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Effects of inducers of drug metabolism on basic hepatic forms of mouse glutathione transferase

Paolo DI SIMPLICIO,* Helgi JENSSON† and Bengt MANNERVIK†‡

*Department of Environmental Biology, University of Siena, 53100 Siena, Italy, and †Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-10691 Stockholm, Sweden

The cytosolic glutathione transferases (GSTs) with basic pI values have been studied in mouse liver after treatment with 2,3-t-butylhydroxyanisole (BHA), cafestol palmitate (CAF), phenobarbital (PB), 3-methylcholanthrene (3-MC) and *trans*-stilbene oxide (*t*-SBO). The cytosolic GST activity was induced by all compounds except for 3-MC. Three forms of GST were isolated by means of affinity chromatography and f.p.l.c. The examination of protein profiles and enzymic activities with specific substrates showed that the three GSTs correspond to those found in control animals, i.e. GSTs MI, MII and MIII. The class Mu GST MIII accounted for the major effect of induction, whereas the class Alpha GST MI and the class Pi GST MII were unchanged or somewhat down-regulated. The greatest induction was obtained with BHA, PB and CAF. The activities of other glutathione-dependent enzymes were also studied. An increase in glutathione reductase and thioltransferase activities was observed after BHA, PB or CAF treatment; glyoxalase I and Se-dependent glutathione peroxidase were depressed in comparison with the control group in all cases studied.

INTRODUCTION

The cytosolic glutathione transferases (GSTs) are a class of enzymes inducible by several agents such as drugs, xenobiotics, food additives or substances normally contained in the diet. For example, 2,3-t-butylhydroxyanisole (BHA), cafestol palmitate (CAF) and many other compounds induce by several fold the GST activity in different organs in the mouse (Benson *et al.*, 1978, 1979; Lam *et al.*, 1981, 1987; Sparnins & Wattenberg, 1981; Sparnins *et al.*, 1982; Prochaska *et al.*, 1985*a*,*b*).

GST induction has been instrumental in screening for the anti-carcinogenic activity of different agents (Wattenberg, 1983), and a correlation between an increase in GST activity and a lower incidence of experimental cancer in rodents has been reported (Sparnins & Wattenberg, 1981; Prochaska *et al.*, 1985*a*). However, the biological role of GST induction is unclear, because in preneoplastic nodules, the transferase activity is increased significantly and is accompanied by the appearance of an isoenzyme not expressed in normal hepatocytes (Jensson *et al.*, 1985).

Three major forms of GST with basic pI values have been isolated from mouse liver (Warholm *et al.*, 1986). They represent the three classes of mammalian cytosolic GSTs, Alpha, Mu and Pi (Mannervik *et al.*, 1985). The mouse isoenzymes are homodimers characterized by different substrate specificities: GST MI (class Alpha) has comparatively high activity with cumene hydroperoxide (CuOOH), GST MII (class Pi) with ethacrynic acid (EA), and GST MIII (class Mu) with 1,2-dichloro-4-nitrobenzene (DCNB) and bromosulphophthalein (BSP).

In spite of the previous work, the nature of GST induction is incompletely understood. For example, in screening for anti-carcinogens, only the total activity with 1-chloro-2,4-dinitrobenzene (CDNB) was measured (cf. Wattenberg, 1983), and up-regulation of one form of GST may be masked by down-regulation of another isoenzyme. It is therefore necessary to investigate the changes in the isoenzyme pattern brought about by different inducers and thereby gain further knowledge about the differential induction of GSTs. The enzymes are encoded by several genes, and establishing that different inducers affect the levels of different isoenzymes would indicate distinct mechanisms of gene regulation of these forms of GST. The present investigation provides such evidence for differential gene regulation.

