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CYTOSOLIC GLUTATHIONE *S*-TRANSFERASES IN *DROSOPHILA MELANOGASTER**

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Abstract—Post-microsomal supernatants from *Drosophila melanogaster* and rat liver homogenates were investigated with respect to their glutathione *S*-transferase (GST) activity. It appeared that the Drosophila transferase did not conjugate the epoxides styrene-7,8-oxide and 1,2 epoxy-3(*p*-nitrophenoxy)-propane.

Attempts to isolate the Drosophila GST isozymes by means of the well-documented method for the purification of the rat liver transferases failed, because the Drosophila transferases did not bind to CM-cellulose. Purification by subsequent ion exchange on DEAE-cellulose, molecular sieving on Sephadex G-100 and hydroxylapatite chromatography, revealed three active fractions from Drosophila cytosol and five active fractions from rat liver cytosol, using 1-chloro-2,4-dinitrobenzene as the electrophilic substrate. None of the Drosophila active fractions catalyzed the conjugation of glutathione with the epoxides mentioned.

It is concluded that there are important differences between the GST systems of both species, resulting in differences in the metabolic fate of chemicals that are substrates for glutathione conjugation. This has to be taken into account in the evaluation of genotoxicity testing in Drosophila of potentially harmful compounds.

The majority of genotoxic chemicals initiate genetic damage only after being activated by metabolic processes to electrophilic reactive agents [1]. Such intermediates are often capable of inducing mutations in suitable biological models. Bioassays based on this principle are frequently used to detect the potential genotoxic activity of chemicals [2]. Of these bioassays, the fruitfly *Drosophila melanogaster* has been shown to exhibit mutations when exposed to a wide array of procarcinogens and promutagens [3, 4]. Obviously the capability of Drosophila to biotransform xenobiotics is an import precondition for its suitability to detect the potential harmful effects of such compounds [5]. Previous studies have demonstrated the presence of several enzyme systems in Drosophila which are capable of metabolizing xenobiotics: cytochrome P-450 containing mono-oxygenases [6], epoxide hydrolase and glutathione *S*-transferase [7], while also indications for the presence of glucose and phosphate conjugations were obtained [8]. Furthermore subcellular fractions from Drosophila homogenates were shown to activate a number of indirectly acting chemicals to mutagenic agents in several test systems [9].

The conjugation of xenobiotics with glutathione (GSH) as catalyzed by the glutathione *S*-transferase system (GST; EC 2.5.1.18) is an important physiological process in the detoxification or toxification of electrophilic compounds [10–12]. In mammals it consists of several isozymes, with overlapping substrate-specificities [10, 13]. Concerning insects far less information is available: the GST of the cockroach was studied by Usui *et al.* [14], while Motoyama and Dauterman [15] and Oppenoorth *et al.* [16] reported on house fly enzymes. Baars *et al.* [7] and Hällström and Grafström [17] described GST activity in *Drosophila melanogaster*.

1-Chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) are substrates for the GSTs of both rat and Drosophila, while no activity was detectable when styrene-7,8-oxide (STOX) or 1,2-epoxy-3(*p*-nitrophenoxy)-propane (ENPP) were used with Drosophila [7, 18].

In the present study this apparent species difference was further investigated by purifying the GSTs from both Drosophila and rat liver and comparing substrate specificities of the isozymes.

MATERIALS AND METHODS

Chemicals. CDNB and DCNB were purissimum grade products of Fluka AG (Buchs, Switzerland), STOX (analytical grade) was obtained from Merck/Schuchardt (Darmstadt, G.F.R.) and ENPP was obtained from Eastman Kodak Company (Rochester, U.S.A.). GSH (reduced form) was produced by Sigma Chemical Company (St. Louis, U.S.A.). DEAE ion exchange cellulose (DE 32) was a product of Whatman (Maidstone, U.K.), Sephadex G-100 was obtained from Pharmacia (Uppsala, Sweden) and hydroxylapatite (Bio-Gel HT) was produced by BioRad Laboratories (Richmond, U.S.A.). All

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other chemicals were of the best quality commercially available. Water was glass-distilled prior to use.

