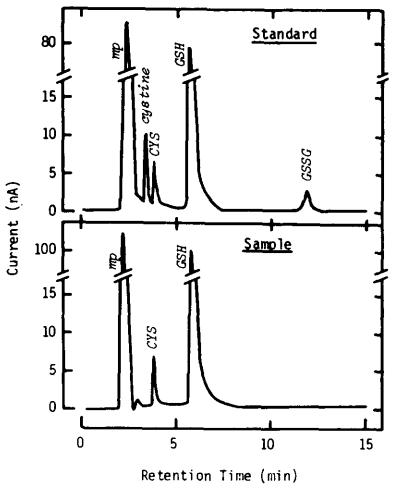

FIG. 1. HPLC profile of an adult mosquito sample. A pooled sample of 15 adult (12-day-old) mosquitoes was processed as described in the text. After dilution in HPLC buffer, a 20-μl aliquot was injected onto the HPLC column and eluted as described in the text. The standard chromatogram represents 60 pmole cystine, 30 pmole cysteine, 480 pmole GSH, and 60 pmole GSSG. Cystine and CSSG were not found. The peak labeled MP is due to meta-phosphoric acid used as the protein precipitant in the sample processing.

Life span profiles of Cys and GSH. The Cys and GSH levels as well as the GSH/Cys ratios during the life span of the adult mosquito are shown in Fig. 2. The levels are expressed per milligram of fresh tissue since our previous studies showed that weight, DNA, and protein content are equivalent biochemical parameters of cell size and number in all stages of the mosquito (10, 19). The GSH content was 12–23 times greater than that of Cys throughout the life span.

The GSH levels decreased from 2.04 ± 0.0676 to 1.10 ± 0.0475 nmole/mg during the first 7 days of adult life, a period representing the end of metamorphosis. From 8 to 48 days, the periods of maturity and senescence, the GSH content decreased 40%. These GSH values confirmed our earlier values (3).

Similarly the Cys levels decreased from 0.143 ± 0.00359 to 0.0901 ± 0.00847 nmole/mg during the first 7 days and decreased 60% during maturity and senescence. The Cys profile paralleled that for GSH as demonstrated by the GSH/Cys ratios which were constant throughout most of the life span. However, in the very old, 47-day mosquitoes a larger decrease in Cys than in GSH resulted in a higher ratio.

The Cys and GSH decreases in the aging mosquito coincided with a rapid decrease in survival, and all decreases were statistically significant ($P < 0.001$). It is unlikely that starvation was responsible for the aging decreases in GSH and Cys because no changes

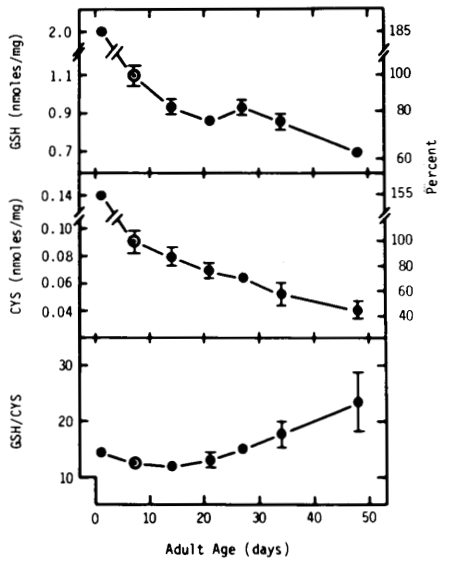

FIG. 2. Correlation of cysteine and glutathione levels during the life span of the mosquito. Cysteine and reduced glutathione levels were determined in pooled samples of 12–15 adult mosquitoes of different ages throughout the life span. Cystine and oxidized glutathione were not found. The relative concentration levels for the CYS, GSH, and GSH/CYS profiles were based on their 7-day values expressed as 100% (⊙). Each point and bar represent the means ± SEM of 4 to 10 samples. Bars were omitted when the SEM was less than the size of the point.

in levels were observed in mature and old adults maintained on water alone for up to 3 days.

Effect of MgTC on Cys and GSH levels. The effects of MgTC and BSO feeding on Cys and GSH levels are shown in Table II. MgTC alone increased the concentration of Cys by 83% and GSH by 39%. BSO feeding caused an increase in Cys of 100% but, in contrast, a decrease in GSH levels of 31% compared to those in the control groups. When a combination of MgTC and BSO was fed, an increase of 160% in Cys was observed, but GSH levels were 25% lower than the controls. This specific block of GSH synthetase provides strong evidence that the MgTC effect on GSH is through Cys.

Discussion. The present results demonstrate that a decrease in Cys levels is the cause of the GSH deficiency of aging in the mosquito. The key findings were the correlation of Cys and GSH concentrations with aging and the concomitant increase in both by feeding a Cys precursor.

