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Glutathione biosynthesis in the aging adult yellow-fever mosquito [*Aedes aegypti* (Louisville)]

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Our previous findings [Hazelton & Lang (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**(6), 2378 (abstr.)] demonstrated a senescence-specific decrease in glutathione (GSH) concentration in the yellow-fever mosquito *Aedes aegypti* (Louisville)]. As a possible mechanism for this change, GSH biosynthesis was investigated in adult mosquitoes of different ages through the life-span. Biosynthesis was measured as the incorporation rate of [^{14}C]glycine or [^{14}C]cystine into glutathione. Essential information to validate the procedure was also obtained on the precursor-amino-acid pool sizes and kinetic parameters such as lag-time and time course of incorporation. Also, synthesis *de novo* rather than exchange was verified using buthionine sulphoximine, a specific inhibitor of GSH biosynthesis. The synthetic rates with either amino acid precursor varied throughout the adult life-span, but the patterns for both precursors were essentially identical. Biosynthesis was high in the newly emerged adult and decreased 62–70% ($P < 0.005$) to a plateau during maturity. From the mature value there was a decrease of 36–41% ($P < 0.005$) to a new plateau during senescence. Glutathione biosynthesis and concentration were correlated throughout maturity and senescence ($r = 0.982$) and thus biosynthesis was proportional to glutathione content. On this basis we concluded that impaired biosynthesis is the major and perhaps sole mechanism for the aging decrease in glutathione content.

The importance of GSH stems from its high concentration in many animal and plant cells. Indeed, this tripeptide (L-γ-glutamyl-L-cysteinylglycine) is the most abundant thiol-reducing agent in tissues. Of greater significance is that glutathione participates in a variety of biosynthetic and detoxification reactions as described in a number of reviews (Jocelyn, 1972; Flohé *et al.*, 1974; Meister, 1975; Meister & Tate, 1976; Arias & Jakoby, 1976; Kosower & Kosower, 1978; Sies & Wendel, 1978).

Previously we found marked life-span changes in GSH content. Of special significance was the lower GSH concentration in the aging adult mosquito (Hazelton & Lang, 1978). Also aging-specific decreases were found in a variety of tissues of the senescent mouse (Abraham *et al.*, 1978; Hazelton & Lang, 1980*a*).

There are several possible metabolic mechanisms for this aging phenomenon, including GSH oxidation, utilization and degradation. Our previous studies indicated that these do not account for the GSH decrease (Hazelton & Lang, 1979, 1980*a*,*b*). Thus the only other major possibility is GSH synthesis.

To our knowledge there has been no systematic investigation of GSH biosynthesis during aging. The only work related to this is the report that enzymes of GSH synthesis were unchanged in aging erythrocytes (Minnich *et al.*, 1971). Also, during rat growth, the rate of GSH biosynthesis in the liver decreased (Lauterburg *et al.*, 1980).

The objective of the present study was to determine the levels of glutathione biosynthesis in aging adult mosquitoes. This aging profile was correlated with the profile of GSH concentrations to determine their possible causal relationship.

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-(5-phenyloxazol-2-yl)benzene.

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Materials and methods

The background and culture conditions for the yellow-fever mosquito, *Aedes aegypti* (Louisville),

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were described in the preceding paper (Hazelton & Lang, 1983).

Materials

[1-^{14}C]Glycine (47.4 mCi/mmol), L-[U-^{14}C]cystine (318 mCi/mmol), Protosol, Liquifluor (PPO/POPOP/toluene concentrate) and Aquasol-2 were obtained from New England Nuclear Corp., Boston, MA, U.S.A. Both labelled amino acids were purified by ion-exchange chromatography on Dowex-1 (acetate form) before use. DL-Buthionine-(*S*,*R*)-sulphoximine was generously given by Dr. O. W. Griffith of Cornell University Medical College, New York, NY, U.S.A. GSH, GSSG, the amino acids, and Dowex-1 (Cl^- form) ion-exchange resin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Other chemicals were reagent grade, and the water used was double-glass-distilled.