MATERIALS AND METHODS

Treatment of mice

Out-bred male NMRI mice (Naval Medical Research Institute, Bethesda, MD, U.S.A.) were housed in stainless steel wire cages; the animals were fed on either a powder or a pellet diet *ad libitum*, depending on drug treatment. BHA (Sigma) and CAF (a gift from Nestec Ltd., Vevey, Switzerland) were added to a powder diet at concentrations of 4 mg/g and 2 mg/g respectively. Phenobarbital (PB; Fluka)-treated animals received the drug in the diet (2 mg/kg) or by daily intraperitoneal injections (80 mg/kg in 0.9% NaCl). *trans*-Stilbene oxide (*t*-SBO; EGA Chemie) (400 mg/kg) and 3-methylcholanthrene (3-MC; Sigma) (20 mg/kg) were dissolved in

Abbreviations used: GST, glutathione transferase; BHA, 2,3-t-butylhydroxyanisole; t-BOOH, t-butyl hydroperoxide; CAF, cafestol palmitate; CuOOH, cumene hydroperoxide; EA, ethacrynic acid; DCNB, 1,2-dichloro-4-nitrobenzene; BSP, bromosulphophthalein; CDNB, 1-chloro-2,4-dinitrobenzene; PB, phenobarbital; 3-MC, 3-methylcholanthrene; *t*-SBO, *trans*-stilbene oxide; *t*-PBO, *trans*-4-phenylbut-3-en-2-one.

‡ To whom correspondence should be sent, at present address: Department of Biochemistry, Biomedical Center, Box 576, University of Uppsala, S-75123 Uppsala, Sweden.

corn oil and given by intraperitoneal injection. All animals were treated for four consecutive days.

Processing of liver tissue for GST determination

For most experiments, livers from groups of 5–6 mice were pooled and homogenized in ice-cold 0.25 M-sucrose by means of an Ultra-Turrax homogenizer to obtain a 20% (w/v) homogenate that was centrifuged at 15000 *g* for 10 min and at 105000 *g* for 60 min at 2 °C. The final supernatant was used to represent the cytosol fraction in the studies. [In a second series of induction experiments (see Table 4), animals were analysed individually by the above procedure.]

All subsequent purification steps were carried out at 4 °C. The supernatant fraction formed by the 105000 *g* centrifugation step was passed through a Sephadex G-25 column (3 cm × 27 cm) packed in 10 mM-Tris/HCl (pH 7.8) containing 1 mM-EDTA and 0.2 mM-dithioerythritol, and the pooled fractions containing GST activity were then applied on to a column of *S*-hexylglutathione–Sepharose 6B (2 cm × 7 cm) equilibrated with the same Tris buffer. The affinity column was then washed with Tris buffer fortified with 0.2 M-NaCl until no protein could be detected in the eluent. GSTs were eluted with 5 mM-*S*-hexylglutathione dissolved in Tris buffer containing 0.2 M-NaCl. The pooled fractions were passed through the Sephadex G-25 column equilibrated with the original Tris buffer lacking NaCl. The sample was finally concentrated to about 10 ml by ultrafiltration on an Amicon PM-10 membrane.

GSTs with basic pI values were separated at 22 °C by f.p.l.c. using the chromatofocusing column Mono P HR 5/20 (Pharmacia Fine Chemicals, Uppsala, Sweden). Before injection, samples (6–9 ml) were adjusted to pH 9 with 25 mM-triethylamine and passed through a 0.22 μm Millipore filter. The column was equilibrated with 25 mM-triethylamine/HCl, pH 10.4. The GSTs were eluted by a pH gradient generated by a buffer composed of 1 ml of Pharmalyte (pH 8.0–10.5) and 5.2 ml of Polybuffer 96 diluted with deionized water and adjusted to pH 8.0 with 1 M-HCl to give a final volume of 300 ml. The flow rate was 1 ml/min. After the gradient elution (pH 8.0), the column was washed with 2 M-NaCl to recover any remaining GST activity.

Determination of GST activity

GST activity with CDNB was assayed at 30 °C (Mannervik & Guthenberg, 1981); assay conditions for DCNB, EA and, *trans*-4-phenylbut-3-en-2-one (*t*-PBO) were those described by Habig & Jakoby (1981) Glutathione peroxidase activity with H_2O_2, t-BOOH and CuOOH was assayed according to Lawrence & Burk (1976), and the conjugation of GSH with BSP was followed as described by Habig *et al.* (1974). Thioltransferase, glyoxalase I and glutathione reductase were determined according to the procedures described by Mannervik *et al.* (1981*a*), Mannervik *et al.* (1981*b*) and Carlberg & Mannervik (1975) respectively.

Protein concentrations were estimated according to Lowry *et al.* (1951) and Kalckar (1947); bovine serum albumin was used as standard. All chemicals were standard commercial products.