We believe that a combined Cys and GSH deficiency is a biochemical determinant of the aging process, since the specific correction of either results in an increased longevity of the mosquito (12). This deficiency is not due to a general nutritional lack of proteins or amino acids, because dietary supplements of cystine, Glu, Gly, Ser, and GSSG had no effect on either the tissue content of GSH or Cys or the life span. GSH and Cys supplements also were ineffective, probably due to the poor transport and rapid autoxidation of these compounds (23).

The aging decrease in Cys content is to our knowledge the first demonstration in any normally aging organism. This decrease occurred concurrently and was well correlated with a decrease in GSH levels ($r^2 = 0.99$).

TABLE II. MgTC IS A SPECIFIC PRECURSOR OF CYSTEINE

Diet group	Cysteine		Reduced glutathione	
	nmole/g	% of control	nmole/g	% of control
Control	68.2 ± 15.9	(100)	816 ± 60.6	(100)
0.05% MgTC	124 ± 15.9*	183	1140 ± 54.2**	139
0.1% BSO	137 ± 23.4*	200	566 ± 48.9*	69
0.05% MgTC + 0.10% BSO	177 ± 12.4**	260	627 ± 40.6	77

Note. Cysteine and reduced glutathione levels were determined in pooled samples of 12–15 adult (12-day old) mosquitoes fed a control diet or a diet supplemented with 0.05% (w/v) MgTC, 0.10% BSO, or both 0.05% MgTC and 0.10% BSO for 8 days. Results were expressed as the means ± SEM for four samples.

* Difference from control is statistically significant, $P < 0.01$.

** Difference from control is statistically significant, $P < 0.001$.

Both the Cys and GSH decreases were unusual, for the free amino acid, protein, and DNA levels are constant in the mature and old mosquito (19).

The cause of the aging deficiency in Cys in the mosquito is unknown but could be due to a decreased rate of formation or an increased rate of utilization or degradation. Regardless of the specific cause, our results showed that the deficiency is corrected by nutritional modification.

A decreased level of Cys, like that of GSH, may also occur in other organisms and account for the GSH deficiency of aging. Indeed our preliminary results suggest that a Cys deficiency also occurs in the aging mouse and can be corrected by Cys precursors. Further investigation is necessary to determine the generality of this phenomenon and its underlying cause.

The causal relationship between the Cys and the GSH deficiencies was demonstrated by MgTC supplementation which caused concomitant increases in Cys and GSH. Further, the specific conversion steps of MgTC to Cys and to GSH were verified by the addition of BSO, an inhibitor of GSH synthetase, which blocked the MgTC enhancement of GSH but not Cys.

A major signficance of the findings is the discovery of a Cys deficiency in aging. Cys is important in protein biosynthesis, as a component of the active sites of many enzymes, and as a major determinant of secondary and tertiary protein structure. Indeed a Cys deficiency could be responsible for the decrease in protein biosynthesis observed in the aging mouse and mosquito (9, 24–26).

An additional effect of the Cys deficiency is that it can give rise to a lack of GSH, which has many well-established metabolic roles. These roles have been reviewed extensively (27–30) and include the maintenance of cell membranes, destruction of metabolic peroxides and free radicals, detoxification of the xenobiotics, maintenance of sulfhydryl groups of enzymes and proteins, control of redox status and disulfide-exchange reactions, regulation of enzyme activities, and translocation of amino acid and peptides across membranes.

The results also provide direct evidence of the *in vivo* conversion of MgTC to Cys. Our earlier results suggested this possibility because MgTC enhanced GSH levels (12). Also as indirect evidence of MgTC conversion to Cys were the findings that it could replace Cys as a nutrient in the rat (13), that MgTC was enzymatically converted to Cys *in vitro* by proline oxidase (14), and that other thiaolidine carboxylic acids such as the 2-oxo and 2-methyl derivatives were converted to Cys *in vitro* and increase GSH levels *in vivo* (31, 32).

Finally, of special interest, these findings together with our earlier results (12) demonstrated that the nutritional modification of Cys affects both GSH levels and longevity. Therefore, the role of dietary Cys and other sulfur amino acids is a fruitful area for future study.

