Drug Induction of Hepatic Glutathione *S*-Transferases in Male and Female Rats*

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The induction of the glutathione *S*-transferases by phenobarbital and polycyclic hydrocarbons was studied in male and female rats. Administration of phenobarbital resulted in 60–80% increase in *S*-aryl and *S*-alkyl enzyme specific activities, whereas the *S*-epoxide and *S*-alkyl activities were increased by 30–40%. In following the sequence of induction, the former two activities were noted to reach peak activities before an increase in the latter two activities was observed. Both 3-methylcholanthrene and 3,4-benzopyrene were shown to induce these four enzymic activities, although without the discrimination between pairs of activities noted with phenobarbital. No change in K_m accompanied the increase in V_{max} after induction by drugs, and no change occurred in K_i for sulphobromophthalein inhibition. Significantly lower enzyme specific activities were found for three of the activities studied in female rats but no difference was observed in the *S*-alkyltransferase activity. However, the proportional increase in the enzymic activities in response to phenobarbital was the same in males and females. These studies demonstrate the drug induction of a group of cytosolic drug-metabolizing enzymes as well as the identification of sex differences in these activities.

The glutathione *S*-transferases, a group of hepatic cytosolic enzymes, catalyse reactions between the thiol group of glutathione and aryl (Booth *et al.*, 1961), aralkyl (Boyland & Chasseaud, 1969), epoxide (Boyland & Williams, 1965; Fjellstedt *et al.*, 1973) and alkyl (Johnson, 1966) substrates. These enzymes are believed to be important in the detoxication of alkylating chemicals and drugs by substituting electrophilic attack on glutathione for the nucleophilic sites of cellular proteins (Boyland & Chasseaud, 1969; Pabst *et al.*, 1973).

In the past the glutathione *S*-transferases have been partially separated on the basis of differences in physical properties. Thus an enzyme preparation obtained after lowering liver supernatant to pH 5, then raising to pH 6.5, leaves 95–100% of activity toward aryl substrates but only 7–10% of activity toward alkyl and 0.3% of activity toward epoxide substrates (Boyland & Williams, 1965). Prolonged dialysis against distilled water results in loss of more than 85% of activity toward alkyl substrates but spares the others (Boyland & Chasseaud, 1969). These observations have suggested that each substrate activity represents a different enzyme.

Three glutathione transferases have been purified to homogeneity (Pabst *et al.*, 1973). These homogeneous proteins are dimers with basic isoelectric points and the same molecular weight. Although

individual proteins have been found to catalyse reactions with more than one class of substrate, the general separation of activities described by earlier workers still seems to apply. For example, two proteins seem to have the bulk of aryltransferase activity (Pabst *et al.*, 1973; Clark *et al.*, 1973), another has the epoxide transferase activity, and alkyltransferase activity is accounted for by two enzyme proteins which are different from that containing the aryltransferase activity (Pabst *et al.*, 1973).

Similarities have been noted in molecular weight and binding properties between one of the glutathione *S*-transferases, *S*-aryltransferase and ligandin (Kaplowitz *et al.*, 1973). Glutathione *S*-aryltransferase is believed to catalyse the glutathione conjugation of sulphobromophthalein (Combes & Stakelum, 1961; Booth *et al.*, 1961), whereas ligandin is believed to be an important cytoplasmic sulphobromophthalein- and organic anion-binding protein (Litwack *et al.*, 1971). More recent experiments using isoelectric focusing have indicated separate identity for glutathione *S*-aryltransferase and ligandin (Ketterer *et al.*, 1974). However, since the glutathione transferases have the same molecular weight (Pabst *et al.*, 1973) and organic anion-binding properties (Kaplowitz *et al.*, 1974), there remains the strong possibility that ligandin might be another of this group of enzymes.

A somewhat unique feature of ligandin, as a presumed non-enzymic and cytoplasmic protein, is its inducibility by drugs (Reyes *et al.*, 1969, 1971).

