Differential induction of class Alpha glutathione *S*-transferases in mouse liver by the anticarcinogenic antioxidant butylated hydroxyanisole

Purification and characterization of glutathione *S*-transferase Ya₁Ya₁

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A novel cytosolic Alpha class glutathione *S*-transferase (GST) that is not normally expressed in mouse liver was found to be markedly induced (at least 20-fold) by the anti-carcinogenic compound butylated hydroxyanisole. This enzyme (designated GST Ya₁Ya₁) did not bind to either the *S*-hexylglutathione–Sepharose or the glutathione–Sepharose affinity matrices, and purification was achieved by using bromosulphophthalein–glutathione–Sepharose. The purified isoenzyme, which comprises subunits of *M_r* 25600, was characterized, and its catalytic, electrophoretic, immunochemical and structural properties are documented. GST Ya₁Ya₁ was shown to be distinct from the Alpha class GST that is expressed in normal mouse liver and is composed of 25800-*M_r* subunits; the Alpha class isoenzyme that is constitutively expressed in the liver is now designated GST Ya₃Ya₃. Hepatic concentrations of GST Ya₃Ya₃ were not significantly affected when mice were treated with butylated hydroxyanisole. Both Pi class GST (subunit *M_r* 24800) and Mu class GST (subunit *M_r* 26400) from female mouse liver were induced by dietary butylated hydroxyanisole. By contrast, hepatic concentrations of microsomal GST (subunit *M_r* 17300) were unaffected.

INTRODUCTION

Dietary 2(3)-*t*-butyl-4-hydroxyanisole (BHA) and other phenolic antioxidants have been shown to protect rodents against chemically initiated carcinogenesis in certain target organs (Wattenberg, 1972, 1986; Wattenberg *et al.*, 1980). Inhibition of neoplasia is thought to be mainly a consequence of elevated cellular concentrations of certain phase II drug-metabolizing enzymes, which include the GSH-conjugating enzymes, glutathione *S*-transferases (GST, EC 2.5.1.18) (Benson *et al.*, 1978, 1979).

To understand the basis of this acquired resistance to chemical carcinogens, the effect of dietary BHA on the expression of GST has been studied in mouse and rat liver (Benson *et al.*, 1978). Although it is clear that dietary phenolic antioxidants elevate hepatic GST activity dramatically, the identification of the particular enzymes that are induced by BHA is not simple because GSTs represent a complex multi-gene family, comprising at least four distinct classes of protein designated Alpha, Mu, Pi and microsomal GST (for definitions see Mannervik *et al.*, 1985). In their early study Pearson *et al.* (1983) demonstrated a marked induction of hepatic Mu class GST after feeding mice with a diet containing BHA. More recently, Pearson *et al.* (1988) have extended their investigation and reported that hepatic concentrations of mRNAs that code for members of the three cytosolic GST gene families are increased after treating mice with BHA. The increases in hepatic Alpha class, Mu class and Pi class mRNAs were 50-fold, 15-fold and

2–5-fold respectively. Hence Pearson *et al.* (1983, 1988) have revealed a remarkable induction of Alpha and Mu class GST message by BHA in the mouse. To date, Pearson and his colleagues have restricted their investigation of the Alpha and Pi class GST to cloning and Northern-blotting experiments and have not studied the inducible Alpha and Pi class GST enzymes as such. However, sequence analysis of the cDNA encoding the mouse Alpha class enzyme induced by BHA showed that it shared 95% amino acid sequence homology with the rat Ya subunit and 70% homology with the rat Yc subunit.

The BHA-inducible GSTs in the mouse have not been studied rigorously at the protein level. The relationship between the enzymes that are expressed constitutively and those expressed in BHA-treated animals is unclear. Hepatic cytosols from untreated female mice contain class Alpha GST (YaYa, subunit *M_r* 25800) and class Mu GST (YbYb, subunit *M_r* 26400) with a trace of class Pi GST (YfYf, subunit *M_r* 24800) (McLellan & Hayes, 1987). By contrast with females, male mice express much higher concentrations (approx. 10-fold greater) of the Pi class Yf subunit in the liver. Quantitatively, YfYf is the major hepatic GST in male mice but represents only a minor form in females. The BHA studies by Benson *et al.* (1978), as well as those by Pearson *et al.* (1983, 1988), were conducted with female, not male, mice, and when relating the data of these workers to others in the field the marked sex-specific expression of mouse GSTs should be borne in mind.

In the present paper we verify the BHA-mediated

Abbreviations used: GST, glutathione *S*-transferase; BHA, 2(3)-*t*-butyl-4-hydroxyanisole; G–Ag, glutathione–Sepharose 6B; HexG–Ag, *S*-hexylglutathione–Sepharose 6B; BSP–G–Ag, bromosulphophthalein–glutathione–Sepharose 6B.

induction of GST YfYf and YbYb mRNA reported by Pearson *et al.* (1988) at a protein level and demonstrate the differential induction of two distinct class Alpha GSTs, one of which is only expressed in measurable quantities in the livers of mice that have been fed with BHA. We describe the purification and characterization of this novel BHA-inducible class Alpha GST.

