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Ethoxyquin-induced resistance to aflatoxin B_1 in the rat is associated with the expression of a novel Alpha-class glutathione *S*-transferase subunit, Yc_2, which possesses high catalytic activity for aflatoxin B_1-8,9-epoxide

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A purification scheme has been devised for two ethoxyquin-inducible Alpha-class glutathione *S*-transferases (GSTs) which possess at least 25-fold greater activity towards aflatoxin B_1 (AFB_1)-8,9-epoxide than that exhibited by the GSTs (i.e. F, L, B and AA) that have been described previously. These two enzymes are both heterodimers and both contain a subunit of M_r 25800. This subunit has been isolated from both of the GST isoenzymes and, after cleavage with CNBr, it has been subjected to automated amino acid sequencing. The primary structure of the M_r 25800 subunit revealed that it forms part of a subfamily of Alpha-class GSTs which possess closest identity (about 92%) with the Yc subunit of apparent M_r 27500, which is encoded by the recombinant cDNA clone pGTB42 [Telakowski-Hopkins, Rodkey, Bennett, Lu & Pickett (1985) J. Biol. Chem. **260**, 5820–5825]. As these two GSTs possess less than 70% sequence identity with the Ya_1 and Ya_2 subunits, both of M_r 25500, the constitutively expressed Yc subunit of M_r 27500 has been renamed Yc_1 and the ethoxyquin-inducible GST of M_r 25800 has been designated Yc_2. Using this nomenclature, the two GSTs with high activity for AFB_1-8,9-epoxide are Ya_1Yc_2 and Yc_1Yc_2. Although evidence suggests that induction of Yc_2 is responsible for the high detoxification capacity of livers from ethoxyquin-treated rats for AFB_1-8,9-epoxide, resistance towards AFB_1 may be multifactorial in this instance as dietary ethoxyquin also induces the Ya_1, Ya_2 and Yc_1 subunits about 2.2-, 10.9- and 2.7-fold respectively. Besides the induction of GST by ethoxyquin, activity towards AFB_1-8,9-epoxide is also elevated in the livers of neonatal rats and in livers that contain preneoplastic nodules. Western blotting experiments show that Yc_2 is not present in hepatic cytosol from adult rats fed on normal diets but is expressed in neonatal rat livers and in the livers of adult rats that contain preneoplastic nodules that have arisen as a consequence of consuming diets contaminated with AFB_1.

INTRODUCTION

Aflatoxin B_1 (AFB_1) is a naturally occurring hepatotoxin and hepatocarcinogen of major clinical significance. It is a mycotoxin produced by the mould *Aspergillus flavus* and is often found in cereal and other crops that are contaminated with *A. flavus* as a consequence of being incorrectly stored.

Throughout the Animal Kingdom, significant variations exist in the susceptibility of different species to AFB_1. Man and rats are sensitive to AFB_1, but mice, by contrast, can tolerate this mycotoxin. The toxicity of AFB_1 has been extensively studied in the rat and the necrotic and carcinogenic effects of AFB_1 have been well documented in this animal (Newberne & Butler, 1969). In man, the ingestion of AFB_1 has been reported to be responsible for an outbreak of hepatitis which resulted in the deaths of 106 out of the 397 patients (Krishnamachari *et al.*, 1975). Epidemiological evidence suggests that AFB_1 is responsible for the high incidence of liver cancer in certain regions of the world (Wogan, 1975; Peers *et al.*, 1976) and may also be involved in hepatic cirrhosis, kwashiorkor and Reye's syndrome (for a review, see Neal, 1987).

The toxicity of AFB_1 is a result of its being metabolized to AFB_1-8,9-epoxide, a reaction catalysed in the rat by cytochrome *P*-450 IIc (C. R. Wolf, D. J. Judah & G. E. Neal, unpublished work). Other cytochrome *P*-450 isoenzymes are responsible for the conversion of AFB_1 into the less toxic metabolites, AFQ_1, AFM_1 and AFP_1. Once formed, AFB_1-8,9-epoxide does not necessarily produce genotoxic or cytotoxic damage as it can also be inactivated through the formation of AFB_1-dihydrodiol, catalysed by epoxide hydrolase, or through the formation of an AFB_1–glutathione conjugate, catalysed by glutathione *S*-transferase (GST). These detoxification reactions are not of equal importance and in the rat the major biliary metabolite is the AFB_1–GSH conjugate (Degen & Neumann, 1978).