Animals. Male rats of the laboratory bred SPF Wistar strain weighing 180–200 g were used for rat liver experiments. They were fed a commercially available diet (Hope Farms, Woerden, The Netherlands). Food and tap water were allowed *ad libitum*. *Drosophila melanogaster*, strain Berlin K was obtained from the Department of Radiation Genetics and Chemical Mutagenesis, Faculty of Medicine, Leiden University. They were provided as adult flies from mass cultures that were reared at 25°.

Preparation of crude glutathione S-transferases. Rats were starved overnight and killed by cervical dislocation. The livers were perfused *in situ* via the portal vein with 50 mM Tris–KCl buffer, pH 7.4, containing 10% v/v glycerol and 0.3 mM EDTA. The removed livers were homogenized in a Potter–Elvehjem tube with a glass pestle. The homogenate was centrifuged for 20 min at 8000 *g* in a Beckman JA-20 centrifuge. The supernatant was filtered through tissue paper to remove floating fat, followed by further centrifugation for 90 min at 100,000 *g* in a Beckman L5-50E ultra centrifuge. The supernatant contained the soluble GST activity. Whole flies from the strain Berlin K were sacrificed by freezing, and homogenized in a Potter–Elvehjem tube with the same buffer as used for rat liver except that 30% v/v glycerol was used. Subcellular fractionation was as described for rat liver homogenate.

Purification of glutathione S-transferases. Firstly, the cytosolic proteins were passed through a column of DEAE-cellulose (3 × 30 cm). The column was eluted with the homogenization buffer and fractions of 7 ml were collected. The active fractions were pooled and the column was regenerated with the elution-buffer, containing 0.3 M NaCl. The partially purified enzymes were stirred with solid $(NH_4)_2SO_4$ to a final concentration of 40%. This preparation was centrifuged at 8000 *g* for 30 min. The precipitate was discarded and the supernatant fluid was brought gradually to a concentration of 90% $(NH_4)_2SO_4$. After centrifugation at 8000 *g* for 30 min, the precipitated proteins were dissolved in a minimal volume of 10 mM potassium phosphate buffer, pH 7.4 with 0.3 mM EDTA and 10% or 30% glycerol for rat liver and Drosophila respectively, followed by dialysis for 24 hr against the same buffer. The dialyzed preparation was applied to a Sephadex G-100 column (3 × 100 cm) and eluted with the same buffer as used for dialysis. Fractions of 5 ml were collected; the fractions with the highest amount of specific activities were pooled and used for further purification. Hydroxylapatite columns (3 × 20 cm) were prepared and equilibrated with 10 mM potassium phosphate buffer, pH 7.4, containing 0.3 mM EDTA and 10% or 30% glycerol for rat liver and Drosophila respectively. Samples containing 15 mg of protein were brought on the column. After adsorption and washing with five times the primary applied volume, a linear buffer gradient of 500 ml was started (10–350 mM with respect to potassium phosphate) to elute the adsorbed isozymes.

All manipulations were performed at 4°.

Protein determination. During all purification steps protein was monitored by measuring the absorbance of the eluted fractions at 280 nm. The protein content of the fractions with enzyme activity was determined according to the method of Lowry *et al.* [19], using bovine albumin as a standard.

Enzyme assays. GST activities were determined using GSH as the first substrate and CDNB, DCNB, STOX or ENPP as the second, electrophilic substrates, in general under saturating conditions with respect to enzyme kinetics. Incubations were for 5 min in a 0.13 M phosphate buffer, pH 7.0 for CDNB and DCNB, pH 8.0 for STOX and pH 6.7 for ENPP as the substrate [20]. Activities from rat liver were measured at 37°, incubations with Drosophila enzymes were done at 27°. CDNB, DCNB and ENPP conjugates were estimated as reported by Habig *et al.* [21], conjugations with STOX were determined by measuring the amount of unreacted GSH with Ellman's reagent [22] as modified by Baars *et al.* [23].