Glutathione biosynthesis

Glutathione biosynthesis was quantified from the initial rates of incorporation of [^{14}C]glycine and [^{14}C]cystine into glutathione after injection. Our general protocol was described for protein and DNA synthesis *in vivo* (Du *et al.*, 1977; Smith, 1971; Mills & Lang, 1976). Incorporation rates were expressed in terms of either pmol of GSH formed or of glycine (or half-cystine) incorporated. These are equivalent according to the stoichiometry of the reaction.

Pulse-labelling

Adult female mosquitoes of known age were cold-inactivated before injection. The injected solutions were prepared in *Aedes* Ringer solution (0.13 M-NaCl/4.7 mM-KCl/1.9 mM-$CaCl_2$/7.0 mM-KH_2PO_4, adjusted to pH 6.8 with KOH) and contained 6 nCi of [^{14}C]glycine or 4 nCi of [^{14}C]-cystine. The solutions (1 μl in volume) were injected into the membranous region between the first and second thoracic spiracles. In some experiments buthionine sulphoximine also was included in the injection solution at a final concentration of 20 mM. Injection needles were made from Pasteur pipettes (Medglass) pulled out to very fine points (approx. 0.1 mm in diameter) over a microflame. Each group of 8–14 injected mosquitoes was kept at 29°C for a specific time and then was quickly inactivated at 0°C, weighed and homogenized.

The injection procedure was evaluated for reproducibility and accuracy by injecting radiolabelled amino acid solutions, with the following results. The mean and S.E.M. of the indicated number of experiments (*n*) were 15381 ± 413 (9) d.p.m./mosquito with [^{14}C]glycine and 8068 ± 377 (9) d.p.m./mosquito with [^{14}C]cystine. Thus the coefficients of variation for [^{14}C]glycine and [^{14}C]cystine were 0.08 and 0.15 respectively. By these criteria the injection technique was highly reproducible. As an estimation of accuracy, portions of the injection solutions were pipetted directly into scintillation vials, counted for radioactivity and compared with the previous values. The results indicated that the amounts recovered in the injected mosquitoes were 95% for glycine and 96% for cystine. Thus the technique was demonstrated to be accurate.

The injection procedure did not affect survival, GSH or amino acid concentrations. Our results indicated that more than 93% of the injected mosquitoes of all ages survived at least 24 h. Also the concentrations of GSH and amino acids were determined in mosquitoes at 0 and 30 min after injection with the pulsing solutions of glycine and cystine. The results indicated that the concentrations were the same in injected and in the uninjected control groups of both mature and senescent mosquitoes.

Standard preparation of samples

For the biosynthesis experiments, cold-inactivated adult mosquitoes were homogenized in 0.5% (w/v) picric acid by using an all-glass Ten-Broeck homogenizer. A portion of the homogenate (3.5–5%, w/v) was centrifuged for 15 min at 14000 *g* at 4°C in a Lourdes LRA centrifuge with a 9RA rotor. The acid-soluble supernatant was removed, and the acid-precipitable material was resuspended in homogenizing medium and re-centrifuged under the same conditions. The resultant washing was combined with the first supernatant. Portions of the homogenate and acid-insoluble fraction were solubilized in 1 ml of Protosol at 55°C, and 10 ml of scintillation fluid were added. To a portion of the acid-soluble fraction was added 10 ml of Aquasol-2. The samples were then counted for radioactivity. The overall recovery of radioactivity in the different fractions ranged from 86 to 112% of the homogenate value. The remainder of the acid-soluble fraction was used for the isolation of radiolabelled glutathione as described below.

The sample preparation for amino acid analyses was similar to the homogenization and centrifugation methods described above, except that 5-sulphosalicylic acid (3%, w/v) was used as the precipitant instead of picric acid.

Isolation of glutathione

Radiolabelled glutathione was isolated and measured quantitatively by our modification of an ion-exchange chromatography method (Griffith, 1981; Lunn *et al.*, 1979; Furano, 1971). To a portion of the 0.5%-picric acid-soluble fraction was added 4 μmol of GSSG and 4 μmol of glycine or cysteine. The sample was applied to a small column (8 mm × 18 mm) of Dowex-1 resin (acetate form), which was eluted sequentially with 0.02 M-(12 ml), 0.2 M-(2 ml), 0.4 M-(2 ml), 0.6 M-(2 ml) and 2.0 M-

