Glutathione *S*-transferase activities in the yellow-fever mosquito [*Aedes aegypti* (Louisville)] during growth and aging

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Our previous findings [Hazelton & Lang (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 37(6), 2378 (abstr.)] demonstrated aging-specific changes in glutathione concentrations in the yellow-fever mosquito [*Aedes aegypti* (Louisville)]. A possible mechanism could be increased utilization via glutathione *S*-transferase. Thus glutathione *S*-transferase activities were measured in mosquito samples from the entire life span, including growth, maturity and senescence. Methods were validated for the quantitative determination of transferase activities with 1,2-dichloro-4-nitrobenzene (DCNB) and 1-chloro-3,4-dinitrobenzene (CDNB) as second substrates. Marked changes occurred during the life span, and the profiles for both DCNB and CDNB activities were identical. The activities increased throughout larval development and reached a maximum in the metamorphosis stage. The activities decreased at the end of metamorphosis in the 5-day-old adult, reached a plateau during maturity (5–20 days), and then decreased 31% ($P < 0.007$) during senescence (after 33 days). This senescence-specific decrease occurred in both sexes and was localized in the abdominal region. Further kinetic analyses indicated that the lower enzyme activities were most likely due to lower amounts of active enzyme rather than a change in kinetic properties. These findings indicate that the capacity for GSH utilization via glutathione *S*-transferase is diminished with aging. This does not explain our previously observed decreases in GSH, but the results suggest that GSH-linked detoxification would be impaired during senescence.

The glutathione *S*-transferases (EC 2.5.1.18) play a salient role in the detoxification of xenobiotics, including chemical carcinogens. This group of enzymes catalyses the conjugation of GSH with xenobiotics to form GSH thiol ethers (Jakoby, 1978*a,b*; Reed & Beatty, 1980; Chasseaud, 1979). These thiol esters are further metabolized to mercapturic acids and excreted (Boyland & Chasseaud, 1969). Also the transferases can function non-enzymically by binding metabolites and foreign compounds and thereby decrease their availability and toxicity (Smith & Litwack, 1980).

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). These results indicated that the lower GSH concentrations found during aging might be the result of increased GSH utilization via the glutathione *S*-transferases. Finally, the overall importance of glutathione *S*-transferases in detoxification suggested a need for a study of these enzymes during aging.

To our knowledge there has been no systematic investigation of glutathione *S*-transferases during the entire life span, including the stages of growth, maturity and senescence. However, there are numerous growth studies of different organisms (Saleh *et al.*, 1978; James & Pheasant, 1978; Kuo & Hook, 1980; Hales & Neims, 1976; Juchau & Namkung, 1974; Chasseaud, 1973; Mukhtar & Bresnik, 1976; Klaassen, 1975; James *et al.*, 1977).

The mosquito was selected and used as an appropriate experimental model because of the following advantages. First, it is a multicellular, eukaryotic organism that resembles mammals in its

Abbreviations used: GSH, reduced glutathione; DCNB, 1,2-dichloro-4-nitrobenzene; CDNB, 1-chloro-2,4-dinitrobenzene.

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biochemical composition and nutritional requirements. Secondly, the mosquito has discrete, synchronizable life-span stages of growth, maturity and aging, which have been characterized biochemically. Thirdly, it can be cultured axenically and aged under controlled and defined nutritional conditions; and fourthly, the short life-span enables the study of the entire life cycle in 6 weeks.

The objective of the present study was to determine the activity of glutathione *S*-transferase in aging adult mosquitoes during the life-span. These activities were correlated with GSH concentrations to determine their possible relationship to GSH-linked detoxification and to senescence.

Materials and methods

Experimental organism and its culture

The experimental organism was the yellow fever mosquito, *Aedes aegypti* (Louisville), which has been colonized in this laboratory for over 17 years (amounting to about 250 generations). Under our standard conditions the larval period lasts approx. 7 days, and the pupal period 2 days.

The chronological and biological ages of adult mosquitoes were as follows: 0–5 days, end of metamorphosis; 6–22 days, mature; 23–35 days, old; 35+ days, very old. These ages were characterized from survival curves and from DNA and protein profiles (Lang *et al.*, 1965) of cohort populations that were cultured and aged under our standard conditions. The median survival time for these mosquitoes was 29 ± 2 days, a value which has been reproducible for several decades.