MATERIALS AND METHODS

Chemicals

S-Hexylglutathione was synthesized by the method of Vince *et al.* (1971). Androst-5-ene-3,17-dione was a gift from Dr. P. K. Stockman (Royal Infirmary, Edinburgh). All other chemicals were of analytical grade and readily available commercially.

Both glutathione-Sepharose 6B (G-Ag) and bromosulphophthalein-glutathione-Sepharose 6B (BSP-G-Ag) affinity matrices were purchased from Sigma Chemical Co., Poole, Dorset, U.K. The *S*-hexylglutathione-Sepharose 6B (HexG-Ag) gel was constructed by using the method of Mannervik & Guthenberg (1981).

Animals

Male and female BALB/c mice were purchased from Bantin and Kingman, Hull, U.K. Those of the 129/OLA strain were from Harlam and Olac, Oxon., U.K.

Animals were received at 7–9 weeks old and were acclimatized for about 1 week before being fed with a diet containing 0.75% (by wt.) of BHA as described by Pearson *et al.* (1983). Control animals were age-matched and were fed with the same diet (Rat and Mouse no. 3 Breeding Diet; SDS, Stetfield, Witham, Essex, U.K.) without the addition of BHA. The mice were fed with the BHA-containing diet for 14 days before they were killed in all experiments, except for those when the effect of BHA on GST concentrations over a period of time was examined. In this instance dietary BHA was given to female BALB/c mice for between 1 and 50 days.

Both control and BHA-treated mice were given food and water *ad libitum* and were killed by being placed in a chamber containing a toxic concentration of CO₂.

Buffers

The pH values of all buffers are quoted for the working temperature, which was either 4 °C or room temperature (20 °C). The buffers were as follows: buffer A, 200 mM-Tris/HCl buffer, pH 7.8, containing 200 mM-NaCl; buffer B, 10 mM-sodium phosphate buffer, pH 6.7, containing 0.2 mM-dithiothreitol; buffer C, 10 mM-Mes/NaOH buffer, pH 6.2, containing 0.2 mM-dithiothreitol; buffer D, 20 mM-sodium phosphate buffer, pH 7.4, containing 100 mM-NaCl.

Analytical

All enzyme assays were carried out at 37 °C. Assays with 1-chloro-2,4-dinitrobenzene as substrate were carried out in a Cobas Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, Herts., U.K.) essentially as described by Hayes & Clarkson (1982). Enzymic activity with androst-5-ene-3,17-dione was measured as described by Benson & Talalay (1976). Selenium-independent glutathione peroxidase activity for organic hydroperoxide was determined by using the method of Reddy *et al.* (1981). All other enzyme assays were performed as described by Habig & Jakoby (1981).

Protein determination for cytosolic material was by the method of Bradford (1976), adapted for use on a centrifugal analyser (A. F. Howie, personal communication). Protein in unsolubilized microsomal fractions was measured by the biuret method, also with the use of a centrifugal analyser.

Peptide mapping

Peptide mapping was carried out by the separation of peptides generated from digestion of proteins with *Staphylococcus aureus* strain V8 proteinase (Heiland *et al.*, 1976), by the use of reverse-phase h.p.l.c. techniques (Hayes, 1984). The freeze-dried peptides (from 0.5 mg of protein) were dissolved in aq. 0.1% (v/v) trifluoroacetic acid, and portions were resolved on a Waters μ Bondapak C₁₈ (10 μ m particle size) column (0.39 cm $\times$ 30.0 cm). The column was developed with a linear gradient of 0–56% (v/v) acetonitrile in aq. 0.1% (v/v) trifluoroacetic acid over a period of 60 min. The flow rate was 1 ml/min and peptides were detected by their absorbance at 214 nm.

Electrophoresis and immunoblotting

The methods used have been described in previous publications from this laboratory (Hayes & Mantle, 1986a,b). Gels for SDS/polyacrylamide-gel electrophoresis routinely contained 12% or 15% acrylamide (C_{Bis} 2.6%) for cytosolic and microsomal proteins respectively, unless otherwise stated.

Preparation of hepatic cytosols and microsomes

Subcellular fractionation was performed as described by McLellan & Hayes (1987).

Enzyme purification

Affinity chromatography. All affinity-chromatography steps were carried out at 4 °C. Both HexG-Ag chromatography and subsequent chromatography of non-adsorbed material on G-Ag were performed by using a similar method to that described by Hayes (1988). The non-adsorbed cytosolic protein, i.e. the flow-through fraction from the second affinity column (G-Ag), was concentrated by (NH₄)₂SO₄ precipitation and subjected to gel-filtration chromatography on 4.4 cm $\times$ 85.0 cm columns of Sephadex G-100 (see the Results section). The GST-containing pool from the Sephadex G-100 column was applied to a BSP-G-Ag column (1.6 cm $\times$ 15 cm) at a flow rate of 36 ml/h, and washed with buffer B (with dithiothreitol omitted) until all non-specifically bound material was removed. Adsorbed protein was eluted with 200 mM-Tris/HCl buffer, pH 9.0, containing 50 mM-GSH. Fractions (4 ml) were collected and protein concentrations and GST activity were measured.