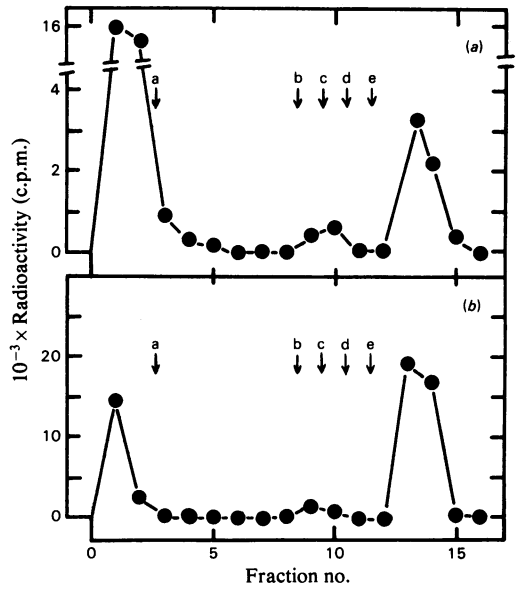

Fig. 1. *Chromatographic isolation of glutathione after radiolabelling with glycine or cystine precursors*

Adult mosquitoes, 6 days old, were pulse-labelled for 30min by injection with [^{14}C]glycine or [^{14}C]-cystine. These samples were processed and chromatographed as described in the text. In brief, each resultant acid-soluble fraction was applied to a Dowex-1 (acetate form) column, which was eluted sequentially with the following acetic acid solutions as indicated by the arrows: a, 0.02M; b, 0.2M; c, 0.4M; d, 0.6M; and e, 2.0M. The upper elution profile (*a*) refers to a pulse of [^{14}C]glycine and the lower (*b*) to [^{14}C]cystine. Glutathione was isolated in the 2.0M-acetic acid fractions, nos. 12–16.

(10ml) acetic acid. Glycine and cystine were not adsorbed and appeared in the 0.02M-acetic acid wash, whereas glutathione was eluted by 2.0M-acetic acid (Fig. 1). Portions of the column fractions were added to 10ml of Aquasol-2 and counted for radioactivity. The procedure was validated with authentic compounds and by quantitative recovery (91–97%) of radiostandards added to tissue samples. In several experiments, portions of the 2.0M-acetic acid eluent from the Dowex-1 column were chromatographed on an amino acid analyser, and fractions were collected from the analyser every 30s. All of the radioactivity appeared in the same fraction as the glutathione standard. Also, portions of the 2.0M-acetic acid wash were spotted on to thin-layer silica-gel (Eastman) sheets and chromatographed in a phenol/water (7:3, v/v) solvent system. All of the radioactivity co-chromatographed with authentic glutathione.

Liquid-scintillation counting

Samples were counted for radioactivity in either a Nuclear Chicago Unilux II counter by using channels-ratio settings or a Beckman LS 7500 counter using the Beckman H-number external standard. The counting efficiencies for various fractions ranged from 34 to 92%.

Amino acid analysis

For the determination of glycine, glutamate and methionine pool sizes, tissue samples (2–10mg-equivalents) were chromatographed by using a Dionex D-300 automatic amino acid analyser with a 4cm × 17cm column and a three-buffer sodium citrate eluent system. The quantitative recoveries of authentic amino acids added to tissue samples at the time of homogenization ranged from 92 to 101%.

For the determination of the half-cystine pool size, tissue samples (30–150mg-equivalents) were chromatographed by using a modified Technicon amino acid analyser with a 10cm × 58cm column and the same buffer system as before. The recoveries of [^{14}C]cysteine and [^{14}C]cystine added to tissue samples at homogenization were 86 and 90% respectively.

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 (Snedecor & Cochran, 1980). Statistical procedures were facilitated by use of a Hewlett–Packard minicomputer.

Results

In the present investigation glutathione biosynthesis was measured by the incorporation rates of two different amino acid precursors. These measurements also required information on the amino acid pools and kinetic parameters such as possible lag times and time course of incorporation.