The sensitivity of rats to AFB_1 can be reduced substantially by pretreatment with anticarcinogenic agents. Such compounds include ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) (Cabral & Neal, 1983), butylated hydroxyanisole (Kensler *et al.*, 1986; Jhee *et al.*, 1988), β-naphthoflavone (Gurtoo *et al.*, 1985) phenobarbital (Lotlikar *et al.*, 1989) and oltipraz (Kensler *et al.*, 1987). All these chemoprotectors appear to confer resistance to AFB_1 through altering the expression of phase I and/or phase II drug-metabolizing enzymes (for reviews, see Wattenberg, 1985; Talalay, 1989; Hayes & Wolf, 1990). Ethoxyquin-induced changes in the metabolism of AFB_1 have been studied in greater detail than those produced by other anticarcinogenic agents. Mandel *et al.* (1987) showed that the dietary administration of ethoxyquin resulted in an approx. 3.5-fold increase in the capacity to detoxify AFB_1 to both AFQ_1 and AFM_1. These workers also reported a modest increase, after

Abbreviations used: GST, glutathione *S*-transferase; ethoxyquin, 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline; AFB_1, aflatoxin B_1; GPx, glutathione peroxidase.

ethoxyquin feeding, of 1.5- to 2-fold in the ability of rat liver microsomes to catalyse the formation of AFB_1-dihydrodiol. However, most significantly, Mandel *et al.* (1987) described a marked increase in the levels of AFB_1–GSH-conjugating activity of about 100-fold in rats fed on ethoxyquin-containing diets.

From the work of Mandel *et al.* (1987) it appears probable that the over-expression of a GST with high activity towards AFB_1-8,9-epoxide is the major factor responsible for the ethoxyquin-induced resistance to AFB_1. The identity of the GST involved in this resistance mechanism is unclear. Coles *et al.* (1985) have shown that the rat YaYa, YaYc and YcYc GSTs can detoxify activated AFB_1. Subsequently, Kensler *et al.* (1986) reported the marked induction of GST YaYa in the rat by ethoxyquin and these workers proposed that the over-expression of Ya represents the resistance mechanism to AFB_1. However, it is now recognized that rat liver normally expresses two distinct Ya subunits, designated Ya_1 and Ya_2 (Hayes *et al.*, 1990), and it is not known whether these constitutive GST subunits are responsible for the increased AFB_1–GSH-conjugating activity or whether an additional novel enzyme is involved.

In the present study we demonstrate that dietary ethoxyquin causes the expression of an Alpha-class Yc-type GST subunit, designated Yc_2, that is not normally found in adult rat liver. The Yc_2-containing GST possesses substantially greater activity for AFB_1-8,9-epoxide than other GSTs. It is therefore probable that the de-repression of Yc_2, rather than the induction of Ya_1 or Ya_2, represents the major resistance mechanism to AFB_1.

MATERIALS AND METHODS

Chemicals and chromatography materials

Ethoxyquin was obtained from Sigma Chemical Co., Poole, Dorset, U.K. The Whatman DEAE-cellulose and CM-cellulose ion-exchangers were purchased from Chromatography Services, Wirral, Merseyside, U.K., and hydroxyapatite was from Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K. The μ-Bondapak C_{18} reverse-phase column (10 μm particle size; 0.39 cm × 30 cm), the Protein PAK Glass 200 SW filtration column (10 μm particle size; 0.8 cm × 30 cm) and the Protein PAK Glass SP-SPW cation-exchange column (10 μm particle size; 0.8 cm × 7.5 cm) were purchased from Waters Chromatography Division, Millipore (U.K.), Watford, Herts., U.K. The h.p.l.c.-grade solvents were obtained from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K. The high-purity water, employed for h.p.l.c. analysis of AFB_1 metabolites, was from a Milli-Q cartridge system (Millipore). All other chemicals were purchased from Macfarlane Robson, Thornliebank, Glasgow, Scotland, U.K.