RESULTS

Effect of inducers on liver weight and protein concentration

The treatment of mice with BHA, PB (intraperitoneal) and 3-MC caused a slight increase in liver weight; CAF and *t*-SBO produced an approx. 30% increase, while PB (oral) resulted in a significantly higher increase (105%) (Table 1).

After CAF and oral PB treatment, the total hepatic cytosolic protein content (per animal) was 171 and 165% of control values respectively. BHA, 3-MC and *t*-SBO treatment gave 123–138% of control protein values, whereas PB (intraperitoneal) did not give any significant increase in total cytosolic protein content. In part, the elevated levels are accounted for by the increased mass of hepatic tissue.

The major part of the protein bound to the affinity matrix is known to consist of GSTs (Warholm *et al.*, 1986). This was also found to be true after treatment with inducers, since essentially no protein peaks lacking GST activity were found in subsequent purification steps. Thus, the amount of protein recovered after affinity chromatography is an approximate measure of the GST content in the sample. Treatment with CAF, PB (oral) or BHA gave significant increases (42–257%) in the cytosolic specific GST contents expressed per mg of total cytosolic protein, as compared with the control values. A small increase (9%) was obtained in the case of *t*-SBO; and a decrease of 16% in the case of 3-MC.

Table 1. Liver weight, cytosolic protein and specific cytosolic GST protein content in mice treated with inducers

Values are expressed as percentages, with controls as 100%. Values for untreated animals (means ± S.D. of three groups of mice formed by 6, 2 and 6 animals) were: weight, 0.97 ± 0.04 g; total hepatic cytosol protein per animal, 88.6 ± 23.0 mg; specific cytosolic GST content 3.7 ± 0.5% (estimated as protein recovered after affinity chromatography). i.p., intraperitoneal.

Treatment	Route of administration	*n*	Liver weight (%)	Total cytosolic protein (per animal) (%)	Specific cytosolic GST content (%)
Control	–	14	100	100	100
CAF	Oral	6	139	171	357
PB	i.p.	5	123	104	142
PB	Oral	6	205	165	289
BHA	Oral	6	108	123	294
3-MC	i.p.	6	116	138	84
t-SBO	i.p.	6	132	132	109

Table 2. GST activity assayed with specific substrates in the cytosolic fraction of livers of mice treated with various inducers

Values are percentages of the control values reported in parentheses in nmol/min per mg of protein (means ± S.D. of three groups of animals formed by 6, 2 and 6 subjects). i.p., intraperitoneal.

Treatment	Route of administration	Substrate...	GST activity (% of control)				
			CDNB	CuOOH	EA	DCNB	*t*-PBO
Control	–		100 (1880 ± 250)	100 (375 ± 70)	100 (50.9 ± 10)	100 (42.3 ± 1.2)	100 (0.33 ± 0.41)
CAF	Oral		180	74	119	223	1084
PB	i.p.		130	82	51	134	66
PB	Oral		260	71	114	780	480
BHA	Oral		256	86	113	792	2070
3-MC	i.p.		95	68	52	69	210
t-SBO	i.p.		130	78	63	188	207

Effect of inducers on GST activity

The GST activity of the cytosolic fraction of the liver was increased significantly after BHA, PB (oral) and CAF treatment. The CDNB activity levels were 256, 260 and 180% of the control values respectively (Table 2). The observed change in CDNB activity seemed to correspond to the change in the amount of protein recovered after affinity chromatography purification, except in the case of CAF treatment.

Differential expression of the different forms of hepatic mouse GST can be monitored by use of alternative substrates, such as CuOOH, EA, DCNB and *t*-PBO (Warholm *et al.*, 1986). In induced animals, the specific activities with DCNB and *t*-PBO were in most cases increased to a much higher degree than that obtained with CDNB. The activity with CuOOH was always lower than that in the control group. The EA activity was essentially unchanged after treatment with CAF, PB (oral) or BHA, and significantly decreased after treatment with PB (intraperitoneal), 3-MC or *t*-SBO (Table 2).