Amino acid pool sizes

The free-amino-acid concentrations in the mosquito during its adult life-span are shown in Fig. 2. In general the pool sizes were high in the newly emerged adult, decreased during maturation and remained constant through maturity and senescence. Glutamate and glycine decreased 21–32% between the newly emerged (1-day) and mature (12-day) adult ($P < 0.05$–0.005). During this period a much greater (85%) decrease was observed for half-cystine ($P < 0.005$). However, the methionine pool was not altered. The concentrations of all these precursors did not change from maturity through senescence (12–54 days), as shown by the fact that the slopes of their age profiles were near zero and ranged from

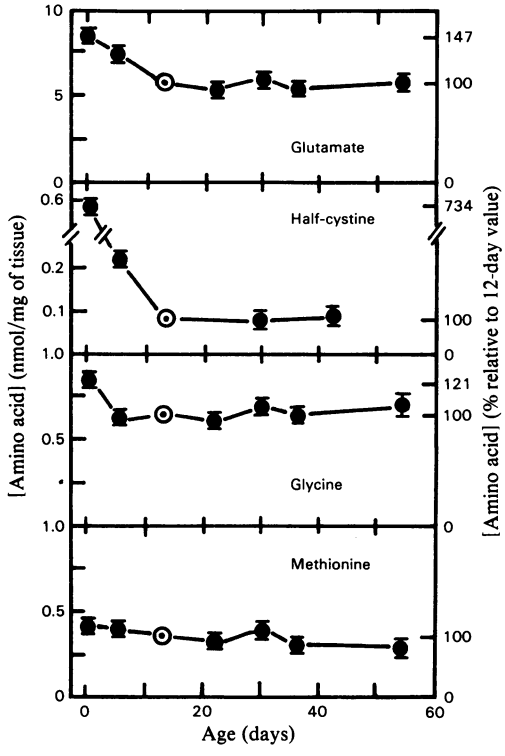

Fig. 2. *Amino acid concentrations in the mosquito during its adult life-span*

The concentrations of glutamate, half-cystine, glycine and methionine were determined in adult mosquitoes of different ages as described in the text. The results are expressed as nmol of amino acid/mg of tissue. Each point and bar represents the mean ± S.E.M. for three to seven samples. The 12-day values, ⊙, were designated 100% and used to calculate the relative values on the right ordinate.

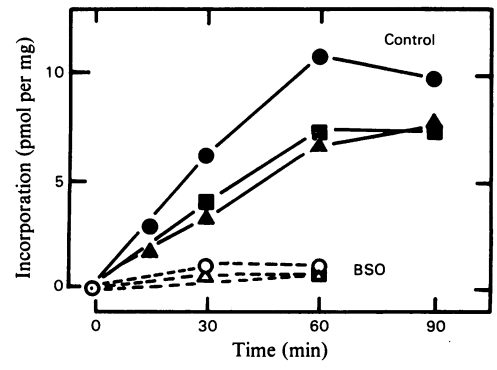

Fig. 3. *Kinetics of [^{14}C]glycine incorporation into glutathione in the aging adult mosquito*

Adult mosquitoes of different ages were injected with [^{14}C]glycine alone (closed symbols) or combined with 20mM-buthionine sulphoximine (BSO) (open symbols) as described in the text. Values are given as pmol of [^{14}C]glycine incorporated into glutathione per mg of tissue. Each point is the mean result for two to three samples, except for the 90-min points, which consist of only one sample. The age groups were: ●, ○, mature (10–12 days); ▲, △, old (30–33 days); and ■, □, very old (48–53 days. ⊙ Represents superimposition of all symbols.

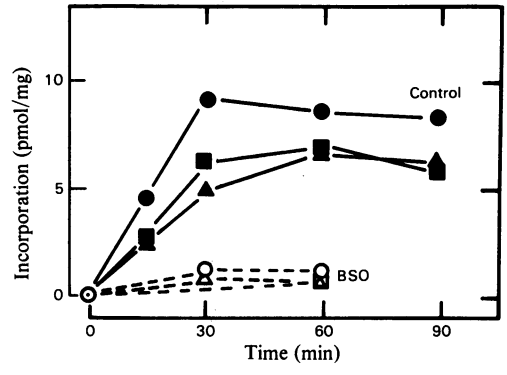

Fig. 4. *Kinetics of [^{14}C]cystine incorporation into glutathione in the aging adult mosquito*

Adult mosquitoes of different ages were injected with [^{14}C]cystine alone (closed symbols) or combined with 20mM-buthionine sulphoximine (BSO) (open symbols) as described in the text. Values are given as pmol of [^{14}C]half-cystine (cysteine equivalent) incorporated into glutathione per mg of tissue. Each point is the mean for two to three samples, except for the 90min points, which consist of only one sample. The age groups were: ●, ○, mature (10–12 days); ▲, △, old (30–33 days); and ■, □, very old (48–53 days). ⊙ Represents superimposition of all symbols.