Hydroxyapatite and cation-exchange chromatography. Hydroxyapatite chromatography with the use of open-column techniques was performed at 4 °C, whereas cation-exchange f.p.l.c. on a Mono S HR 5/5 column was carried out at room temperature (20 °C). These procedures are described in the Results section.

Hydroxyapatite chromatography on h.p.l.c. was performed at room temperature as described previously (McLellan & Hayes, 1987; Hayes *et al.*, 1987).

Table 1. Effect of dietary BHA on hepatic cytosolic GST activity

Measurements of activity towards 1-chloro-2,4-dinitrobenzene were carried out in duplicate. The within-batch coefficient of variation for this assay, performed with a centrifugal analyser, is less than 5%.

Mouse strain	Sex	Treatment	Activity towards 1-chloro-2,4-dinitrobenzene ($\mu\text{mol}/\text{min per mg}$)
129/OLA	Male	None	7.7
	Male	BHA	30.6
	Female	None	4.2
	Female	BHA	26.9
BALB/c	Male	None	8.1
	Male	BHA	24.3
	Female	None	4.3
	Female	BHA	24.6

Fig. 1. Electrophoretic analysis of cytosol preparations from BHA-treated and untreated mice

Hepatic cytosols (30 μg of protein each), from male and female mice of the 129/OLA strain that had been fed with either a BHA-containing or an additive-free diet, were analysed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). The gel was loaded as follows: lane 1, cytosol from untreated male mice; lane 2, cytosol from BHA-treated male mice; lane 3, cytosol from untreated female mice; lane 4, cytosol from BHA-treated female mice; lane 5, rat lung GST isoenzyme mixture [Y_f (M_r 24800), Y_b (M_r 26300) and Y_c (M_r 27500)].

Lithocholic acid binding

Cytosols (100 mg of protein) from control and BHA-treated female BALB/c mice were made 85% saturated with $(\text{NH}_4)_2\text{SO}_4$ at 4 °C. The protein pellets were resuspended in 3 ml of ice-cold buffer D, and applied to Sephadex G-75 (superfine grade) columns (1.6 cm $\times$ 90 cm) that had been equilibrated with the same buffer (4 °C). The columns were developed with buffer D at a flow rate of 13.8 ml/h. Fractions (3 ml) were collected and GST activity in the column eluates was measured. The GST-containing fractions from control and BHA-treated mice were pooled separately, and 0.5 μM -lithocholic acid containing 0.5 μCi of [^{14}C]lithocholic acid, as a solution in ethanol, was added to each. After being mixed and incubated for 30 min, each solution was applied to a Sephadex G-75 (superfine grade) column (1.6 cm $\times$ 90 cm) and the columns were developed with buffer D as described above. Column eluates were monitored for GST activity and radioactivity.

RESULTS

Comparison of hepatic cytosols from control and BHA-treated mice

The effect of dietary BHA on the GST complement was investigated in livers of males and females from two separate inbred strains of mice, BALB/c and 129/OLA. The hepatic cytosol from BHA-treated and untreated (control) animals ($n = 8$) of each strain and sex were examined by SDS/polyacrylamide-gel electrophoresis. This showed a marked increase in the intensity of two cytosolic polypeptides of M_r 25600 and M_r 26400. The induction of these two polypeptides was observed in both BALB/c and 129/OLA mice of both sexes and was accompanied in all instances by a dramatic increase in GST activity (Table 1). The 25600- M_r band was found to be essentially absent from control mice, whereas the 26400- M_r band was found to be expressed constitutively at moderately high concentrations (Fig. 1).

To permit the induction of the separate classes of mouse GST by BHA to be better understood, the hepatic GST enzymes from treated and untreated animals were

Fig. 2. Separation of HexG-Ag-affinity-purified hepatic GST from BHA-treated and untreated male BALB/c mice on hydroxyapatite h.p.l.c.