RESULTS

The enzymatic activities as determined in the cytosolic fraction of Drosophila and rat liver homogenates are given in Table 1. Rat liver has generally a higher activity than Drosophila when expressed per mg of protein. However, as insects lack a specific organ with high metabolizing capacity, activities were also expressed per g body weight and then activities per g body weight are considerably higher

Table 1. Glutathione *S*-transferase activities in post-microsomal supernatants of *Drosophila melanogaster* and rat liver

Enzyme activity	Substrate	Drosophila	Rat liver
Per mg cytosolic protein	1-Chloro-2,4-dinitrobenzene	111 ± 15 (6)	1240 ± 160 (11)
	1,2-Dichloro-4-nitrobenzene	14 ± 3 (6)	300 ± 13 (5)
	Styrene-7,8-oxide	n.d. (4)	70 ± 6 (11)
	1,2-Epoxy-3(*p*-nitrophenoxy)-propane	n.d. (8)	78 ± 9 (4)
Per g body weight	1-Chloro-2,4-dinitrobenzene	6100 ± 850 (6)	2200 ± 380 (11)
	1,2-Dichloro-4-nitrobenzene	765 ± 165 (4)	532 ± 24 (5)
	Styrene-7,8-oxide	n.d. (4)	127 ± 18 (11)
	1,2-Epoxy-3(*p*-nitrophenoxy)-propane	n.d. (8)	142 ± 16 (8)

Enzyme activities are given in nmoles of conjugate produced per minute, as mean ± S.D., with the number of experiments in brackets.

n.d.: not detectable.

Table 2. Purification of glutathione S-transferases of *Drosophila melanogaster* and rat liver

	Drosophila			Rat liver		
	Post-microsomal supernatant	DEAE eluate	Sephadex G-100 eluate	Post-microsomal supernatant	DEAE eluate	Sephadex G-100 eluate
Volume (ml)	74	156	51	41	122	114
Protein content (mg/ml)	6.48	1.35	2.35	17.50	2.36	1.78
Glutathione S-transferase activity (V_{max}; μmoles/min per mg)	0.26	0.56	0.67	2.27	5.47	6.25
Yield (% of total activity)	100	94	64	100	97	78

in Drosophila (Table 1). No activity towards epoxide substrates could be shown in Drosophila post-microsomal supernatant.

Mammalian GST isozymes are generally isolated by subsequent chromatography on DEAE-cellulose, Sephadex G-100 and CM-cellulose [10]. However, attempts to purify the GST isozymes of Drosophila by this method failed, because they did not bind to CM-cellulose (results not shown). As chromatography on DEAE-cellulose and Sephadex G-100 was able to remove a considerable amount of contaminating protein, these steps were maintained. Subsequent use of hydroxylapatite chromatography allowed the isolation of a number of isozymes from both species. Details of the purification procedure are given in Table 2; after molecular sieving on Sephadex G-100 the total amount of protein is decreased by a factor 4, while still 64% of the total GST activity is present. The hydroxylapatite chromatograms of Drosophila and rat liver isozymes are shown in Fig. 1. Using CDNB as the electrophilic substrate, 3 active peaks from Drosophila material were obtained, eluting between 20 and 100 mM potassium phosphate, while with DCNB as the substrate, an additional small fourth peak was observed. From rat liver material five active peaks were seen with CDNB as the substrate; these fractions started to elute at a potassium phosphate concentration >100 mM. When DCNB was used as the substrate the main activities eluted only at potassium phosphate concentrations >200 mM. The isozymes from Drosophila thus obtained were not active in catalyzing the conjugation of STOX or ENPP with GSH.