Separation of hepatic GSTs

The separation by chromatofocusing on f.p.l.c. of the different forms of GST with basic pI values is reported in Fig. 1. The protein as well as CDNB activity profiles of treated animals were always different from those of the control group in all cases examined. Three major peaks of GST were obtained in samples from control as well as from treated animals. In the cases of BHA (Fig. 1*b*), PB (oral) (results not shown) and CAF (Fig. 1*f*), peak III (in the order of elution from the column) was considerably higher than peak II.

Table 3 reports the protein content in each of these peaks as a percentage of the total amount of protein recovered from the column. These values give a measure of the relative amounts of protein contributed by the different GSTs. The activity with CDNB (Fig. 1) in the fractions after separation by f.p.l.c. showed the same general pattern as did the protein concentration; the specific activity measured with CDNB was higher in peak III than in peak I, in accordance with results with untreated animals.

After washing the Mono P column with 2 M-NaCl, two additional peaks of protein with GST activity were obtained (results not shown). These fractions were also active with substrates other than CDNB. The percentage of the total CDNB activity collected in this fraction from treated animals was sometimes higher than that of the control group (6% in control group, 10% for CAF-treated and 11% for BHA-treated mice) but we could not establish whether this activity represented residual portions of the basic forms or induced forms of GST with lower pI values (< 8.0).

Characterization of the separated GSTs

Different substrates which are comparatively more specific for forms MI, MII and MIII were used to identify the GSTs after f.p.l.c. separation.

In Fig. 2, the activity profiles of GSTs in a sample from BHA-treated animals are reported. CuOOH produced the highest relative activity in protein peak I. EA gave the highest value in peak II; and DCNB, BSP (results not shown) and *t*-PBO gave the highest values in peak III. The same relative values were observed in samples obtained from mice subjected to the other treatments. The results of the substrate specificity studies suggest that in induced liver, peak I corresponds to MI in control liver; peak II to MII and peak III to MIII (Warholm *et al.*, 1986).

Inhibition studies with selective inhibitors did not show any significant differences between GSTs from treated mice in comparison with those from control animals. The IC_{50} values measured in peaks I, II and III using triphenyltin chloride and tributyltin acetate (characteristic for MI and MIII) and Cibacron Blue (characteristic for MII) (Warholm *et al.*, 1986) were the same in control and in BHA-treated mice (results not shown).

In order to study possible individual variations in different animals in their response to the treatments, a second series of induction experiments was carried out. Two inducers, PB and BHA, were used and GST activities were measured in the hepatic cytosol fraction from individuals rather than from groups of animals. The results (Table 4) are in agreement with those reported in Table 2 with pooled livers: CDNB, DCNB, BSP and *t*-PBO activities were increased in treated animals. The t-BOOH activity that measures both GST and GSH peroxidase activity was depressed or unchanged.

In spite of the general agreement regarding the substrate specificities of the corresponding forms of GST in control and treated animals, it was found that ratios