Affinity-purified GST (7.3 mg and 4.3 mg of protein from control and BHA-treated mice respectively), which had been dialysed against 10 mM-sodium phosphate buffer, pH 6.7, containing 0.2 mM-dithiothreitol, was subjected to chromatography on Bio-Gel HPHT. Protein was eluted from the hydroxyapatite column at 0.5 ml/min with a linear gradient of 10–350 mM-sodium phosphate buffer containing 0.2 mM-dithiothreitol and 0.4 mM- CaCl_2 throughout. Absorbance at 280 nm was monitored continuously. Panels (a) and (b) show the separation of GST from untreated and BHA-treated male BALB/c mice respectively. The identities of the GST isoenzymes in the eluates are indicated.

purified by affinity chromatography on HexG-Ag and separated into their classes by hydroxyapatite h.p.l.c.; this strategy has been used previously to isolate and quantify changes in expression of soluble mouse GST (McLellan & Hayes, 1987; Hayes *et al.*, 1987). In these experiments the livers from animals of each strain and sex were combined separately and used for enzyme purification. The separation on h.p.l.c. of the affinity-purified GST from BHA-treated and control male BALB/c mice is shown in Fig. 2. Hydroxyapatite h.p.l.c. showed that, after BHA treatment, Mu class GST represents the major enzyme(s) in the affinity-purified pool from both strains and sexes. The relative proportions of GST from male BALB/c control livers were estimated to be YfYf 70%, YaYa 10% and YbYb 20%, whereas after BHA treatment the proportions changed to YfYf 24%, YaYa 5% and YbYb 71%. Surprisingly, despite the report by Pearson *et al.* (1988), the class Alpha GST that is purified by HexG-Ag affinity chromatography did not appear to be induced by BHA in either male or female mice. Most significantly, it was found that the inducible 25600- M_r polypeptide did not bind to HexG-Ag. Attempts were made to purify this BHA-inducible polypeptide because it may represent the Alpha class GST cloned by Pearson *et al.* (1988). In this case the 25600- M_r polypeptide would provide the first evidence at the protein level of multiplicity in the Alpha class GST in the mouse.

Purification of a BHA-inducible GST with subunit M_r 25600

Cytosol (approx. 6.2 g of protein) from 56 g of liver from 30 female BHA-fed BALB/c mice was dialysed for 18 h at 4 °C against two changes, each of 2 litres, of

buffer A before being subjected to two separate affinity-chromatography steps on first HexG-Ag and then G-Ag, as described by Hayes (1988). Most of the inducible class Mu GST as well as YaYa and YfYf were removed from cytosol by HexG-Ag (McLellan, 1988).

The small amounts of these previously characterized GST that failed to bind to HexG-Ag and were eluted in the flow-through fractions were removed by G-Ag. Material that bound to this second affinity matrix was found to contain Ya (M_r 25800), Yb (M_r 26400) and Yf (M_r 24800) subunits; the putative Alpha class GST subunit (i.e. the 25600- M_r polypeptide) was not adsorbed on either of the above affinity matrices.

The non-adsorbed protein from the two affinity columns was made 90% saturated with $(\text{NH}_4)_2\text{SO}_4$ (4 °C). The precipitated material was harvested by centrifugation (20000 g for 30 min at 4 °C) and the pellet obtained was dissolved in a minimum volume of ice-cold buffer B. The re-solubilized protein was subjected to gel-permeation chromatography at 4 °C on a 4.4 cm $\times$ 85.0 cm column of Sephadex G-100 at 36 ml/h in buffer B. The protein with subunit M_r 25600 was found to be co-eluted with a peak of 1-chloro-2,4-dinitrobenzene-GSH-conjugating activity of M_r approx. 50000, consistent with it originating from a dimeric protein.

The fractions that contained GST activity were combined and applied to a column of BSP-G-Ag as described in the Materials and methods section. The adsorbed protein, which contained the putative GST comprising 25600- M_r subunits, was eluted from the affinity matrix with 50 mM-GSH in 200 mM-Tris/HCl buffer, pH 9.0. The affinity-purified protein was collected and dialysed for 18 h (4 °C) against two changes, each of 2 litres, of buffer B, and applied to a 1.6 cm $\times$ 55 cm column of

Fig. 3. Resolution of BSP-G-Ag-affinity-purified GST

The GSTs that had been isolated from cytosols from BHA-treated mice by BSP-G-Ag chromatography were subjected to chromatography on a hydroxyapatite column (1.6 cm × 55 cm) as described in the text. The flow rate was 13.8 ml/h and 4.6 ml fractions were collected. Absorbance at 280 nm (○), GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) (▲) and sodium phosphate concentrations (■) were measured. The pooled fractions are indicated by the horizontal bars.

hydroxyapatite at 4 °C. The column, which had also been equilibrated with buffer B, was washed with half a column volume of buffer B before being developed with a gradient of 10–300 mM-sodium phosphate buffer, pH 6.7, containing 0.2 mM-dithiothreitol. Four peaks of GST activity were eluted, at approx. 60 mM-, 105 mM-, 110 mM- and 135 mM-sodium phosphate, and were named peak I, peak IIa, peak IIb and peak III respectively (Fig. 3). These were pooled separately. SDS/polyacrylamide-gel electrophoresis showed that peaks I and III contained subunits with M_r 24800 (Yf-type) and M_r 26400 (Yb-type) respectively, whereas peaks IIa and IIb contained the novel 25600- M_r subunit as the major

component. Small amounts of the 25800- M_r subunits (i.e. constitutive Ya-type GST) were also observed in peaks IIa and IIb.