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Aside from phenobarbital induction of several NADP-requiring soluble enzymes (Bresnick & Yang, 1964), little is known about the drug induction of cytosolic enzymes. Studies were performed to evaluate the inducibility of the glutathione *S*-transferases by phenobarbital and polycyclic aromatic hydrocarbons in male and female rats in order to examine further the similarities between these enzymes and ligandin. These studies were also designed to identify a potentially inducible cytosolic enzyme system of importance in drug metabolism.

Materials and Methods

Animals, treatment and preparation of liver cytosol

Sprague-Dawley rats (160–180g) were given phenobarbital sodium (Sigma Chemical Co., St. Louis, Mo., U.S.A.) (2–8mg in 0.9% NaCl) and 3,4-benzopyrene (Sigma Chemical Co.) or 3-methylcholanthrene (Calbiochem, San Diego, Calif., U.S.A.) (1mg in corn oil intraperitoneally twice daily). Controls received 0.9% NaCl or corn oil by the corresponding route. Animals were killed under light ether anaesthesia 24h after the last drug dose. The liver was perfused *in situ* with 0.01M-sodium phosphate buffer (pH7.4)–0.25M-sucrose. Homogenates (20%, w/v) were prepared with the same buffer, and supernatant fractions (cytosol) harvested after centrifugation at 105000g for 60min in a Beckman L2-65B ultracentrifuge (Beckman Instruments Inc., Fullerton, Calif., U.S.A.). Specimens were stored at –15°C for up to several months without significant loss of activity.

Determination of enzymic activities

Glutathione *S*-aryl-, *S*-aralkyl- and *S*-epoxide-transferase activities were measured kinetically by described techniques (Booth *et al.*, 1961; Pabst *et al.*, 1973) in duplicate in 2.0ml (0.1M-sodium phosphate) reaction mixtures containing 10mM-GSH (Sigma Chemical Co.) and the following substrates respectively: 1.0mM-3,4-dichloronitrobenzene (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.), 0.5mM-*p*-nitrobenzyl chloride (Aldrich Chemical Co.) and 0.5mM-1,2-epoxy-(*p*-nitrophenoxy)propane (Aldrich Chemical Co.), to which were added 10–200μl of liver supernatant. The *S*-aryl activity was determined at pH8.0 and the *S*-aralkyl and *S*-epoxide activities at pH6.5. Assays were carried out at 37°C in a Beckman DK-2A spectrophotometer. The initial rate of reaction was determined by measurement of the production of a GSH conjugate of the aryl, aralkyl and epoxide substrates at 344, 310 and 360nm respectively. Spontaneous interaction of substrates was negligible under these experimental conditions and enzymic activity was a linear function of time and protein concentration.

Glutathione *S*-alkyltransferase activity was measured by using 1.7mM-[¹⁴C]methyl iodide (New England Nuclear Corp., Boston, Mass., U.S.A.) and 10mM-GSH in a modification of a method described by Johnson (1966). Assays were performed in duplicate at 25°C in 3.0ml of 66mM-sodium phosphate–2mM-EDTA buffer, pH7.0. The addition of liver cytosol (200μl) initiated the reaction. Portions (0.5ml) were withdrawn at 0 and 3 min and blown to dryness with gentle heating in liquid-scintillation counting vials. Hot distilled water (1.0ml) was added, followed by 15ml of Aquasol (New England Nuclear Corp.). Samples were counted for 10min in a Packard Tri-Carb liquid-scintillation spectrometer. Under these conditions reactions were shown to be linear for more than 5min; spontaneous interaction of substrates was negligible.

Protein concentration was determined by the method of Lowry *et al.* (1951). Enzyme specific activities were expressed as nmol of product formed/min per mg of cytosolic protein.

Results of determinations of enzymic activity in treatment and control groups were compared by unpaired *t* test (Batson, 1956).

Results

Induction by phenobarbital

Phenobarbital administration (8mg/100g) for 7 days to male rats resulted in significant increases in the four transferase activities (Table 1). The increases of *S*-aryl and *S*-aralkyl activities were very similar (68 and 62% respectively) and approximately twofold greater than the increase in the epoxide and alkyl activities (32 and 39% respectively). The route of administration (i.e. intraperitoneal or subcutaneous) did not affect the differential increases in these activities.