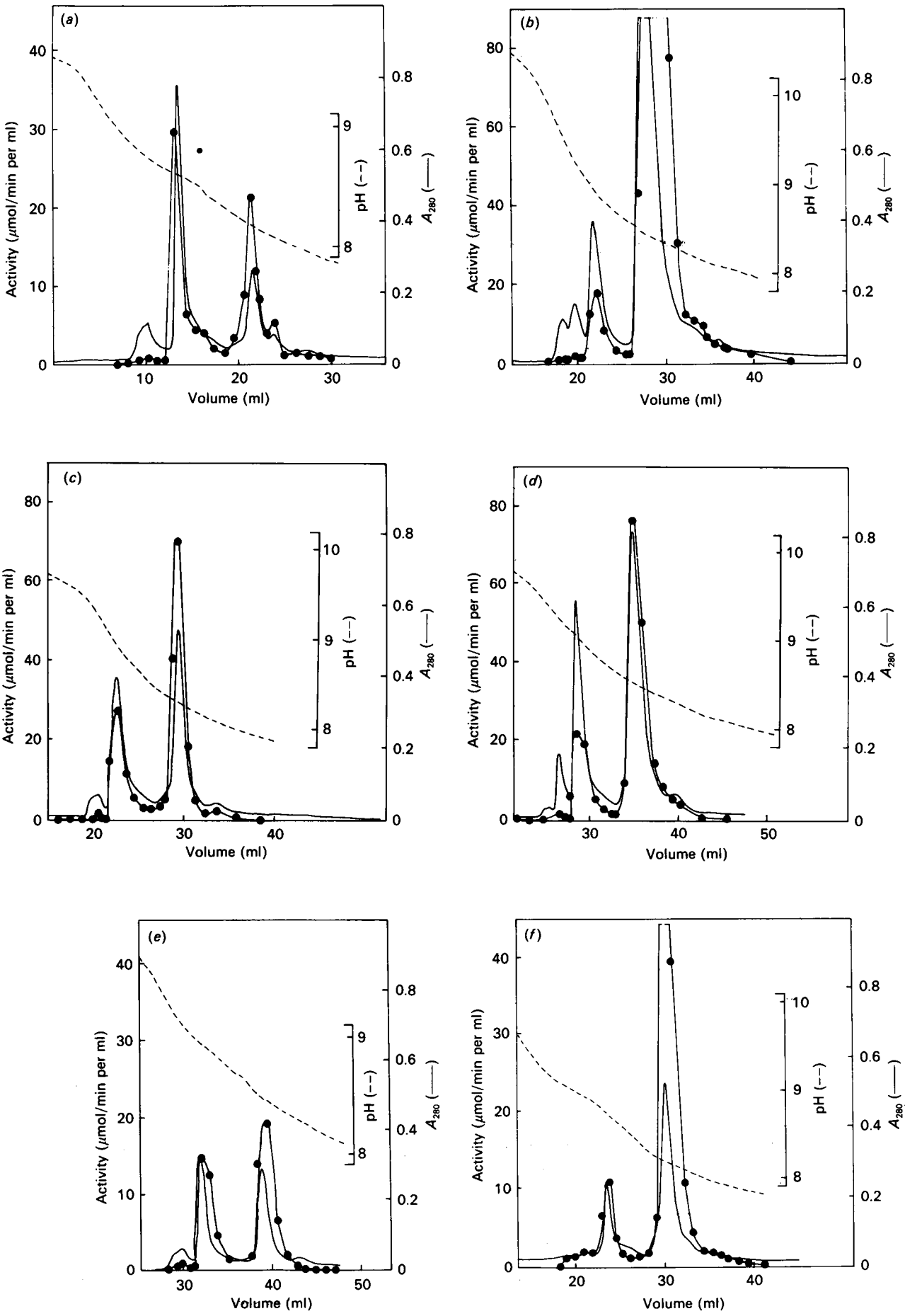
(a)
(b)
(c)
(d)
(e)
(f)
Activity (μmol/min per ml)
Volume (ml)
pH (--)
A_{280} (——)

Table 3. Percentage of total protein recovered in the three major GST-containing peaks eluted from the f.p.l.c. Mono P column

Protein concentration was estimated by integration of the A_{280} profile. See also Fig. 1. i.p., intraperitoneal.

Treatment	Route of administration	Peak...	Protein (% of total) I	II	III
Control*	-		11.3±2.8	48.7±3.4	31.1±4.4
CAF	Oral		4.9	24.8	60.3
PB	i.p.		6.4	40.3	45.6
PB	Oral		6.7	17.2	72.9
BHA	Oral		7.0	16.1	70.6
3-MC	i.p.		9.3	41.7	44.6
t-SBO	i.p.		7.1	31.0	60.0

* Means±S.D. of three groups of mice formed by 6, 2 and 6 animals.