Final purification of the novel 25600- M_r subunit was achieved by using f.p.l.c. on a Mono S HR 5/5 cation-exchange column. The protein pools from peaks IIa and IIb were dialysed separately for 18 h at 4 °C against two changes, each of 2 litres, of buffer C before application to the Mono S HR 5/5 column, which had been equilibrated with buffer C at room temperature (20 °C). The column was developed with a linear salt gradient of 0–0.15 M-NaCl in buffer C over 1 h at a flow rate of 0.75 ml/min, followed by a linear gradient of 0.15–1.0 M-NaCl in buffer C over 15 min at the same flow rate. This resulted in the elution of one major peak of pure protein (Fig. 4), which comprised subunits with M_r 25600 from peaks IIa and IIb. This protein was found to be eluted in an identical position of the salt gradient for both peak IIa and peak IIb. Furthermore, analytical isoelectric focusing demonstrated that both had an identical pI value of > 9.3. The two pure proteins from peaks IIa and IIb are presumed to be identical. All analytical studies were performed on the purified protein from peak IIb. The purification scheme described above resulted in the isolation of 4.5 mg of pure GST (subunit M_r 25600) from 56 g of liver.

Fig. 4. Purification of a BHA-inducible GST with subunit M_r 25600

The protein in peak IIa and peak IIb obtained from hydroxyapatite chromatography was separately applied to a Mono S HR 5/5 cation-exchange column and subjected to f.p.l.c. as described in the text. The column was developed with a linear salt gradient of 0–0.15 M-NaCl in the running buffer over 1 h, followed by 0.15–1.0 M-NaCl over 15 min. The flow rate was 0.75 ml/min and 0.75 ml fractions were collected. Absorbance at 280 nm was monitored continuously. Chromatography of protein from peak IIb is depicted, and pooled fractions are indicated by the horizontal bar.

Identification and characterization of a class Alpha GST induced by BHA

The purified protein with subunit M_r 25600 was examined for cross-reactivity with antisera raised against various GSTs by Western blotting. No cross-reactivity was observed with anti-(mouse YbYb) IgG, anti-(mouse YfYf) IgG or anti-(rat YkYk) IgG, but it did cross-react weakly with anti-(mouse YaYa) IgG. Antiserum raised to rat YaYa (i.e. 'ligandin') was found to cross-react strongly with the 25600- M_r subunit, suggesting that this protein, which has not been purified before, is a GST belonging to the Alpha class.

The constitutive mouse GST YaYa was compared with the inducible class Alpha GST by peptide mapping by the use of reverse-phase h.p.l.c. techniques. The elution

Fig. 5. Peptide maps of mouse Alpha class subunits

Peptide mapping was performed with the use of reverse-phase h.p.l.c. techniques as described in the text. Panel (a) shows the peptide map of the constitutive mouse Ya subunit. Panel (b) shows the peptide map of the inducible 26500- M_r subunit.

pattern of peptides from the μ Bondapak C_{18} column showed significant differences between the two Alpha class GSTs (Fig. 5). Furthermore, antisera raised against the inducible 26500- M_r subunit cross-reacted only very weakly with the constitutive Alpha class GST. Similarly,

Fig. 6. Variable electrophoretic mobility of class Alpha GST subunits in polyacrylamide gels containing different concentrations of cross-linker

The mouse BHA-inducible GST (subunit M_r 25600) was analysed by electrophoresis in SDS/polyacrylamide gels where the resolving gel comprised (a) 12% (w/v) acrylamide containing 0.32% (w/v) NN' -methylenebisacrylamide or (b) 17.25% (w/v) acrylamide containing 0.1% (w/v) NN' -methylenebisacrylamide (Hayes & Mantle, 1986b). In both panels the GSTs were applied in the same order, which was as follows: lanes 1, rat Yk (M_r 25000); lanes 2, rat Yf (M_r 24800); lanes 3, mouse BHA-inducible subunit (M_r 25600); lanes 4, mouse constitutive Ya (M_r 25800); lanes 5, mouse Yb (M_r 26400). Subunit M_r values were estimated from the relative electrophoretic mobility of subunits in polyacrylamide gels containing 12% (w/v) acrylamide (C_{Bis} 2.6%).

a weak immunological cross-reactivity was also seen between anti-(mouse YaYa) IgG and the inducible 25600- M_r protein. This suggests that, although the proteins belong to the same gene family, their amino acid sequences must be sufficiently diverse to provoke the production of dissimilar sets of polyclonal antibodies in rabbits.