When phenobarbital was administered intraperitoneally in various doses (0–8mg/100g) to male rats (Fig. 1), induction of the four activities was apparent at lowest doses (2mg/100g). At higher doses (6–8mg) increases in the activities were noted to couple in a similar fashion to that indicated in Table 1.

The sequence of induction of the four enzymic activities was studied by administering phenobarbital subcutaneously for 7 consecutive days, followed by 6 days without phenobarbital (Fig. 2a). Four treated and four control (injected with 0.9% NaCl) animals were killed daily. Parallel increases in *S*-aryl and *S*-aralkyl activities were found preceding and reaching near-maximum values at a time (day 3) when the other activities were at control values. By day 7 of phenobarbital administration, the *S*-epoxide and *S*-alkyl activities were noted to be at maximum values which were significantly lower than the increases in the former activities. After cessation of phenobarbital,

Table 1. Drug induction and sex differences of four glutathione *S*-transferase activities from rat liver cytosol

Enzyme specific activities are expressed as mean $\pm$ S.E.M. in units of nmol/min per mg of protein. For experiments with male animals groups of ten were used and for female animals groups of six were used. Results of the statistical comparisons of enzyme activities from drug-treated versus control groups are given below the value for that group. Ratios of female to male control activities are 0.45 ± 0.03 ($P < 0.001$) for *S*-aryltransferase, 0.62 ± 0.04 ($P < 0.001$) for *S*-aralkyltransferase, 0.75 ± 0.04 ($P = 0.005$) for *S*-epoxidettransferase, and 1.01 ± 0.09 (not significant) for *S*-alkyltransferase. See the text for details of assay procedures and treatment of animals.

	Glutathione <i>S</i> -transferase activity					
	Male				Female	
	Control	Phenobarbital-treated	3-Methylcholanthrene-treated	3,4-Benzopyrene-treated	Control	Phenobarbital-treated
Aryl	113 $\pm$ 5	190 $\pm$ 6 ($P < 0.001$)	151 $\pm$ 6 ($P < 0.001$)	142 $\pm$ 9 ($P = 0.020$)	51.4 $\pm$ 1.5	81.7 $\pm$ 3.3 ($P < 0.001$)
Aralkyl	169 $\pm$ 6	274 $\pm$ 17 ($P < 0.001$)	240 $\pm$ 7 ($P < 0.001$)	233 $\pm$ 12 ($P < 0.001$)	105 $\pm$ 6	183 $\pm$ 8 ($P < 0.001$)
Epoxide	11.6 $\pm$ 0.7	15.2 $\pm$ 1.0 ($P < 0.001$)	14.7 $\pm$ 0.7 ($P = 0.005$)	14.2 $\pm$ 1.0 ($P = 0.025$)	8.7 $\pm$ 0.4	12.3 $\pm$ 0.8 ($P < 0.001$)
Alkyl	16.6 $\pm$ 0.6	23.1 $\pm$ 0.9 ($P < 0.001$)	20.7 $\pm$ 1.1 ($P = 0.01$)	18.9 $\pm$ 0.8 ($P = 0.05$)	16.8 $\pm$ 1.0	23.7 $\pm$ 1.3 ($P < 0.001$)

Fig. 1. Dose-related increase in the glutathione *S*-transferase activities after phenobarbital administration

Groups of four male rats received phenobarbital (0–8 mg/100 g) or 0.9% NaCl intraperitoneally for 7 days and were killed 24 h after the last injection. The following enzyme activities from liver cytosol are expressed as mean % increase of specific activity for the phenobarbital versus control groups: glutathione *S*-aryltransferase ($\blacktriangle$), *S*-aralkyltransferase ($\triangle$), *S*-epoxidettransferase ($\bullet$), and *S*-alkyltransferase ($\circ$). See text for details of assay conditions.

enzymic activities remained at peak values for several days and then gradually declined. Fig. 2(b) indicates the increase in liver wt./body wt. ratio as a marker of the effective action of phenobarbital. Prolongation of treatment with phenobarbital for up to 14 days resulted in no greater increases in enzymic activities than after 7 days.