Standard culture

Our standardized culture procedure with defined conditions was used to produce mosquitoes of all ages through the life cycle (Kao *et al.*, 1976). The procedure is outlined as follows. Eggs stored at 29°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 used in axenic media. An aq. 10% (w/v) suspension of desiccated pig liver (Teklad, Madison, WI, U.S.A.) 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 with cotton pads moistened with 10% (w/v) sucrose solution, a normal diet except for egg production. No blood meals were offered to the experimental colonies, to suppress the added variable of ovarian development. An environmental temperature of $29 \pm 1^\circ\text{C}$ was maintained throughout the life cycle.

Axenic culture

Mass cultures of mosquito larvae were grown axenically on the defined medium described by Lang *et al.* (1972). Axenic and standard (non-axenic) mosquitoes have the same rates of growth, pupation and adult emergence.

Tissue preparation

The protocol for each daily experiment included samples from different age groups, and all processing was carried out at 0–4°C. Preliminary experiments demonstrated that glutathione *S*-transferase activities were located only in the soluble fraction of larval and adult tissues. Therefore 9% (w/v) homogenates were prepared in ice-cold 0.1 M-Tris/HCl buffer, pH 8.5, saturated with phenylthiourea, with an all-glass Ten-Broeck homogenizer. Portions of the homogenates (1.0 ml) were centrifuged at 14000 *g* for 10 min in a Lourdes LRA centrifuge with a 9RA rotor. The supernatants were further centrifuged at 100000 *g* for 60 min in a Beckman model L ultracentrifuge with a 50.1 swinging-bucket rotor. The soluble fractions were filtered through glass wool and used directly for glutathione *S*-transferase assay.

In some experiments, samples of head, thorax and abdomen were obtained from cold-inactivated mosquitoes, which were dissected at the head–thorax and thorax–abdominal junctions with stainless-steel needles. Pooled samples of each body region were processed as described above.

Enzyme assays

Preliminary experiments indicated that enzyme activities were stable for at least 6 h. Therefore each sample was assayed at two or more concentrations for glutathione *S*-transferase activities within 2 h of sample preparation. Only initial rates were measured, and endogenous activity (without added substrates or cofactors) was less than 10% of the total activity for all tissues.

Glutathione *S*-transferase activities were measured by the spectrophotometric procedures of Habig *et al.* (1974), with DCNB and CDNB as second substrates. These compounds were chosen since they are substrates for a wide variety of insect and mammalian glutathione *S*-transferases (Motoyana & Dauterman, 1977; Jakoby, 1978a). For the assay with DCNB the reaction mixture (volume 1.0 ml) contained: 5 mM-GSH, 1.25 mM-DCNB, 0.1 M-sodium phosphate, pH 7.5, and 1–10 mg-equivalents of tissue, and the rate of *S*-(2-chloro-4-nitrophenyl)glutathione formation was determined from the increase in A_{345} . For the assay with CDNB the reaction mixture (volume 1.0 ml) contained: 5 mM-GSH, 1.25 mM-CDNB, 0.1 M-sodium phosphate, pH 6.5, and 0.1–1 mg-equivalents of

tissue, and the rate of *S*-(2,4-dinitrophenyl)glutathione formation was determined from the increase in A_{340} .

All spectrophotometric measurements were carried out in a Zeiss PMQII spectrophotometer at 37°C in 1.0 ml quartz cuvettes with a light-path of 1.0 cm. Specific activities were expressed as μmol of DCNB or CDNB conjugated/min per mg of protein, using the absorption coefficients $8.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ and $9.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for *S*-(2-chloro-4-nitrophenyl)-glutathione and *S*-(2,4-dinitrophenyl)glutathione respectively (Habig *et al.*, 1974). Protein concentrations were determined by the method of Lowry *et al.* (1951), with human serum albumin (Cohn fraction V) as the standard.

Enzyme kinetics

Supernatant fractions from 10-day and 49-day adults were prepared, desalted on Sephadex G-25 columns, and used for the following measurements. The apparent Michaelis–Menten parameters (K_m , V_{max}) were determined for GSH, CDNB and DCNB by varying the concentration of GSH (0.625–5.00 mM) at fixed concentrations of CDNB (0.417, 0.833, 1.25 mM) or DCNB (0.625, 0.833, 1.25 mM). These results were analysed by using least-squares fits to Lineweaver–Burk plots and their secondary plots (Segal, 1975).