The BHA-induced Alpha class GST was shown to behave in a similar manner to rat Ya during SDS/polyacrylamide-gel electrophoresis in gels with either a low or a high relative concentration of NN' -methylenebisacrylamide cross-linker (C_{Bis} 0.6% or 2.6%). When the 25600- M_r subunit was analysed by SDS/polyacrylamide-gel electrophoresis in gels that contained 12% acrylamide and 0.32% cross-linker (C_{Bis} 2.6%) it had a slower anodal mobility than the Yf subunit (M_r 24800). However, when SDS/polyacrylamide-gel electrophoresis was performed with gels incorporating 17.25% acrylamide and 0.1% cross-linker (C_{Bis} 0.6%), the 25600- M_r subunit had a faster anodal

Table 2. Substrate-specificities of mouse BHA-inducible GST Ya₁Ya₁: comparison with other Alpha class GSTs

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; tPBO, *trans*-4-phenylbut-3-en-2-one; BSP, bromosulphophthalein; pNPA, *p*-nitrophenyl acetate; EA, ethacrynic acid; CH, cumene hydroperoxide; AD, androst-5-ene-3,17-dione; ENPOP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; N.D.R., no detectable rate; N.D., not determined.

GST	Specificity activity (μ mol/min per mg)								
	CDNB	DCNB	tPBO	BSP	pNPA	EA	CH	AD	ENPOP
Mouse Ya ₁ Ya ₁	3.14 $\pm$ 0.1	N.D.R.	N.D.R.	N.D.R.	0.70 $\pm$ 0.03	0.86 $\pm$ 0.03	1.06 $\pm$ 0.04	0.05 $\pm$ 0.005	N.D.R.
Mouse Ya ₃ Ya ₃ *	15.1	0.1	N.D.	0.01	0.01	0.1	12.7	0	N.D.
Rat YaYa†	96	0.11	0	0	1.32	0.4	3.6	2.4	N.D.
Rat YcYc†	51	0.06	0	0	0.58	3.5	11.7	0	N.D.

* Data for all substrates except androst-5-ene-3,17-dione are taken from McLellan & Hayes (1987). The datum for androst-5-ene-3,17-dione is taken from Hayes *et al.* (1987).

† Data for all substrates except cumene hydroperoxide and androst-5-ene-3,17-dione are taken from Hayes (1986). Data for cumene hydroperoxide and androst-5-ene-3,17-dione are taken from Hayes *et al.* (1987).

Fig. 7. Effect of dietary BHA on hepatic concentrations of individual mouse GSTs

The levels of expression of individual GSTs in livers from female mice that had been fed with BHA for various lengths of time were examined by Western blotting (Towbin *et al.*, 1979) with antisera raised against purified mouse and rat GSTs. Hepatic cytosols or microsomal fractions were isolated from either control (untreated) mice, where samples were taken at corresponding time intervals to those that were fed with BHA, or mice that had been treated with BHA for 2–29 days. The samples are named according to the time elapsed (days) since feeding with BHA was commenced. Protein (20 μ g or 100 μ g from cytosols or microsomal fractions respectively) was applied to SDS/polyacrylamide gels as follows: lane 1, the antigen against which the relevant antiserum was raised; lane 2, control, day 2; lane 3, control, day 8; lane 4, control, day 29; lane 5, control, day 50; lane 6, BHA-treated, day 2; lane 7, BHA-treated, day 4; lane 8, BHA-treated, day 6; lane 9, BHA-treated, day 8; lane 10, BHA-treated, day 12; lane 11, BHA-treated, day 16; lane 12, BHA-treated, day 20; lane 13, BHA-treated, day 29. After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose paper and the resolved cytosolic proteins [panels (a)–(e)] or microsomal proteins [panel (f)] were probed for cross-reactivity with antisera raised against (a) mouse $Y_{a_1}Y_{a_1}$, (b) rat $Y_{a_1}Y_{a_1}$, (c) mouse $Y_{a_3}Y_{a_3}$, (d) mouse $Y_{b_1}Y_{b_1}$, (e) mouse $Y_{f_1}Y_{f_1}$ or (f) rat microsomal GST.

mobility than Yf (Fig. 6). Both rat Y_{a_1} and rat Y_{k_1} subunits have been shown to behave in this fashion (Hayes & Mantle, 1986b). In either gel system, the constitutive mouse Y_{a_1} subunit has a slower mobility than the Yf subunit.

Because of the electrophoretic and immunochemical similarities between the rat Y_{a_1} subunit and the mouse BHA-inducible Alpha class GST subunit, we have designated the mouse 25600- M_r polypeptide as Y_{a_1} . The Alpha class subunit of M_r -25800, which is expressed constitutively in mouse liver, is now called Y_{a_3} .

Specific activities of the novel mouse GST $Y_{a_1}Y_{a_1}$ were determined for a variety of substrates (Table 2). For every substrate examined the conjugation rate was fairly

low. This is particularly apparent with the specific activity towards 1-chloro-2,4-dinitrobenzene when the value of 3.1 μ mol/min per mg is compared with the rates observed for constitutive mouse GST. Values of 175.5, 75.4 and 15.1 μ mol/min per mg have been reported for mouse $Y_{b_1}Y_{b_1}$, $Y_{f_1}Y_{f_1}$ and $Y_{a_3}Y_{a_3}$ respectively (McLellan & Hayes, 1987).