Animals

Fischer 344 rats were used throughout this study and were bred on site at the MRC Toxicology Unit. Rats were fed on either a normal diet (powdered MRC 41B rat diet supplemented with 2% arachis oil), an ethoxyquin-containing diet or an AFB_1-containing diet, as described previously (Mandel *et al.*, 1987; Neal *et al.*, 1987). For preparative purposes, the livers of 150 g male F344 rats were used. For analytical purposes, GST expression in the livers from F344 rats of various ages was investigated; the ages of the animals studied are described in the Results section.

Enzyme assays

Activation of AFB_1 was achieved using quail liver microsomes and was based on published methods (Moss *et al.*, 1983), although GST availability imposed the following variations. Incubations each contained the equivalent of potassium phosphate buffer at pH 7.4 (20.3 μmol), $MgCl_2$ (1.27 μmol), NADP (0.11 μmol), glucose 6-phosphate (1.58 μmol) and glucose-6-phosphate dehydrogenase (0.08 unit type XV from baker's yeast) and water estimated to give a final incubation volume of 244 μl. These common ingredients were incubated at 37 °C for 5 min before GSH (12.7 μl containing 79.3 μmol) and AFB_1 (2.54 μl containing 11 nmol) were added to each incubation mixture. Finally, a 20 μl suspension of quail liver microsomes (equivalent to 12.5 mg of protein) and the GST source (the volume of which varied from 10 to 50 μl) were added to the assay constituents described above to give a final volume of 244 μl. The enzymic reaction was allowed to proceed at 37 °C for 10 min and was terminated by the addition of 1 ml of ice-cold methanol. Precipitated material was removed by centrifugation (45 min, −20 °C, 1500 *g*) before the reaction mixtures were dried in a Savant vacuum concentrator (Stratech Scientific, Luton, Beds., U.K.), to maximize recoveries, and stored at 4 °C until assayed. The residues were dispersed in 200 μl of 0.1 M-HCl/methanol (1:1, v/v) and the samples were clarified by centrifugation (45 min, −20 °C, 1500 *g*) before 20 μl portions were subjected to automated h.p.l.c. analysis. The chromatographic method is essentially as described by Moss *et al.* (1983) except a reverse-phase C_{18} glass cartridge system (0.3 cm × 10 cm; particle size 8 μm) (Chrompack, London, U.K.) was used for improved mass sensitivity. Chromatographic data were acquired on an IBM-compatible computer using the PC Integrator Pack (Kontron, Watford, Herts., U.K.). Fluorescence data for the conjugate were individually normalized against the u.v. and fluorescence response for unmetabolized AFB_1 to compensate for fluctuation with temperature using Quattro Professional (a spreadsheet program from Borland U.K., which was generously given by the company's agents) to process files generated by the acquisition software.

GST activity towards 1-chloro-2,4-dinitrobenzene was carried out at 37 °C using a centrifugal analyser (Hayes & Clarkson, 1982) and oxosteroid isomerase activity of GST for Δ^5-androstene-3,17-dione was measured at 37 °C using a manual method (Habig & Jakoby, 1981).

Glutathione peroxidase activity towards cumene hydroperoxide was determined at 37 °C by the method of Paglia & Valentine (1967), as adapted for use on a centrifugal analyser (Howie *et al.*, 1990). To allow the effect of ethoxyquin on both the selenium-dependent glutathione peroxidase (GPx) and on the selenium-independent glutathione peroxidases (i.e. GST) to be measured separately, GPx and GST were resolved by gel-filtration chromatography. This was achieved by subjecting portions of cytosol (0.1 ml, 1.4 mg of protein) to f.p.l.c. on a Protein PAK Glass 200 SW filtration column in a Waters 650E Advanced Protein Purification System; using this system GPx and GST were eluted at 9.3 and 10.8 ml respectively.

Protein assay

This was carried out by the dye-binding method of Bradford (1976).

Purification of GST

The first purification step involved anion-exchange chromatography on columns (4.4 cm × 90 cm) of DEAE-cellulose equilibrated with 10 mM-Tris/HCl buffer, pH 8.1. The GST activity towards AFB_1-8,9-epoxide in the hepatic 10000 *g* supernatant from ethoxyquin-treated rats failed to bind to this anion-exchanger and the flow-through fractions were collected and the protein was concentrated by $(NH_4)_2SO_4$ precipitation (for an overview, see Scheme 1).

