Effect of catalase inactivation on levels of inorganic peroxides, superoxide dismutase, glutathione, oxygen consumption and life span in adult houseflies (*Musca domestica*)

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The effects of total inhibition of catalase, induced by 3-amino-1,2,4-triazole, on the adult housefly (*Musca domestica*) were examined. The lack of catalase activity had no effect on the longevity of the houseflies. Inorganic-peroxide concentration was elevated at younger ages, but declined in older flies. The rate of oxygen consumption by the flies was greatly decreased and the levels of oxidized as well as reduced glutathione were augmented. Superoxide dismutase activity showed a slight increase. This study suggests that loss of catalase activity does not affect survival of houseflies due to adaptive responses.

H₂O₂ is a normal product of oxygen metabolism in cells (Chance *et al.*, 1979). If not eliminated, it can cause cytotoxic effects, apparently by reacting with superoxide (O₂^{•-}) radicals in the presence of iron to produce the highly reactive hydroxyl radical (OH[•]):

Hydroxyl radicals can potentially cause a variety of deleterious changes in biological systems (Fong *et al.*, 1976; Fridovich, 1978; Halliwell, 1981). The enzymic degradation of H₂O₂ is catalysed by catalase and glutathione peroxidase; the latter enzyme is, however, absent in insects (Smith & Shrift, 1979). There is some evidence to suggest that H₂O₂ may also be non-enzymically degraded by glutathione (Finley & Lundin, 1980).

Paradoxically, Nicolosi *et al.* (1973) found that 90% inactivation of catalase in the fruit-fly *Drosophila melanogaster* by administration of herbicide 3-AT had no apparent effect on the life span of the flies. This would seem to suggest the involvement of mechanisms which minimize the effects of loss in catalase activity, possibly by modulating the production and the elimination of H₂O₂. The objective of the present study was to identify compensatory responses induced by catalase inhibition with 3-AT. 3-AT irreversibly binds with the protein moiety of the enzyme (Margoliash *et al.*, 1960). Specifically, the effects of

3-AT on the life span, metabolic rate, catalase activity, superoxide dismutase activity, concentration of inorganic peroxides and levels of oxidized and reduced glutathione in the adult male housefly, *Musca domestica*, were examined.

Experimental

Reagents

Light petroleum (b.p. 35–60°C) was obtained from Baker Scientific Supply Co., Phillipsburg, NJ, U.S.A. All other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Rearing and maintenance of insects

Eggs were obtained from 7–10-day-old houseflies belonging to a stock originally obtained from the Department of Zoology, University of Cambridge, Cambridge, U.K. Larvae were fed on moist CSMA (Chemical Specialties Manufacturer's Association) fly larval medium obtained from Ralston Purina Co., Richmond, IN, U.S.A. After emergence from pupae, flies were segregated by sex and males were housed in 0.028 m³ (1 ft³) cages with 200 flies/cage. Test groups were administered 2 mm-3-AT in their drinking water; controls were provided with distilled deionized water. All groups of flies were fed on sucrose and maintained at 25°C with a relative humidity of about 40%.

Metabolic Rate

The rate of O₂ consumption of whole populations of flies was measured by placing cages, containing

Abbreviations used: 3-AT, 3-amino-1,2,3-triazole; GSH, reduced glutathione; GSSG, oxidized glutathione.

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the flies, within a Plexiglas respiration chamber and determining the decline in oxygen concentration during a 3 h period. O₂ concentration was monitored with a Beckman OM14 O₂ analyser.

Superoxide dismutase

Superoxide dismutase activity was measured by the indirect method suggested by Crapo *et al.* (1978). Insects were homogenized in 10 vol. (v/w) of 50 mM-potassium phosphate buffer, pH 7.8, containing 0.1 mM-EDTA. The homogenate was centrifuged at 4°C and 3000g for 5 min; the pellet was discarded. The supernatant was centrifuged at 50000g for 30 min and the pellet was discarded again. The supernatant obtained after the second centrifugation was dialysed for 12 h against cold iso-osmotic potassium phosphate buffer, pH 7.8, containing 0.1 mM-EDTA. To a reaction mixture containing 2.1 ml of phosphate buffer, 0.3 ml of 0.1 mM-cytochrome c, 0.3 ml of 0.5 mM-xanthine and 0.3 ml of 0.1 mM-KCN, was added a 10 µl portion containing enough xanthine oxidase to produce a ΔA_{550} of 0.020/min. Increasing amounts of tissue homogenate were added to the reaction mixture to determine the maximum inhibition which could be caused by the presence of homogenate. The activity of cyanide-insensitive superoxide dismutase was determined by using the above reaction mixture, except that 10 mM-rather than 0.1 mM-KCN was used. Superoxide dismutase activity was calculated as follows:

$$\text{Units} = \frac{\Delta A \text{ of reaction mixture without homogenate minus } \Delta A \text{ of reaction mixture with homogenate}}{(\Delta A \text{ of reaction mixture without homogenate}/2) \times (\text{mg of protein/ml of reaction mixture})}$$

Catalase

Catalase activity was determined by the method of Luck (1965). A 10% (v/w) homogenate was made by using 0.066 M-potassium phosphate buffer/0.1% Triton-X, pH 7. The homogenate was centrifuged at 4°C and 3000g for 5 min and the pellet discarded; the supernatant was centrifuged at 50000g for 30 min and the pellet discarded. The time required for the ΔA_{240} of a mixture of homogenate, H₂O₂ and buffer to change from 0.45 to 0.40 was used as a measure of catalase activity.