Effects of dietary BHA on mouse hepatic GST concentrations

A study of the effects of dietary BHA on GST concentrations over different periods of time was undertaken. Levels of GST activity towards 1-chloro-2,4-dinitrobenzene were measured in hepatic cytosols

Fig. 8. Effect of dietary BHA on the lithocholic acid-binding capacity of mouse GST

Mouse hepatic cytosols were subjected to gel filtration on Sephadex G-75 (superfine grade) as described in the text, and the GST-containing fractions were incubated with $0.5 \mu\text{M}$ -lithocholic acid containing $0.5 \mu\text{Ci}$ of $[^{14}\text{C}]$ lithocholic acid ($[^{14}\text{C}]\text{LCA}$). The cytosolic mixtures from (a) untreated mice and (b) BHA-treated mice were applied to Sephadex G-75 (superfine grade) columns ($1.6 \text{ cm} \times 90 \text{ cm}$) equilibrated with 20 mM -sodium phosphate buffer, pH 7.4, containing 100 mM -NaCl. Columns were developed with the equilibration buffer at a flow rate of 13.8 ml/h . Fractions (3 ml) were collected and radioactivity ($\square$) and GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) ($\blacktriangle$) were measured.

from female mice that had been fed with BHA for 1–50 days. Hepatic samples ($n = 5$) were analysed every day for the first 6 days, then every second day for a subsequent 14 days. Further samples were examined on days 29, 36, 43 and 50. Activity towards 1-chloro-2,4-dinitrobenzene was seen to increase after 2 days of BHA feeding from 3.0 to $9.9 \mu\text{mol/min per mg}$ of protein, then continue to rise until day 12, whereupon GST activity ceased to rise further, but remained elevated at about $20 \mu\text{mol/min per mg}$ of protein until day 50. Corresponding control samples taken at days 1, 8, 14, 29 and 50 showed little variation in GST activity over the same time interval.

The effect of dietary BHA on individual members of the mouse GST family, including the microsomal GST, over a period of 29 days was examined by Western blotting (Fig. 7). The blots show the rapid induction (within 2–4 days) by BHA of the Yb subunit, the Yf subunit and the novel Alpha class Ya_1 subunit, but little, if any, induction of the constitutive Ya_3 subunit. The samples probed with anti-(rat YaYa) IgG also show the induction of a class Alpha subunit, which is presumed to share identity with the $25600\text{-}M_r$ subunit described in this study. No corresponding increase in the concentrations of microsomal GST were observed over the time period of BHA feeding.

The effects of BHA on the lithocholic acid-binding capacity of the GST complement of mouse liver cytosol was investigated by using radiolabelled lithocholic acid and gel filtration. The elevation of GST activity in hepatic cytosol after BHA treatment was found to be accompanied by an increase in the amount of lithocholic acid bound over the GST fraction (Fig. 8). It is, however, as yet unclear which of the mouse hepatic GSTs is responsible for lithocholic acid binding.

DISCUSSION

The effects of dietary BHA on the GST phenotype of adult mice has been investigated, and it has been demonstrated that the concentration of a hitherto undescribed GST (subunit M_r 25600) is elevated in the hepatic cytosols of mice that have been treated with BHA. This novel isoenzyme has been purified and identified as belonging to the cytosolic Alpha class of GST. It is distinct from the constitutively expressed hepatic Alpha class GST, which is thought to be identical with MI described by Warholm *et al.* (1986) and has been described in previous publications from this laboratory where it has been called YaYa or P4 (McLellan & Hayes, 1987; Hayes *et al.*, 1987). The $25600\text{-}M_r$ subunit cross-reacted strongly with antisera raised to rat YaYa, more so than did the mouse Alpha class $25800\text{-}M_r$ GST subunit that is expressed constitutively in the liver. Accordingly, the novel $25600\text{-}M_r$ subunit has been designated Ya_1 , whereas the subunit that was formerly known as Ya has been renamed Ya_3 .

Mouse Ya_1Ya_1 appears to be more closely related to rat YaYa than is mouse Ya_3Ya_3 in other respects. The mouse subunit with M_r 25600 behaves more like rat Ya (M_r 25500) than mouse Ya_3 (M_r 25800) during SDS/polyacrylamide-gel electrophoresis; like rat Ya, mouse Ya_1 displays the same cross-linker-dependent electrophoretic mobility. In gels that contained 12% acrylamide and 0.32% (w/v) *NN'*-methylenebisacrylamide (C_{Bis} 2.6%) rat Ya and Yk and mouse Ya_1 and Ya_3 all had an anodal mobility that is less than that of the Yf subunit. However, in gel comprising C_{Bis} 0.6%, rat Alpha class Ya and Yk as well as mouse Ya_1 possess faster electrophoretic mobilities than Yf. By

contrast, the mobility of mouse Ya₃ was relatively unchanged by alterations in the degree of cross-linking of the resolving gel used during SDS/polyacrylamide-gel electrophoresis.