The precipitated material was dissolved in a minimal volume of 10 mM-sodium phosphate buffer, pH 6.7, which contained

1 mM-dithiothreitol, and was dialysed against six changes, each of 2 l, of the same buffer. After dialysis, the precipitated protein was removed by centrifugation (30 min, 10000 *g*). The resulting supernatant was applied to a column (3.2 cm × 90 cm) of CM-cellulose which was equilibrated with 10 mM-sodium phosphate buffer, pH 6.7, and developed with a linear gradient of 0–120 mM-NaCl, formed in a 1-litre mixing reservoir. The eluate from this column was assayed for peroxidase activity towards cumene hydroperoxide and for GST activity towards both 1-chloro-2,4-dinitrobenzene and AFB_1-8,9-epoxide. Previous work has demonstrated that GSTs are eluted from CM-cellulose in the order, D (Yb_2Yb_2), F (Ya_1/Ya_2 dimer), L (Ya_1/Ya_2 dimer), C (Yb_1Yb_2), B (a hybrid between Ya_1/Ya_2 and Yc), A (Yb_1Yb_1) and, finally, AA (YcYc) [for further details about purification schemes and nomenclature, see Hayes (1983), Sheehan & Mantle (1984), Hayes & Mantle (1986*a*) and Hayes *et al.* (1990)].

Electrophoresis

SDS/PAGE, used to identify GST subunits, was performed by the method of Laemmli (1970). The resolving gels employed 12% polyacrylamide which contained 0.32% *NN*-methylene-bisacrylamide [see Hayes & Mantle (1986*c*) for a description of the effect of cross-linker concentration on the electrophoretic mobility of GST].

Western blotting

The protocol used to transfer GST electrophoretically to nitrocellulose paper and to visualize immunoreactive subunits has been described elsewhere (Hayes & Mantle, 1986*b*). The method by which antibodies against purified rat and mouse GST were obtained has been reported previously (Hayes & Mantle, 1986*b*; McLellan & Hayes, 1989).

Reverse-phase h.p.l.c.

The method and equipment used to resolve and quantify GST subunits [devised originally by Ostlund Farrants *et al.* (1987)] has been described elsewhere (Hayes *et al.*, 1990).

Amino acid sequencing

This was performed on purified CNBr fragments of the Yc_2 GST subunit using an Applied Biosystems (Warrington, Cheshire, U.K.) 477A instrument with a 120A on-line phenylthiohydantoin analyser as described previously (Hayes *et al.*, 1989).

RESULTS

Induction of GST activities in ethoxyquin-treated rats

Previous work has demonstrated that the chemoprotector, ethoxyquin, can produce a substantial increase in the ability of rat liver to conjugate GSH with activated AFB_1 (Mandel *et al.*, 1987). In an attempt to establish whether this hepatic increase in capacity to detoxify activated AFB_1 is due to the induction of Alpha-, Mu- or Pi-class GSTs, the increase in enzyme activity towards model substrates that display selectivity for different GST subunits was investigated. Table 1 shows the level of GST activity and peroxidase activity in cytosols from the livers of control rats and rats administered dietary ethoxyquin. Comparisons of the hepatic enzyme activities between the two groups of rat reveal substantial differences. In this experiment, dietary ethoxyquin produced an increase of about 6-fold in GST activity towards AFB_1-8,9-epoxide. By contrast, a smaller increase, of only 4-fold, in activity towards the general substrate 1-chloro-2,4-dinitrobenzene was observed. Ethoxyquin produced an elevation of 2.2-fold and 1.5-fold in activity towards 1,2-dichloro-4-nitrobenzene and *trans*-4-phenyl-3-buten-2-one respectively, indicating that the induction of neither the Yb_1 nor the Yb_2 subunit is responsible for the increase in GST activity towards AFB_1-8,9-epoxide. The ethacrynic acid–GSH-conjugating activity also failed to be induced to the same extent as the AFB_1-8,9-epoxide–GST activity; the GST activity towards ethacrynic acid was elevated about 2.1-fold but this compound is not specific and serves as a substrate for Yc (class Alpha), Yf (class Pi) and Yk (class Alpha) subunits (Hayes, 1986).