−0.00190 to 0.00393 ($P < 0.475$). Also, during this period our findings and the work of others indicated that the total free-amino-acid content as well as total protein content, total nitrogen content and body weight are constant during the mature and senescent periods (G. A. Hazelton & C. A. Lang, unpublished work; Lang *et al.*, 1965; Thayer & Terzian, 1962, 1970; Terzian *et al.*, 1957).

Glutathione biosynthesis

(*1*) *Kinetics of precursor incorporation into glutathione.* Information on the kinetics of incorporation with each precursor was required before standardized times of sampling and other conditions could be established for routine analysis.

The lag times of amino acid incorporation were determined from Figs. 3 and 4 by extrapolation of

the initial linear portions of the curves to the abscissa. With both glycine and cystine, and at all ages, the lag times ranged from −3.63 to 0.078 min and thus were considered as zero.

The kinetics of [^{14}C]glycine incorporation into glutathione in mature, old and very old adult mosquitoes is shown in Fig. 3. In all age groups, incorporation was proportional to time for the first 60 min. In the mature (10–12-day) adult the rate was 0.195 pmol/min per mg of tissue. In the old (30–33-day) and very old (48–53-day) adults, the rates were 35–42% lower.

Glycine can also be incorporated into glutathione by an exchange reaction catalysed by glutathione synthetase (Griffith, 1981; Minnich *et al.*, 1971). To determine if glycine incorporation in the mosquito was due to this exchange, buthionine sulphoximine, a potent and specific inhibitor of glutathione biosynthesis, was injected (Griffith & Meister, 1979*a*,*b*). The rates of glycine incorporation in all age groups were inhibited more than 89%. It was concluded that glutathione synthesis *de novo* and not the exchange reaction was measured in the absence of the inhibitor.

The kinetics of [^{14}C]cystine incorporation into glutathione in aging adult mosquitoes is shown in Fig. 4. In all age groups, [^{14}C]cystine incorporation was proportional with time for the first 30 min, a shorter time than for glycine. In the mature (10–12-day) adult the incorporation rate was 0.293 pmol/min per mg of tissue. In the old (30–33-day) and very old (48–53-day) adults the rates were 35–47% lower. Also, cystine incorporation at all ages was due to glutathione synthesis *de novo*, for in the presence of buthionine sulphoximine the rates of [^{14}C]cystine incorporation were inhibited more than 94%.

(*2*) *Synthetic rates.* With the previous kinetic information, synthetic rates in different age samples were determined with standardized procedures using a 30 min pulse period for glycine and a 15 min period for cystine.

The rates of glutathione biosynthesis during the adult life-span of the mosquito are shown in Table 1. In general a similar pattern of decrease was found for either amino acid precursor, whether expressed per mg of tissue or per mosquito.

The profile of the GSH-biosynthetic rates during the developing, mature and senescent periods of the adult life-span are shown more clearly in Fig. 5(*a*). The highest rate was in the newly emerged 1-day adult, which was still undergoing metamorphosis. This decreased rapidly and reached a plateau in the 12–22-day mature period. Next, there was a marked and specific decrease in the aging adult from 22 to 33 days old. Finally, the rate reached a plateau in the old to very old adult (33–51 days), suggesting a critical biosynthetic level for survival.

The magnitude of these changes was a 62–70% maturation decrease between the newly emerged and mature adults ($P < 0.005$) and a 36–43% aging decrease between the mature and old adults ($P < 0.005$).

Of special importance were the similar patterns of GSH-biosynthetic rates and GSH concentration shown in Fig. 5. During the mature and senescent periods, from 12 to 50 days, these two parameters were closely correlated ($r = 0.982$). Thus the biosynthetic rate was proportional to GSH content and also to GSH + GSSG, since the GSH concentration was more than 97% of the total (Hazelton & Lang, 1978).