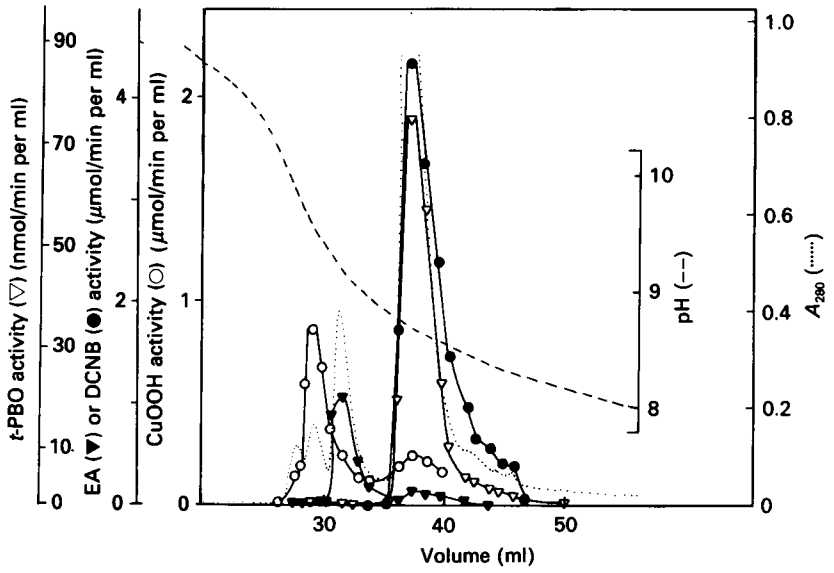

Fig. 2. Activity profile with specific substrates after chromatofocusing of hepatic cytosolic affinity-purified GSTs in BHA-treated mice

Substrates: ○, CuOOH; ▼, EA; ●, DCNB; ▽, *t*-PBO; ····, A_{280}; ----, pH.

between some activities were significantly changed after different treatments. In particular, in comparing the BSP (or DCNB) and *t*-PBO activities, the relative increase in the induced liver is much higher for *t*-PBO. This substrate is characteristic for class Mu (Mannervik *et al.*, 1985), and the expression of a new class Mu transferase is suggested.

Induction of other GSH-dependent enzymes

The activities of GSH-dependent enzymes other than GSTs were studied in the cytosol fraction after induction. In Table 5 the activities of glutathione reductase, glyoxalase I, glutathione peroxidase (Se-dependent) and thioltransferase are reported as percentages of the control values. Glyoxalase I and glutathione peroxidase activities were lower in all treated groups than in controls. The results obtained with glutathione reductase and thioltransferase showed an increase in activity after CAF, PB (oral) and BHA treatment, thus qualitatively reflecting the results of induction of GSTs.

Fig. 1. Chromatofocusing of hepatic cytosolic GST from mice treated with inducers

The samples (5–9 ml) separated by f.p.l.c. on a Mono P column were cytosolic fractions purified by affinity chromatography on *S*-hexylglutathione–Sepharose 6B. The enzyme activity was monitored with CDNB and the three peaks of activity were designated I, II and III in order of elution. —— A_{280}; ---- pH. (*a*) Control, sample containing 5.7 mg of protein; (*b*) BHA treatment, sample containing 14 mg of protein; (*c*) PB (intraperitoneal) treatment, sample containing 5.5 mg of protein; (d) *t*-SBO treatment, sample containing 6.1 mg of protein; (*e*) 3-MC treatment, sample containing 3.0 mg of protein; (*f*) CAF treatment, sample containing 3.8 mg of protein.

Table 4. GST activity assayed with different substrates in the cytosolic fraction of the liver after treatment with PB and BHA

Values are means ± S.D. of 4–9 animals per group.

Treatment	*n*	Substrate...	GST activity (μmol/min per g of liver)					
			CDNB	DCNB	BSP	*t*-PBO	EA	t-BOOH
Control	9		401 ± 88	4.92 ± 1.05	0.519 ± 0.091	0.060 ± 0.020	17.3 ± 3.3	73.5 ± 9.9
PB (2 mg/g, oral)	4		634 ± 30*	12.4 ± 1.4*	1.51 ± 0.24*	0.252 ± 0.044*	15.1 ± 1.8	45.6 ± 0.8*
BHA (4 mg/g, oral)	4		833 ± 107*	17.1 ± 2.4*	1.78 ± 0.16*	0.390 ± 0.122*	21.4 ± 3.3*	49.8 ± 8.8*
BHA (6 mg/g, oral)	6		1602 ± 264*,†	39.7 ± 7.5*	4.56 ± 0.87*,†	1.07 ± 0.31*,†	20.5 ± 3.2	71.0 ± 6.4†

* Statistically significant with respect to the control group; $P < 0.05$; Student's *t* test;
† Statistically significant with respect to the BHA 4 mg/g group; $P < 0.05$; Student's *t* test.

Table 5. Activities of hepatic cytosolic glutathione reductase (GR), glyoxalase I (GLY), glutathione peroxidase (Se-dependent; GSH-Px) and thioltransferase (TT) after treatment of mice with various inducers

Control values (nmol/min per mg) are reported in parentheses (means ± S.D. of three groups of mice formed by 6, 2 and 6 animals).