A closer examination of the effects of ethoxyquin on the activity of the Alpha-class GST was made with substrates that are specific for this family, because of the report by Coles *et al.* (1985) that these subunits are able to detoxify activated AFB_1. It was found that the total hepatic glutathione peroxidase activity towards cumene hydroperoxide was marginally reduced in rats fed ethoxyquin-containing diets. However, when this activity was divided, by gel-filtration chromatography, into that contributed by the selenium-dependent peroxidase (GPx) and that contributed by the selenium-independent peroxidase (Alpha class GST), it was found that the ethoxyquin treatment resulted in GPx activity being reduced to 44% of control levels, whereas a 2.8-fold increase in Alpha-class GST peroxidase activity was observed in rats administered dietary ethoxyquin. The specific activity towards Δ^5-androstene-3,17-dione, which is highly selective for the Ya_1 and/or the Ya_2 subunit (Benson *et al.*, 1977; Beale *et al.*, 1982), was increased by ethoxyquin treatment to a similar extent (2.9-fold) as the selenium-independent glutathione peroxidase activity. These results indicate that the suggestion of Kensler *et al.* (1986) that the over-expression of Ya subunits (presumably Ya_1 and/or Ya_2) is responsible for the marked increase in GST activity towards AFB_1-8,9-epoxide may not be entirely correct. Our results in Table 1 would favour the hypothesis that the dietary administration of ethoxyquin stimu-

Table 1. Levels of GST activity in hepatic cytosol from control and ethoxyquin-treated Fischer 344 rats

The 100000 *g* supernatant fraction was prepared from the livers of rats fed on control or ethoxyquin-containing diets. All analyses were performed at 37 °C. All results are expressed as means ± S.D. for four determinations. Peroxidase activity with cumene hydroperoxide was resolved into the selenium-dependent (GPx) and the selenium-independent forms (non-GPx) by gel-filtration chromatography, as described in the text. Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; tPBO, *trans*-4-phenyl-3-buten-2-one; EA, ethacrynic acid; Δ^5-Add, Δ^5-androstene-3,17-dione; CuOOH, cumene hydroperoxide.

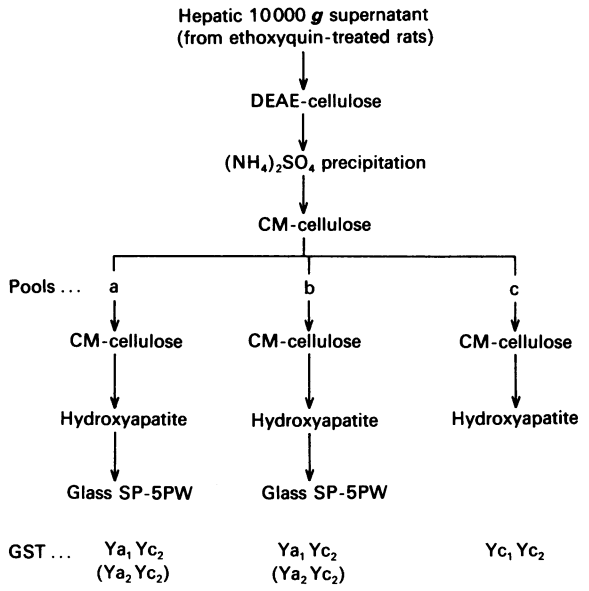
	Specific activity (nmol/min per mg of protein)								
	AFB_1-8,9-epoxide	CDNB	DCNB	tPBO	EA	Δ^5-Add	CuOOH (total)	CuOOH (GPx)	CuOOH (non-GPx)
Control	0.0247 ± 0.0009	1376 ± 77	52.7 ± 4.5	11.6 ± 1.2	44.1 ± 3.8	14.3 ± 0.3	1097 ± 8	920 ± 15	175 ± 8
Ethoxyquin-treated	0.1302 ± 0.0027	5419 ± 44	117.3 ± 3.3	16.9 ± 0.8	91.2 ± 3.2	41.9 ± 0.7	902 ± 7	406 ± 9	496 ± 11

lates the synthesis of a hepatic GST, which exhibits a high specific activity for AFB_1-8,9-epoxide but is not normally expressed in adult rat liver.