Chemicals

CDNB and DCNB were obtained from Aldrich Chemical Co. and were recrystallized twice from ethanol/water before use. Solutions then were prepared in ethanol. GSH was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Other chemicals were of reagent grade, and the water was double-glass-distilled.

Statistical methods

Student's *t*-test was used for statistical comparisons (Snedecor & Cochran, 1980). Statistical procedures were facilitated by use of a Hewlett–Packard minicomputer.

Results

Validation of the methods

Essential to the present study was the validation of enzyme assays for mosquito tissues. The initial rates and time courses of glutathione *S*-transferase activities in larval and adult tissues were determined, and both DCNB and CDNB activities were proportional to time for only the initial 2 min. Thus for routine assays the initial rates were determined during the first 1.5 min.

The proportionality of glutathione *S*-transferase activities to sample size in larval and adult tissues was determined also. Each sample was assayed

Fig. 1. Glutathione *S*-transferase activities during the life-span of the mosquito

Larvae, pupae and adults of different ages were analysed for glutathione *S*-transferase activities with DCNB (●) and CDNB (○) as substrates. The larvae and pupae were obtained by axenic culture, and adults were maintained by standard non-axenic culture. Each point with bar represents the mean $\pm$ S.E.M. for four to eight samples. Bars are omitted if the S.E.M. is less than the size of the point. Each sample consisted of 20–30 mosquitoes.

routinely at two or more levels to ensure proportionality. Experiments in which samples of high and low activity were mixed were carried out to determine the possible presence of activators or inhibitors. None was found, for the results were additive.

Glutathione *S*-transferase activities

The glutathione *S*-transferase activities during the life-span of the mosquito are shown in Fig. 1. The data were expressed as specific activities (per mg of protein). Previously we showed that protein content is constant through the larval stages and increases to another constant level through the adult life span (Lang *et al.*, 1965). In general, the life-span profiles of glutathione *S*-transferase activities with either DCNB or CDNB were essentially identical. The specific activities increased 2-fold during the larval and pupal stages and reached a maximum in the newly emerged adult. Thereafter glutathione *S*-transferase activities decreased sharply, reached a plateau by 5 days adult age and were constant until 33 days, when a 31% decline occurred in the senescent adult ($P < 0.001$).

Of special importance was the relationship between glutathione *S*-transferase activities and glutathione concentrations (Hazelton & Lang, 1978) at different biological ages of the adult mosquito (Fig. 2). The decrease in transferase activities occurred at a later age, 33–45 days, than the decrease in GSH concentrations at 15–33 days. However, both decreases occurred during senescence as defined by survival criteria.

Fig. 2. Glutathione *S*-transferases, glutathione and survival of the aging mosquito

(a) Adult mosquitoes of different ages were analysed for glutathione *S*-transferase activities as described in the text. Activities with both DCNB (●) and CDNB (○) activities were based on their 12-day values (expressed as 100%): 3.45 and 122 μmol conjugated/min per mg of protein respectively. (b) The GSH data (●) are replotted from those of Hazelton & Lang (1978) and are based on the 12-day value (1.07 n-equiv. of GSH per mg of tissue) expressed as 100%. Each point with bar represents the mean $\pm$ S.E.M. for five to seven samples and bars are omitted if the S.E.M. is less than size of the point. The survival data (○) are expressed as percentage survival of a population of 53 mosquitoes. The median survival time (ST_{50}) was 29.1 days.

To explore the possibility of a sex difference in glutathione *S*-transferase activities, adult male and female mosquitoes of mature (10–12 days) and senescent (31–44 days) biological ages were compared (Table 1). There was no sex difference, for the specific activities were the same despite the 2-fold greater weight of the female. Also in both sexes there was a similar aging-specific decrease of 30–34% in the enzyme activities.