Treatment	Route of administration	Activity (% of control)			
		GR	GLY	GSH-Px	TT
Control	–	100 (55.2 ± 7.8)	100 (1160 ± 130)	100 (303 ± 50)	100 (7.32 ± 1.3)
CAF	Oral	152	52	72	135
PB	i.p.	91	86	79	95
PB	Oral	155	86	62	290
BHA	Oral	205	73	80	172
3-MC	i.p.	107	73	68	86
t-SBO	i.p.	110	88	75	82

DISCUSSION

The induction of GSTs in rodents has been studied previously. A common mechanism for several inducers of phase I and II detoxication enzymes has been proposed (Prochaska *et al.*, 1985*a*) and studies of the transcriptional activation of a GST gene have recently been performed (Telakowski-Hopkins *et al.*, 1988). In general, however, little is known about the effects of inducers on individual forms of GST. Limited information is available for mouse tissues (Benson *et al.*, 1978; Parchment & Benson, 1984; Adams *et al.*, 1987).

In the present investigation it was shown that after treatment of mice with several inducers, three major hepatic cytosolic GSTs previously characterized in normal male mice (Warholm *et al.*, 1986) are present. Moreover, the relative amounts of the different forms of enzyme change with treatment (Fig. 1). Qualitatively, the class Mu representative, MIII, was the form most affected by induction. By examination of the EA activity (see Tables 2 and 4), which is largely accounted for by MII, it can be concluded that this class Pi enzyme was essentially unaffected or somewhat down-regulated by the compounds used. It should be taken into account that MIII, which is clearly induced, also has activity with EA (Fig. 2). Likewise, the activities obtained with CuOOH, t-BOOH and H_2O_2 (Tables 2, 4 and 5; Fig. 2) show that the class Alpha representative, MI, is unaffected or down-regulated; the activity with H_2O_2 allows correction for the contribution of the Se-dependent GSH peroxidase. Thus, the major effect of the inducers is the elevation of the concentration of the class Mu GST MIII. This is evident from the chromatograms (Figs. 1 and 2) as well as from the activities with the class Mu characteristic substrates DCNB, BSP and *t*-PBO (Tables 2 and 4).

It has been found that BHA and CAF have anticarcinogenic activities (Wattenberg, 1983). Therefore, the present investigation shows that this effect, if due to GST induction, should be ascribed to an elevated concentration of class Mu GST. The finding that class Mu GSTs are preferentially induced is also notable for the reason that elevated levels of class Mu enzymes have been found in rat tumour cells to be associated with acquisition of resistance to 1,3-bis(2-chloroethyl)-1-nitrosourea (Smith *et al.*, 1989).

In contrast with the present studies with inducers, it has been found that in multi-drug-resistant mouse SEWA tumour cells, the class Pi GST is elevated by 2-fold (Dahllöf *et al.*, 1987). Also, in a mouse mastocytoma cell line, the class Alpha GST is down-regulated to an undetectable level (Söderstrom *et al.*, 1987). Thus the 'induction' of GSTs is complex, and qualitatively and

quantitatively distinct under different conditions. An extreme case of differential expression is found in rat ascites hepatoma cells, in which essentially all of the normally occurring hepatic GSTs are undetectable and are replaced by a class Pi GST not expressed in normal rat hepatocytes (Tahir *et al.*, 1989).

The effect of the inducers on GSH-dependent enzymes other than GSTs was a down-regulation of glyoxalase I and of Se-dependent GSH peroxidase (Table 5). The activities of glutathione reductase and thioltransferase were essentially unaffected or increased. Induction of glutathione reductase has previously been observed in the mouse (Cha *et al.*, 1982) and in the rat (Guthenberg *et al.*, 1980; Carlberg *et al.*, 1981). Decreased levels of Se-dependent GSH peroxidase were also observed by Carlberg *et al.* (1981).

In conclusion, inducers of drug metabolism have differential effects on the levels of various GSH-linked enzymes in an organism. It remains to quantitatively assess the major contributing factors affecting the GSH-dependent metabolism of xenobiotics and endogenous substrates.

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