Evidence that novel inducible GSTs are involved in the detoxification of activated AFB_1

The GST-purification protocol which was undertaken to establish whether ethoxyquin treatment induces a novel isoenzyme(s) with high detoxification capacity for activated AFB_1 is summarized in Scheme 1. The method employed is adapted from the original GST-purification scheme of Habig *et al.* (1974) as described later by Hayes (1983). These earlier protocols were used to isolate GST from normal rat liver. However, during the present study it was necessary to employ two CM-cellulose steps as well as an additional cation-exchange f.p.l.c. step because of the relatively larger amounts of GST present in liver cytosol which resulted from administering dietary ethoxyquin; it proved necessary to process relatively large amounts of liver because the GSTs with high detoxification capacity for activated AFB_1 are not abundant enzymes. Scheme 1 indicates that three fractions of GST activity towards AFB_1-8,9-epoxide, pools a, b and c, were obtained using the purification method. These three pools were prepared in parallel and evidence suggests that pools a and b are interconvertible whereas pool c is separate (see below for further details). The catalytic properties of the pools that were collected during the course of the purification are shown in Table 2.

On the basis of their relative activities for cumene hydroperoxide and 1-chloro-2,4-dinitrobenzene it can be concluded that the enzyme-containing peaks shown in Fig. 1 eluted at fractions 69, 91, 101, 114, 135, 143 and 160 represent GST D, F, L, C, B, A and AA respectively. By contrast with the large number of peaks of chlorodinitrobenzene and cumene hydroperoxide activity that were eluted from CM-cellulose, the GST activity towards AFB_1-8,9-epoxide was eluted in only three major peaks which were designated, according to their elution order, as pools a, b and c. The most important feature of the column profile shown in Fig. 1 is that the GST activity for AFB_1-8,9-epoxide is not co-eluted with the GST activity for chlorodinitrobenzene. For example, pool a was eluted with the descending fractions of GST C, pool b was eluted with the ascending fractions of GST B and pool c was eluted immediately before GST AA. These data suggest that the enzymes that detoxify AFB_1-8,9-epoxide are distinct from the forms described previously in adult rat liver.



Scheme 1. GST-purification protocol

Table 2. Summary of the purification of GST with high activity towards AFB_1-8,9-epoxide

GST purification was undertaken from approx. 350 g of liver from Fischer 344 rats which had been fed on an ethoxyquin-containing diet for the 5 days before being killed. It should be noted that by contrast with the analytical experiment shown in Table 1, in which 100000 *g* supernatants were studied, the purification of AFB_1-metabolizing GST utilized hepatic post-mitochondrial supernatants (i.e. 10000 *g* supernatants) as enzyme source. For details of the chromatography steps and enzyme assays, see the text and also Scheme 1. The specific activities for 1-chloro-2,4-dinitrobenzene (CDNB) and cumene hydroperoxide (CuOOH) are expressed as μmol/min per mg of protein. The specific activities for AFB_1-8,9-epoxide are expressed as nmol/min per mg of protein. The percentage yield of the different activities during the purification are shown in parentheses.

Purification step	Total protein (mg)	Specific activity		
		CDNB	CuOOH	AFB_1-8,9-epoxide
Rat liver 10000 *g* supernatant	34200	3.9 (100)	1.1 (100)	0.04 (100)
DEAE-cellulose flow-through	8328	14.6 (92)	4.8 (110)	0.19 (120)
$(NH_4)_2SO_4$ precipitate	7072	12.0 (65)	4.7 (92)	0.09 (49)
CM-cellulose (CM-52)				
Pool a	325	18.8 (4.6)	1.3 (11)	0.61 (15)
Pool b	463	11.8 (4.2)	2.7 (3.4)	0.23 (8.2)
Pool c	101	7.3 (0.6)	2.1 (0.6)	0.86 (6.8)
Second CM-52				
Pool a	38	33.2 (0.1)	3.5 (0.4)	4.84 (14)
Pool b	153	15.7 (1.8)	5.0 (2.1)	0.31 (3.7)
Pool c	14	23.0 (0.2)	3.8 (0.1)	3.82 (4.2)
Hydroxyapatite				
Pool a	7.2	18.7 (0.1)	7.4 (0.1)	12.10 (6.8)
Pool b	6.1	28.4 (0.1)	8.8 (0.1)	2.06 (1.0)
Pool c	2.3	13.7 (0.02)	15.5 (0.1)	6.30 (1.1)
SP-5PW h.p.l.c.				
Pool a	1.29	24.1 (0.02)	7.5 (0.03)	10.4 (1.0)
Pool b	0.2	27.0 (0.004)	8.0 (0.004)	6.2 (0.1)