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1. Abraham EC, Taylor JF, Lang CA. Influence of mouse age and erythrocyte age on glutathione metabolism. Biochem J **174**:819–825, 1978.
2. Hazelton GA, Lang CA. Glutathione levels during the mosquito life span with emphasis on senescence. Proc Soc Exp Biol Med **176**:249–256, 1984.
3. Hazelton GA, Lang CA. Glutathione contents of tissues in the aging mouse. Biochem J **188**:25–30, 1980.
4. Naryshkin S, Miller L, Lindeman R, Lang CA. Blood glutathione: A biochemical index of human aging. Fed Proc **40**:3179, 1981.
5. Schneider D, Naryshkin S, Lang CA. Blood glutathione, a biochemical index of aging women. Fed Proc **41**:7671, 1982.
6. Stephan JK, Acree DW, Lang CA. NADP-linked enzyme levels in young and old mouse tissues. Gerontologist **6**:14, 1966.
7. Lang CA, Stephan JK. Nicotinamide adenine dinucleotide phosphate enzymes in the mosquito during growth and aging. Biochem J **102**:331–338, 1967.
8. Lang CA, Acree DW. Nicotinamide adenine dinucleotide phosphate coenzyme levels during the life span of the mosquito. Gerontologist **7**:13, 1967.
9. Du JT, Beyer TA, Lang CA. Protein biosynthesis in aging mouse tissues. Exp Gerontol **12**:181–191, 1977.
10. Mills BJ, Lang CA. The biosynthesis of DNA during the life span of the mosquito. Biochem J **154**:481–490, 1976.

11. Hazelton GA, Lang CA. Glutathione biosynthesis in the aging adult yellow fever mosquito. Biochem J **210**:289–295, 1983.
12. Richie JP Jr, Mills BJ, Lang CA. Correction of a glutathione deficiency in the aging mosquito increases its longevity. Proc Soc Exp Biol Med **184**:113–117, 1987.
13. DeBey HJ, Mackenzie JB, Mackenzie CG. The replacement by thiazolidine carboxylic acid of exogenous cystine and cysteine. J Nutr **66**:607–619, 1958.
14. Muldoon WP, Nagasawa HT. Thiazolidine-4-carboxylic acid and other anti-metabolites: Oxidation by proline oxidase. Fed Proc **40**:847, 1981.
15. Richie JP Jr, Lang CA. The determination of glutathione, cyst(e)ine, and other thiols and disulfides in biological samples using high-performance liquid chromatography and dual electrochemical detection. Anal Biochem **163**:9, 1987.
16. Lang CA, Basch KJ, Storey RS. The growth, composition and longevity of the axenic mosquito. J Nutr **102**:1057–1066, 1972.
17. Kao PC, Beyer CF, Lang CA. A DNA replication intermediate in the young mosquito. Biochem J **154**:471–480, 1976.
18. Richie JP Jr, Mills BJ, Lang CA. A verification of a mammalian toxicant classification using the mosquito. Fundam Appl Tox **4**:1029–1035, 1984.
19. Lang CA, Lau HY, Jefferson DJ. Protein and nucleic acid changes during growth and aging in the mosquito. Biochem J **95**:372–377, 1965.
20. Putnam P, Shannon RC. The biology of *Stegomyia* under laboratory conditions. Proc Enterol Soc Wash **36**:217–242, 1934.
21. Clements AN. The Physiology of Mosquitoes. New York, Pergamon, pp116–129, 1963.
22. Snedecor GW, Cochran WG. Statistical Methods. Ames, Iowa State Univ Press, 7th ed., 1980.
23. Meister, A. Selective modification of glutathione metabolism. Science **220**:472–477, 1983.
24. Du JT, Hoffman JL, Lang CA. Leucine pool size in liver and heart of mature and old mouse. Gerontologist **12**:27, 1972.
25. Smith ER, Lang CA. Protein metabolism in the aging adult mosquito. Gerontologist **11**:16, 1972.
26. Lang CA, Smith ER. Protein metabolism in the aging adult mosquito. Fed Proc **31**:878, 1972.
27. Meister A, Anderson ME. Glutathione. Annu Rev Biochem **52**:711–760, 1983.
28. Larsson A, Orrenius S, Holmgren A, Mannervik B, Eds. Functions of GSH. New York, Raven Press, 1983.
29. Sakamoto Y, Higashi T, Tateishi N, Eds. Glutathione. Urecht, The Netherlands, VNU Science Press, 1983.
30. Ziegler DM. Role of reversible oxidation-reduction of enzyme thiols-disulfides in metabolic regulation. Annu Rev Biochem **54**:305–329, 1985.
31. Williamson JM, Meister A. Stimulation of hepatic glutathione formation by administration of L-2-oxothiazolidine-4-carboxylate, a 5-oxo-prolinase substrate. Proc Natl Acad Sci USA **78**:936–942, 1981.
32. Nagasawa TN, Goon DJ, Zera RT, Yuzon DL. Prodrugs of L-cysteine as liver-protective agents: 2(RS)-methylthiazolidine-4(R)-carboxylic acid, a latent cystine. J Med Chem **25**:489–491, 1982.

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