DISCUSSION

The present study focused on the differences between the soluble GSTs from Drosophila adults as compared to rat liver, using hydroxylapatite purified enzyme fractions. Interestingly Drosophila GST did not bind on CM-cellulose columns, although this has proved to be effective in the purification of the rat liver enzymes [10]. Motoyama and Dauterman [15] described a purification of housefly GST, in which binding to CM-cellulose did occur, but no details were given on the amount of activity that was bound. Oppenoorth, however, investigating housefly GST (personal communication) found that only a minor fraction of the total GST activity was bound on CM-cellulose columns. The lack of binding of the Drosophila GST as reported here may be due to differences in isoelectric points between the GSTs of rat liver and Drosophila respectively, which is indicative of relatively important differences between the enzymes of the two species. In addition, marked differences were obvious from the hydroxylapatite elution profiles, which also strongly suggests differences between the enzymes from rat liver and Drosophila in isozyme composition as well as in their nature. This in principle is not surprising bearing in mind the evolutionary distance between both species. The hydroxylapatite elution profile of the rat liver GST can be compared with that reported by Guthenberg *et al.* [24]: they were able to characterize the isozymes in order of elution as GST-B, -C and

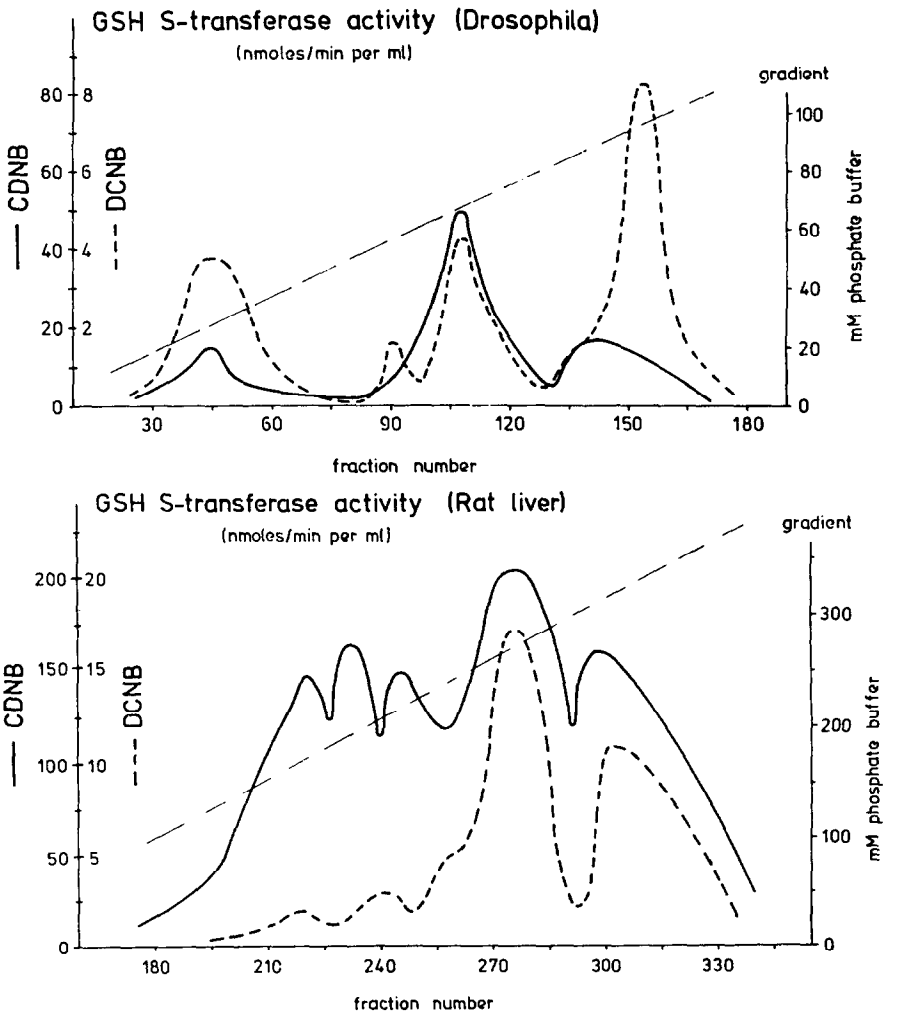

Fig. 1. Hydroxylapatite chromatography of a prepurified post-microsomal supernatant from *Drosophila melanogaster* homogenate (upper part) and from rat liver homogenate (lower part). The cytosolic fractions were firstly purified by DEAE ion exchange chromatography, ammoniumsulfate precipitation followed by dialysis, and molecular sieving on Sephadex G-100 (see Materials and Methods for details). Enzymatically active fractions were eluted from the hydroxylapatite column with a potassium phosphate buffer gradient (— — —). CDNB (——): enzyme activity with 1-chloro-2,4-dinitrobenzene as the substrate; DCNB (– – –): enzyme activity with 1,2-dichloro-4-nitrobenzene as the substrate.

-A respectively, using the nomenclature of Jakoby and coworkers [10]. Our results show a cluster of three peaks of activity with CDNB eluting first (fractions 200–255), followed by two more peaks. The first eluting three peaks could perhaps reflect the heterogeneity of the isozymes formerly characterized as GST-B in rat liver cytosol [13, 25].

The individual GST activities of Drosophila as separated in this report are as yet not further characterized with regard to their molecular properties. However, in view of the present knowledge on the general constitution of the GST system in animal organisms [26] there is no indication that the Drosophila GST would be fundamentally different in that respect from the GST of other species.