The specific activities of Ya₁Ya₁ towards several substrates were found to be somewhat lower than those reported for rat YaYa (Mannervik & Jensson, 1982; Hayes *et al.*, 1987). Mouse Ya₁Ya₁ did, however, display some activity towards cumene hydroperoxide (1.1 µg/min per mg) and androst-5-ene-3,17-dione (0.05 µg/min per mg), substrates towards which class Alpha GST have been shown to be active (Mannervik *et al.*, 1985). Comparison of the catalytic properties of GST Ya₁Ya₁ with those of mouse GSTs that have been purified by others (Lee *et al.*, 1981; Agius & Gidari, 1985; Warholm *et al.*, 1986; Hatayama *et al.*, 1986; Singh *et al.*, 1987) supports our contention that the mouse enzyme that we have purified with subunit M_r 25600 is a novel GST.

It is notable that mouse Ya₁ failed to be adsorbed on either of the commonly used affinity matrices for GST purification, HexG-Ag and G-Ag. Hayes (1988) has demonstrated that the Ya subunit from rat has a low affinity for HexG-Ag, a feature that may be common to this type of GST subunit. In the case of mouse Ya₁, a marginally lower affinity than rat Ya for HexG-Ag may manifest itself in complete failure to bind to the affinity matrix.

Pearson *et al.* (1988) have described the BHA-mediated induction of a class Alpha mRNA that would code for a protein sharing 95% amino acid sequence homology with rat Ya. This mRNA was found to be present in very low concentrations of control livers, but was increased 50-fold after mice had been treated with BHA. It is likely that Ya₁Ya₁ described in the present study is the class Alpha protein for which this BHA-inducible mRNA would translate. The two distinct class Alpha subunits from mouse hepatic cytosol that have been described in the present paper do not undergo concomitant induction by BHA. Dietary intake of this compound appears to have little effect on the concentrations of Ya₃Ya₃, whereas concentrations of Ya₁Ya₁, which are virtually undetectable in hepatic cytosols before BHA treatment, are considerably elevated after 2–4 days of feeding mice with BHA (Fig. 7). Owing to the lack of a perceptible basal level of expression of GST Ya₁Ya₁, it is not possible to be precise about the level of induction of this isoenzyme. Its concentration is estimated to be elevated more than 20-fold.

Daniel *et al.* (1987) have reported the sequence of a mouse Ya gene that is closely similar to but not identical with (96% amino acid sequence homology) the sequence described by Pearson *et al.* (1988). It is, as yet, unclear if the nucleotide sequence reported by Daniel *et al.* (1987) represents the code for Ya₃ or, alternatively, for a further mouse Ya that is highly homologous to Ya₁. The latter supposition is much more likely, as the immunochemical results and peptide-mapping experiments described in the present paper suggest that it is improbable that only trivial differences exist between Ya₁ and Ya₃.

An explanation for the fact that basal hepatic levels of expression of mouse Ya₁ are exceptionally low may be obtained from the work of Telakowski-Hopkins *et al.* (1988). This group of workers have reported that, in mouse hepatoma cells, a trans-acting protein(s) that is required for basal levels of transcription of the rat Ya gene, comparable with those seen in rat and human cells,

appears to be missing. It must be emphasized that these workers were studying rat, not mouse, GST genes, and at this stage it is unknown if the 5' promoting regions of mouse Ya₁ and rat Ya genes are similar. However, the lack of such a trans-acting factor for basal expression of the mouse Ya₁ gene would explain the virtual absence of this GST from untreated mouse liver.

During the present study the effects of dietary BHA on the other classes of GST isoenzymes have been examined. In accord with the report by Pearson *et al.* (1988), who described increases in mRNA from all three classes of cytosolic GST, both the class Pi GST and class Mu GST were found to be induced. Like mouse Ya₁Ya₁, the concentrations of these enzymes were substantially elevated within 2–4 days from commencing BHA treatment. It must be noted that Western-blotting experiments were performed with female mice, and although we have also shown that class Mu GSTs are induced by BHA in male mice (Fig. 2) it is still not clear if BHA has an effect on hepatic concentrations of Yf in males. It is possible that this subunit may be completely de-repressed in the livers of untreated male mice.

Concentrations of microsomal GST were unaffected by dietary BHA. Earlier studies have shown that GST activity is elevated in hepatic microsomal fractions from the mice fed with BHA (Morgenstern & Dock, 1982), but the present study demonstrates that the elevation of microsomal GST activity is not based on a detectable induction of the *N*-ethylmaleimide-activatable GST originally described by Morgenstern *et al.* (1982). To date there are no reports of exogenous compounds that are capable of inducing increased hepatic concentrations of the microsomal GST.

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