Discussion

The present investigation was the first systematic study of glutathione biosynthesis during the adult life-span of any organism. Distinct decreases in the

Table 1. *Glutathione biosynthesis in the mosquito during its adult life-span*

The rates of [^{14}C]glycine and [^{14}C]cystine incorporation into glutathione were determined in adult mosquitoes of different ages as described in the text. Results are expressed as means ± S.E.M. (number of samples). Values are given as pmol of GSH formed/30 min per mosquito or per mg of tissue for [^{14}C]glycine and as pmol of GSH formed/15 min per mosquito or per mg of tissue for [^{14}C]cystine. Statistical comparisons with the 12-day group were: $^{*}P < 0.05$ and $^{**}P < 0.005$.

	GSH-biosynthetic rate			
	Precursor ... Glycine		Cystine	
Age (days)	(per mosquito)	(per mg)	(per mosquito)	(per mg)
1	45.2 ± 7.24 (3)**	18.1 ± 2.89 (3)**	37.6 ± 5.58 (3)**	15.1 ± 2.25 (3)**
5	21.6 ± 3.40 (4)*	9.84 ± 1.56 (4)*	16.7 ± 1.79 (3)**	7.61 ± 0.812 (3)**
12	15.0 ± 0.366 (5)	6.85 ± 0.167 (5)	9.88 ± 0.911 (5)	4.51 ± 0.416 (5)
22	12.8 ± 0.855 (4)	5.88 ± 0.490 (4)	8.65 ± 0.691 (4)	3.97 ± 0.317 (4)
33	8.44 ± 0.421 (5)**	3.92 ± 0.194 (5)**	5.74 ± 0.487 (4)**	2.66 ± 0.227 (4)**
51	9.20 ± 0.300 (4)**	4.35 ± 0.144 (4)**	5.10 ± 0.158 (4)**	2.42 ± 0.176 (4)**

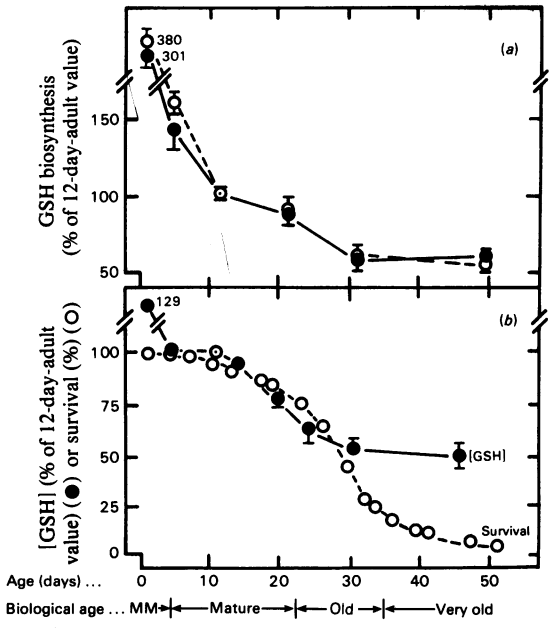

Fig. 5. *Glutathione biosynthesis and glutathione concentrations in adult mosquitoes of different biological ages*

(*a*) The relative rates of glutathione biosynthesis in adult mosquitoes of different ages were calculated from the data in Table 1. These rates for glycine (●) or cystine (○) precursors were based on their respective 12-day values (taken as 100%) and were: 6.85 pmol of GSH formed/30 min per mg of tissue for glycine and 4.51 pmol of GSH formed/15 min per mg of tissue for cystine. (*b*) the GSH concentrations were from the preceding paper (Hazelton & Lang, 1983) and based on the 12-day value of 1.07 n-equiv. of GSH/mg of tissue (expressed as 100%) (⊙). Each point and bar represents the means ± S.E.M. for five to seven samples. Bars are omitted when the S.E.M. is less than the size of the point. The survival curve was determined with a population of 53 female mosquitoes. Their median survival time (ST_{50}) was 29.1 days, which was consistent with our well-established survival rates. The biological stage metamorphosis is denoted MM. Both the GSH and survival data are from Hazelton & Lang (1978). Superimposed symbols are shown thus: ⊙.

rate of biosynthesis were found in the aging adult mosquito.