The ratio of the total enzyme activity with CDNB and DCNB respectively, which for the Drosophila enzyme is twice that of the rat liver enzyme, suggests relatively more enzyme activity towards DCNB in rat liver. Interestingly, the Drosophila GST was not active towards two epoxide substrates, STOX and ENPP, while epoxides in general are well-accepted substrates for mammalian GSTs. In this respect there seems to be some resemblance with the human GST system, for which Kamisaka *et al.* [27] and Awashti *et al.* [28] could not detect activity towards ENPP. However, Pacifici *et al.* [29] reported activity towards STOX, while recently Warholm *et al.* [30] described a specific human liver GST isozyme, which was present only in about 60% of the livers investigated and which conjugates STOX as well as ENPP. Pilot experiments with the Drosophila strains Oregon K and 91R indicated that also in these strains no ENPP-conjugating GST activity is present. In addition, preliminary investigations with the GST of strain Berlin K did not reveal conjugating activity with benzo(*a*)pyrene-4,5-oxide, cyclohexene oxide and butylene-1,2-oxide. Taken together, these results

suggest that the lack of epoxide-conjugating GST activity of Drosophila is a general characteristic of this species.

There is increasing interest in the function of GSTs in biotransformation [26, 31]. Recent work from our laboratory [32] demonstrated the inducibility of soluble and membrane-associated GSTs, while we [11] and others [33] showed its involvement in the activation of certain dihalogenated compounds into mutagenic species.

In earlier studies we reported the presence of soluble GST activity in *Drosophila melanogaster* adults [7], an activity which is of importance in the function of Drosophila in mutagenicity testing. We also pointed to possible differences between the Drosophila GST and the similar enzyme system in rat liver [5]. Hällström and coworkers [17] reported GST activity and inducibility in Drosophila larvae, while they recently were able to demonstrate the inducibility of this activity also in adult flies (I. Hällström, personal communication).

Our results indicate an inability of Drosophila to conjugate certain epoxides with GSH. Together with the observed differences between the GST system of Drosophila and the rat as representative mammal, it points to obvious differences in the qualitative and quantitative metabolic fate of chemicals that are biotransformed via GSH conjugation in the two species. Consequently, caution is needed in extrapolating results from one species to another, especially in the evaluation of genotoxicity testing.

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