Inorganic peroxides

The level of inorganic peroxides was measured by the method of Bernt & Bergmeyer (1976). Flies were homogenized in 10 vol. (v/w) of 5% (w/v) HClO₄ for deproteinization. The resulting homogenate was centrifuged at 400g for 5 min and the pellet discarded. A 0.1 ml portion of supernatant was added to a reaction mixture containing 2.5 ml of 0.1 mM-sodium phosphate buffer, pH 7, 0.125 mg of dianisidine and 100 µg of horseradish peroxidase. The concentration of peroxide in the homogenate was determined by comparing the A_{436} of this

mixture with that of a mixture containing known amounts of H₂O₂.

Glutathione

The total amount of glutathione, i.e. reduced plus oxidized forms, was measured by a modification of the method suggested by Tietze (1969). Groups of ten flies were homogenized in 5 ml of 0.01 M-HCl. A 0.5 ml portion of homogenate was diluted three times with 0.01 M-HCl (final vol. 2 ml), deproteinized with an equal volume of 10% HClO₄ and centrifuged at 3000g for 10 min. Portions (0.2 ml) of homogenate were added to a reaction mixture consisting of 1.2 ml of 1 mM-5,5'-dithiobis-(2-nitrobenzoic acid) dissolved in 0.1 M-potassium phosphate buffer, pH 7.5, containing 5 mM-EDTA, 0.2 ml of glutathione reductase (10 units/ml) and 0.4 ml of 1 mM-NADPH. The concentration of total glutathione was determined by comparing the ΔA_{412} which occurred in 6 min at 30°C with a standard curve constructed with known concentrations of GSSG. The level of GSSG in the homogenate was measured by incubating the deproteinized homogenate with an equal volume of 2 mM-N-ethylmaleimide for 1 h to remove GSH. Because N-ethylmaleimide is extremely inhibitory to glutathione reductase, it was necessary to remove traces of this compound that had not reacted by extracting ten times with light petroleum. After such an extraction the procedure described above could be used to determine the concentration of GSSG.

Results

Mortality

The mean life span of the houseflies in control populations was 20.7 ± 5.9 days, and 20.9 ± 8.2 days in populations administered 3-AT. These results confirm the previously reported observation of Nicolosi *et al.* (1973) that the longevity of *D. melanogaster* was unaffected by 3-AT.

Metabolic rate

The rate of oxygen utilization was measured in populations of flies that were 4, 6 and 9 days of age. As Table 1 shows, the rate of oxygen consumption in 3-AT-administered flies was lower than in the controls at all three ages.

Table 1. Effect of 3-AT on the rate of O₂ consumption in adult male houseflies

Age of flies (days)	Rate of O ₂ consumption (µl/h per mg wet wt.)	
	Control	3-AT-administered
4	8.8	5.3
6	10.7	6.1
9	12.1	6.1

Table 2. *Effect of 3-AT on activities of catalase and superoxide dismutase in adult male houseflies*Results are means $\pm$ S.D. ($n = 4$). Significance levels: * $P < 0.05$; ** $P < 0.01$.

Group	Age (days)	Catalase (units/mg of protein)	Superoxide dismutase (units/mg of protein)	
			Total	Cyanide-insensitive
Control	3	34.57 $\pm$ 2.25	—	—
	7	29.02 $\pm$ 0.23	77.1 $\pm$ 5.0	61.4 $\pm$ 2.5
	9	29.95 $\pm$ 1.62	105.8 $\pm$ 1.5	86.9 $\pm$ 4.4
	12	—	113.3 $\pm$ 1.5	86.5 $\pm$ 0.1
3-AT-administered	3	Undetectable	—	—
	7	Undetectable	80.9 $\pm$ 3.2	66.2 $\pm$ 0.1*
	9	Undetectable	113.7 $\pm$ 1.8**	99.4 $\pm$ 0.1**
	12	—	121.5 $\pm$ 2.8**	98.5 $\pm$ 0.1**

Table 3. *Effect of 3-AT on concentration of inorganic peroxides in adult male houseflies*Results are means $\pm$ S.D. ($n = 4$). Significance levels:* $P < 0.05$; ** $P < 0.01$.