Induction by polycyclic aromatic hydrocarbons

The action of 3-methylcholanthrene and 3,4-benzopyrene was compared with that of phenobarbital (Table 1) as enzyme inducers. Groups of ten animals received one drug intraperitoneally for 10 days. In comparison with the response to phenobarbital, 3-methylcholanthrene and 3,4-benzopyrene administration resulted in a significantly smaller increase in *S*-aryl- and *S*-aralkyl-transferase activities but comparable induction of the *S*-epoxide activity. A diminished effect on the *S*-alkyltransferase activity was noted in rats receiving 3,4-benzopyrene. Two control groups receiving daily injections of either 0.9% NaCl or corn oil showed no differences.

Sex differences in enzymic activity and drug induction

In comparing the enzymic specific activities in male versus female rats of the same size and same proportional liver weight, significantly lower *S*-aryl-, *S*-aralkyl- and *S*-epoxide-transferase activities were observed (Table 1) for the female group. However, no difference in *S*-alkyltransferase activity could be identified.

The effect of sex difference on enzyme induction was evaluated after 7 days of phenobarbital administration (Table 1). As in male animals, increase in the

Fig. 2. Sequential induction of the glutathione *S*-transferases

Groups of male rats were given phenobarbital or 0.9% NaCl subcutaneously for 0–7 days, and four animals from each group were killed daily 24 h after the last injection for 13 days. The glutathione *S*-transferases were measured in liver cytosol. See the text for details of assay conditions. The mean % increases in specific activities for the phenobarbital versus control groups are given in (a) for glutathione *S*-aryltransferase (▲), *S*-aralkyltransferase (△), *S*-epoxide-transferase (●) and *S*-alkyltransferase (○). The mean % increases in liver weight/body weight ratio for the phenobarbital versus control rats are given in (b).

four glutathione *S*-transferases in response to phenobarbital pre-treatment was observed in the females. The proportional increase in enzymic activities in females was closely similar to the effect in males, showing a twofold difference in the induction of *S*-aryl- and *S*-aralkyl- versus *S*-epoxide- and *S*-aralkyl-transferase activities.

Effect of drugs on enzyme kinetics

Studies were performed to evaluate the effects of drugs on the kinetics of the four enzymic activities. Thus, as shown in Fig. 3 by the Lineweaver–Burk (1934) method of plotting, with glutathione *S*-

Fig. 3. Kinetics of drug induction of glutathione *S*-aryltransferase

Enzyme activities (v , $\mu\text{mol per mg of protein/min}$) in liver cytosol from animals treated with 0.9% NaCl (■), 3,4-benzopyrene (○), 3-methylcholanthrene (●) and phenobarbital (△) were measured at 37°C in 0.1M-phosphate buffer, pH 8.0, containing 10mM-GSH and 0.4–1.2mM-3,4-dichloronitrobenzene.

aryltransferase as an example, cytosol obtained after phenobarbital, 3-methylcholanthrene and 3,4-benzopyrene administration exhibited an increase in the maximum velocity (V_{max}) of glutathione 3,4-dichloronitrobenzene conjugation without influencing the Michaelis constant (K_m). Similar results were found for the three other activities. In addition, the inhibitory kinetics of sulphobromophthalein were compared in phenobarbital-treated and control cytosol. There was no difference in the inhibitor constant (K_i) for sulphobromophthalein inhibition of the four glutathione *S*-transferases. In each instance inhibition was found to be competitive.

Discussion

A number of pharmacological agents have been shown to induce microsomal drug-metabolizing NADP-requiring enzymes (for review see Conney, 1967), but there are few examples of the drug induction of cytoplasmic enzymes and proteins. Phenobarbital has been shown to effect a twofold rise in the NADP-requiring soluble enzymes, D-glucose 6-phosphate–NADP oxidoreductase, 6-phospho-D-glucuronate–NADP oxidoreductase (Bresnick & Yang, 1964) and UDP-glucose–NAD oxidoreductase (Conney *et al.*, 1961; Touster & Hollmann, 1961). In contrast with the present work, the phenobarbital-induced effect of the NADP oxidoreductases was evident at day 2 and maximum at day 3 after initiation of treatment, and polycyclic hydrocarbons were ineffective. Another cytosolic enzymic activity which is responsible for sulphobromophthalein conjugation with glutathione, i.e. glutathione *S*-aryltransferase,