The K_m and V_{max} values were determined to evaluate the kinetics of the glutathione *S*-transferase reactions in mature and senescent adults. As shown in Table 2, the maximal enzymic rates (V_{max}) of conjugation for both CDNB and DCNB decreased 34–38% in the senescent adult. However, the

Table 1. Lack of sex difference in glutathione *S*-transferase activities of mature and senescent adult mosquitoes

Adult mosquitoes raised under our standard conditions were sexed by visual examination of their antennae. Biologically mature and senescent mosquitoes were compared. Pooled samples of 10–25 males or females were analysed for glutathione *S*-transferase activities as described in the text, and specific activities are expressed as μmol of substrate conjugated/min per mg of protein. The results are given as means $\pm$ S.E.M. of three to six samples. The glutathione *S*-transferase activities of the senescent groups were statistically different from those of the mature groups ($P < 0.05$).

Sex	Adult age (days)	Weight (mg per mosquito)	Specific activity	
			DCNB	CDNB
Male	10	1.12 $\pm$ 0.120	3.52 $\pm$ 0.402	128 $\pm$ 7.25
	31	1.03 $\pm$ 0.101	2.46 $\pm$ 0.115	87.0 $\pm$ 8.01
Female	12	2.16 $\pm$ 0.015	3.44 $\pm$ 0.339	122 $\pm$ 9.15
	44	2.11 $\pm$ 0.183	2.32 $\pm$ 0.187	80.5 $\pm$ 7.05

Table 2. Kinetic parameters for glutathione *S*-transferase activities in mature and senescent adult mosquitoes

The kinetic parameters for glutathione *S*-transferase activities in mature (10 days) and senescent adults (49 days) were determined as described in the text. The K_m values for GSH were obtained with CDNB as second substrate. The results are given as the means of two determinations.

Age (days)	Apparent kinetic parameters				
	K_m (mM)			V_{max} ($\mu\text{mol}/\text{min}$ per mg of protein)	
	GSH	CDNB	DCNB	CDNB	DCNB
10	1.13	3.65	0.853	765	5.80
49	1.04	3.47	0.911	511	4.01

apparent K_m values for all substrates were unchanged during aging.

To determine whether the decreases in the aging adult were localized or general, different body regions were analysed for these enzyme activities. Glutathione *S*-transferase activities were found in all body regions of the adult, but >50% of the activity was localized in the abdomen. Also, the adult abdomen exhibited the highest specific activity, followed by the thorax and then the head regions. Notable were the changes that occurred in the body regions during the adult aging period, as shown in Fig. 3. Comparison of the mature (13-day) and senescent (43-day) adults indicated that the aging decrease was localized in the abdomen and accounted entirely for the decrease in the whole organism.

Fig. 3. Body-region differences in glutathione *S*-transferase activities in the aging mosquito

Adult female mosquitoes of different ages were trisected into head, thorax and abdominal regions. Each sample consisted of 200–250 mosquitoes. The dissection procedure and methods for determination of glutathione *S*-transferase activities are described in the text. The percentage activities were based on the 13-day adult values ($\equiv 100\%$). These activities, expressed as μmol of (a) DCNB [or (b) CDNB] conjugated/min per mg of protein, were: ●, whole organism, 3.46 (122); □, head, 2.67 (88.5); △, thorax, 2.34 (78.5); ○, abdomen, 5.10 (195).

To determine whether starvation was a causative factor for the aging decreases, groups of mature, 14-day and old 40-day adults were maintained on water or on their usual sucrose diet for several days. At intervals samples were removed and analysed for enzyme activity. In starved mosquitoes of both ages the enzyme activities were the same as the control starting values (Fig. 4). Thus the aging decreases in glutathione *S*-transferase activities were not due to starvation.

Discussion

This investigation is the first systematic study of glutathione *S*-transferase activities during the life-span of any multicellular, eukaryotic organism. The results demonstrated that changes in activity occurred during the different biological stages of the mosquito.

Fig. 4. Lack of starvation effect on the glutathione *S*-transferase activities of mature and senescent mosquitoes

Mature (14 days) and senescent (40 days) mosquitoes were starved by replacing their sucrose diet with water. Glutathione *S*-transferase activities were determined as described in the text, and specific activities are expressed as μmol of substrate conjugated/min per mg of protein. Each point consists of one to three samples of 10–20 mosquitoes. Samples were: ●, control; ○, starved.