The most important change occurred during senescence, when there was a 36–43% decrease in the biosynthetic rate. The age of occurrence and magnitude were the same as for the 46%-lower GSH concentrations found previously (Hazelton & Lang, 1978). For these reasons the major and perhaps sole mechanism for the GSH decrease is impaired biosynthesis.

Other mechanisms we have investigated were unchanged with aging or changed in a direction that did not explain the aging decrease in GSH content. For example, the capacity for glutathione degradation as measured by the rate-limiting enzyme, γ-glutamyl transpeptidase, was constant throughout the entire adult life-span of the mosquito (Hazelton & Lang, 1980*b*). Also, glutathione utilization decreased with aging rather than increased, on the basis of the activity of mosquito glutathione *S*-transferases and mouse glutathione peroxidase and glutathione reductase (Hazelton & Lang, 1979, 1981). However, it is possible that other pathways of utilization we have not measured may contribute to the decline in GSH content.

A decline in GSH biosynthesis could be due to lower concentrations of GSH precursors such as glutamate, cystine and glycine. However, this does not occur, for their concentrations were constant through maturity and senescence. Other possibilities that need to be investigated are changes in the biosynthetic enzyme systems. These include studies of the ATP or magnesium concentrations or the activities of the enzymes γ-glutamylcysteine synthetase and glutathione synthetase. There is suggestive evidence that lower amounts of enzymes may occur, for erythrocytes of sheep with a glutathione deficiency have concomitant decrease in γ-glutamylcysteine synthetase activity (Smith *et al.*, 1973). Also, similar deficiencies of glutathione and these enzymes have been observed in humans (Meister, 1978). However, it should be pointed out that these latter findings were in rare metabolic disorders and thus may not reflect the GSH status in normative aging.

The broad significance of this aging decrease in GSH biosynthesis and GSH is exemplified by the central role GSH plays in a variety of metabolic processes. Thus decreased GSH concentrations could alter many important functions: maintenance of SH groups of proteins and small molecules, biosynthetic reactions, membrane and cellular integrity, and detoxification of peroxides and xenobiotics (Meister, 1975, 1978; Kosower & Kosower, 1978). Indeed, with respect to detoxification, it is conceivable that during senescence there is an inability to resynthesize GSH rapidly after depletion via xenobiotics (Reed & Beatty, 1980), and this could lead to tissue damage.

Equally important may be the very high level of GSH biosynthesis in the newly emerged adult. This was expected, for the first 6 days of adult life, which is still a metamorphic stage, is a period of high protein- and DNA-biosynthetic activity (Lang *et al.*, 1965; Mills & Lang, 1976; Smith, 1971). Although glutathione has been implicated in these biosynthetic processes, its exact function is unknown (Kosower & Kosower, 1978).

The level of GSH biosynthesis in a given tissue

may be directly related to its biosynthetic capacity. Evidence for this view is obtained by comparison of different tissues. For example, in order of increasing biosynthetic potential 0.25% of the GSH pool in erythrocytes is synthesized in 30 min, 0.65–0.85% in the mature mosquito, and 9% in rat liver (Griffith, 1981; Lauterburg & Mitchell, 1981).

There was a difference in incorporation rates with the two precursors, for the rate with cystine was 11–66% higher than it was with glycine. A possible explanation is that the cysteine/cystine pool is more static and is involved with fewer pathways than glycine, which participates in many reactions. Further investigation is needed to clarify this view.

In the mosquito the glycine-exchange reaction played a minor role, for our results with the inhibitor buthionine sulphoximine indicated that the exchange rate could account for, at most, 10% of GSH incorporation. In contrast, in human erythrocytes the rate of GSH biosynthesis using [^{14}C]glycine is overestimated, because glycine is incorporated into erythrocyte glutathione primarily by an exchange reaction catalysed by glutathione synthetase (Griffith, 1981; Minnich *et al.*, 1971).

In summary, the present study demonstrated that age-specific changes occur in the rates of GSH biosynthesis. The findings of lower synthetic levels during aging account for the lower GSH concentrations we previously observed. Further investigations will be needed to elucidate the specific factors of biosynthesis that result in the lower synthetic rates.

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