has been reported to increase by 23% after treatment with phenobarbital for 3 days (Klaassen & Plaa, 1968). In addition to these phenobarbital-induced increases of cytosolic enzyme activities, there is evidence for drug-induced increases in a previously presumed non-enzymic cytosolic protein, i.e. ligandin (Litwack *et al.*, 1971). Both sulphobromophthalein-binding capacity and immunoprecipitable ligandin increase up to twofold in response to phenobarbital and polycyclic hydrocarbons (Reyes *et al.*, 1969, 1971).

Studies by Darby & Grundy (1972) and Grover & Sims (1964) failed to demonstrate significant induction of glutathione *S*-aryltransferase activity (for 3,4-dichloronitrobenzene). In contrast with the present work, where drugs were administered for 7–10 days, Darby & Grundy (1972) administered phenobarbital for only 2 days, measuring enzymic activity 2 days later, whereas Grover & Sims (1964) used a single injection of phenobarbital or aromatic hydrocarbons, after which pairs of animals were killed daily for 10 days. In addition, both studies used larger animals of different species from those used in the present study.

The findings in the present report demonstrate the drug inducibility of four glutathione *S*-transferase activities. Two types of inducing agents, i.e. phenobarbital and polycyclic aromatic hydrocarbons (3-methylcholanthrene and 3,4-benzopyrene), which are believed to induce microsomal enzymes by different mechanisms (Conney *et al.*, 1959, 1960; Creaven & Parke, 1966), were found to induce the four glutathione *S*-transferase activities. The increase in $V_{\max}$ in response to drug treatment was unaccompanied by an effect on the K_m value indicating that no alteration of substrate affinity for enzymes had occurred as a consequence of the drugs. In addition, sulphobromophthalein, a known competitive inhibitor of *S*-aryltransferase (Boyland & Grover, 1967; Kaplowitz *et al.*, 1974), had an indistinguishable K_i value or inhibitory affinity for control and phenobarbital-treated enzyme samples. Therefore on the basis of these findings, it seems reasonable to consider these drugs as affecting an increased quantity of enzyme.

Pabst *et al.* (1973) have described the preparation of homogeneous glutathione transferases, each of which catalyses reactions with several overlapping classes of substrates. Although on the basis of their work it may be difficult for us to interpret exactly which enzymic proteins are induced, the present data do show consistent features indicating the separability of drug-induced activities into two pairs each of which may represent one or more enzymic proteins. The *S*-aryl- and *S*-alkyl-transferase activities increased to a similar extent which differed significantly from the parallel increases of *S*-epoxide- and *S*-alkyl-transferase activities, and

the induction of *S*-aryl and *S*-alkyl activities preceded the induction of the other two activities (Fig. 2). Further discrimination of these pairs of activities is suggested by the decreased induction of the *S*-alkyltransferase activity by 3,4-benzopyrene as compared with 3-methylcholanthrene and phenobarbital and by the differences between male and female activities.

Differences in microsomal enzyme activities relative to sex in the rat are well known (Quinn *et al.*, 1958; Murphy & DuBois, 1958; Kuntzman *et al.*, 1966). Darby & Grundy (1972) found a diminished glutathione *S*-aryltransferase activity in female rat liver compared with that of the male. We also have identified sex differences in this soluble enzyme system; three of the four glutathione *S*-transferase activities were lower in female animals than in males (Table 1). However, the proportional induction of enzymic activities after phenobarbital treatment was virtually identical in male and female animals.

Similarities between the glutathione transferases and ligandin have been described (Kaplowitz *et al.*, 1974), particularly with respect to molecular weight and organic anion-binding properties. That these enzymic activities and ligandin are similarly drug-induced further supports the possibility that ligandin is one of this family of enzymes. Further, ligandin concentrations have been reported to be the same in male and female rats (Reyes *et al.*, 1971). It is noteworthy therefore that glutathione *S*-alkyltransferase was the only activity which was as great in females as in males, suggesting a particularly close relationship between this activity and ligandin.

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