Of special importance were the decreases from age 32 days onwards during the adult aging or senescent period. These changes were aging-specific, for they coincided with the biological period of senescence. The lower specific activities were due to changes in enzymic activity rather than to increases in tissue protein or free-amino-acid contents, which were constant. This conservation of nitrogenous compounds in the adult mosquito has been noted by Terzian *et al.* (1957). Also the decreases in enzyme activities were independent of changes in cell mass or number, for DNA, RNA and protein contents were constant during adult aging (Lang *et al.*, 1965). Finally the decreases were not due to starvation, for the enzyme activities were unchanged in starved, mature and old adults.

The decreases in the glutathione *S*-transferase activities were probably due to decreases in active-enzyme concentrations, since optimal assay conditions were used and no inhibitors or activators were detected. The kinetic data indicated that, whereas the maximal velocities (V_{max}) of the DCNB and CDNB reactions decreased, the affinity constants (K_m) for the various substrates were unchanged. These results indicate that the amounts of these enzymes decreased

in some unknown way. However, if mosquito tissues are like mammalian tissues (Habig *et al.*, 1974; Kamisaka *et al.*, 1975), there are several glutathione *S*-transferase enzymes, and further experimentation will be required to determine if all or a select population of these enzymes decline with aging.

The aging decrease in glutathione *S*-transferase activities does not explain the lower GSH contents observed in the senescent adult (Hazelton & Lang, 1978). Indeed, the decrease in glutathione *S*-transferase activities would be expected to result in an increase in GSH. Thus other mechanisms such as decreased synthesis, increased degradation, or an increase in other pathways of utilization of glutathione must be responsible. However, the decrease in transferase activities suggests a decrease in GSH utilization via the mercapturic acid pathway.

A major consequence of the lower transferase activity is that the capacity for GSH-linked detoxification of xenobiotics is diminished during senescence. The findings are even more significant because of the concomitant decrease in the steady-state GSH concentrations in senescence (Hazelton & Lang, 1978). Indeed the decrease in GSH content may predominate and modulate glutathione *S*-transferase activities, because the steady-state level of GSH is about 1.1 mM, which is similar to its K_m value of 1.04–1.13 mM for glutathione *S*-transferase(s). Thus there is a decrease in the overall potential for conjugation of foreign compounds, which then are metabolized and excreted as mercapturic acids.

For these reasons old organisms would be at a higher risk to toxicants than young organisms. In support of this view our recent findings showed that the toxicity of acetaminophen, a model compound for glutathione-conjugation reactions, was 8-fold higher in senescent compared with mature mosquitoes (Richie & Lang, 1982).

To our knowledge the findings of lower glutathione *S*-transferase activities during senescence is the first demonstration that a phase-2 reaction of the detoxification process is altered during senescence. Others have shown that decreases in phase 1, microsomal cytochrome *P*-450-dependent reactions, occur with aging (Kato & Takanaka, 1968; Schmucker, 1979; Schmucker & Wang, 1980).

The glutathione *S*-transferase activities were found predominantly in the adult abdomen. Also the aging decrease in these enzymes was specifically in this body region. An explanation for these findings is that the mosquito abdomen contains the midgut, fat-body, and Malpighian tubules, which are analogous to mammalian organs that have high transferase levels and are essential for absorption of nutrients and the excretion of waste products (Chasseaud, 1979; Jerina & Bend, 1977). Thus the high enzyme concentration in this region may be related to their functional roles.

Equally important are the developmental changes in glutathione *S*-transferase activities that were observed during larval growth and metamorphosis. The activity increased through larval development and reached a maximum during metamorphosis, which includes the late-larval, pupal and adult periods. A similar pattern occurred for GSH concentrations (Hazelton & Lang, 1978). The significance of both high transferase and GSH concentrations during metamorphosis is that it is a period of high biosynthetic activity (Lang *et al.*, 1965; Mills & Lang, 1976; Smith, 1971). Also these results suggest that, during growth, a large capacity for detoxification is needed to prevent xenobiotic damage to the many sensitive, biosynthetic processes that are occurring and to remove endogenous toxic compounds that might accumulate. A similar view for glucuronidation conjugation reactions in developmental systems has been proposed by Dutton (1978).

In summary, the present study demonstrated that age-specific changes occur in glutathione *S*-transferase activities during growth and senescence. The findings of low activities in the senescent adult do not explain the low GSH concentrations that occur at this time. However, these decreases in glutathione *S*-transferase activities and the metabolic concentration of GSH indicate a diminished capacity for GSH-linked detoxification in senescence.

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