Age of flies (days)	Concentration of inorganic peroxides ($\mu\text{g/g}$ wet wt.)	
	Control	3-AT-administered
4	21.0 $\pm$ 2.8	38.4 $\pm$ 4.2**
6	34.5 $\pm$ 1.7	64.2 $\pm$ 1.9**
9	40.0 $\pm$ 2.2	45.6 $\pm$ 3.2*
11	43.4 $\pm$ 1.0	34.5 $\pm$ 2.5**

Inorganic peroxides

The concentration of inorganic peroxides was measured at 4, 6, 9 and 11 days of age. The peroxide level was found to increase linearly with age in the control flies; however, flies administered 3-AT exhibited an increase in peroxide concentration between 4 and 6 days of age, followed by a decline between 6 and 11 days of age. The concentration of inorganic peroxides in the experimental flies was significantly higher than in the controls at 4, 6 and 9 days of age, but was significantly lower than the control level at 11 days of age (Table 3).

Glutathione

The levels of total (GSH + GSSG) glutathione were measured in 6-, 8-, 10- and 13-day-old flies. GSH concentration was determined by subtracting the amount of GSSG from the total amount. The total glutathione and GSSG concentrations tended to increase in both control and 3-AT-administered flies between 6 and 10 days of age, but declined between 10 and 13 days of age. The levels of GSSG and total glutathione were, however, significantly greater in 3-AT-administered flies than in the controls at all ages examined. The GSH concentration was also greater in treated flies than in the controls at all ages examined (Table 4).

Table 4. *Effect of 3-AT on the oxidized, reduced and total levels of glutathione in adult male houseflies*Results are means $\pm$ S.D. ($n = 3$). Significance levels:* $P < 0.05$; ** $P < 0.01$.

Group	Age (days)	Glutathione (mg/g wet wt.)		
		GSSG	GSH	Total
Control	6	0.97 $\pm$ 0.01	2.08	3.05 $\pm$ 0.05
	8	1.08 $\pm$ 0.11	3.75	4.83 $\pm$ 0.04
	10	1.16 $\pm$ 0.03	3.89	5.05 $\pm$ 0.19
	13	1.02 $\pm$ 0.04	2.52	3.54 $\pm$ 0.13
3-AT-administered	6	1.05 $\pm$ 0.10	3.00	4.05 $\pm$ 0.08**
	8	1.40 $\pm$ 0.6*	3.85	5.25 $\pm$ 0.10**
	10	1.32 $\pm$ 0.01**	4.35	5.67 $\pm$ 0.28*
	13	1.12 $\pm$ 0.02*	3.33	4.45 $\pm$ 0.03**

Superoxide dismutase

The activity of superoxide dismutase was examined in 7-, 9- and 12-day-old flies. 3-AT-administered flies had higher levels of superoxide dismutase activity than the controls at all three ages examined (Table 2).

Catalase

The 3-AT-administered flies did not exhibit detectable levels of catalase activity at 3, 7 or 9 days of age (Table 2).

Discussion

The results of the present study indicate that the initial elevation in the concentration of inorganic peroxides after 3-AT-induced catalase inhibition was not maintained throughout life, and a gradual decline in inorganic peroxide levels occurred after 6 days of age. Whereas administration of 3-AT markedly decreased the rate of oxygen utilization, the concentration of GSH was greatly augmented. Life spans of the flies were unaffected, and superoxide dismutase activity was only slightly increased.

The apparent lack of a deleterious effect by 3-AT on the life span of the flies and the decline of

inorganic-peroxide levels after 6 days of age may be due to the adaptive responses observed in GSH levels and rate of oxygen consumption. Decreased levels of oxygen consumption would be expected to decrease the H_2O_2 production resulting from dismutation of $\text{O}_2^{\cdot -}$. Our previous studies have indicated that the rate of oxygen consumption in the houseflies is decreased after experimental treatments designed to increase putative levels of free radicals, e.g., exposure to ionizing radiation (Allen & Sohal, 1982), and inhibition of superoxide dismutase by administration of diethyl diethiocarbamate (Sohal *et al.*, 1982).

The significance of the elevation in GSH levels after 3-AT administration is unclear, since elimination of peroxides by GSH is usually catalysed by GSH peroxidase; however, insects are known to lack this enzyme (Smith & Shrift, 1979). It is possible that GSH reacts directly with inorganic peroxides; at least two mechanisms for the direct reaction of GSH with H_2O_2 have been proposed (Finley & Lundin, 1980). Interestingly, GSH levels were also found to be elevated after the inhibition of superoxide dismutase by diethyl dithiocarbamate administration (Sohal *et al.*, 1982).

Bray *et al.* (1974) have reported that superoxide dismutase activity is strongly inhibited by H_2O_2 *in vitro*. Although the flies administered 3-AT exhibited elevated levels of inorganic peroxides at early ages, superoxide dismutase activity in treated flies was never found to be lower than in the controls. It is possible that the concentration of inorganic peroxides in the treated flies did not reach a level sufficient to inhibit superoxide dismutase activity.

In conclusion, the results of the present study suggest that a complex balance exists between the products of oxygen metabolism and antioxidant defences. Experimentally induced imbalances evoke compensatory responses.

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