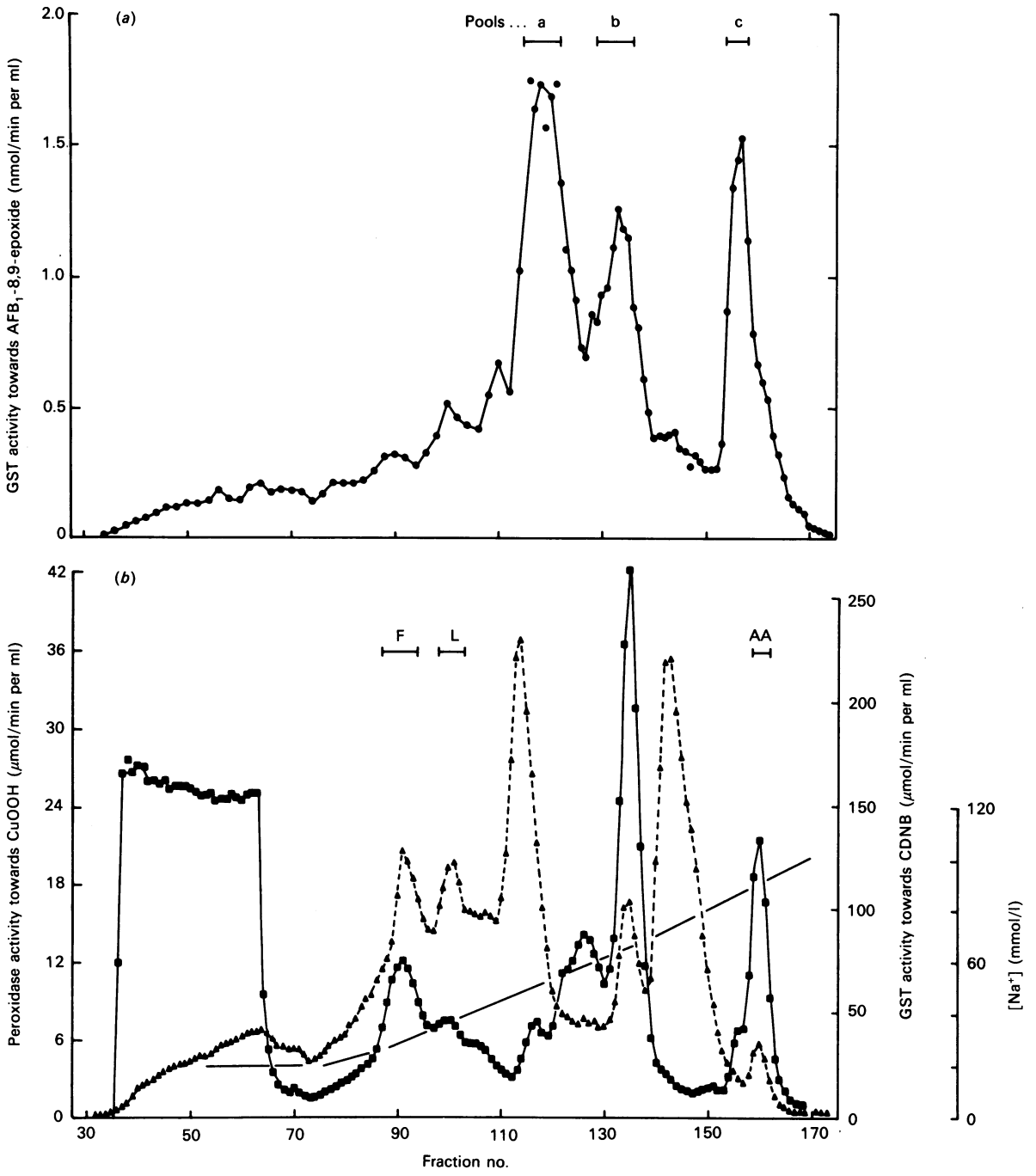


Fig. 1. Cation-exchange chromatography of hepatic GSTs from rats administered dietary ethoxyquin

The basic GSTs, from the livers of Fischer 344 rats that had been fed on an ethoxyquin-containing diet, were obtained by DEAE-cellulose chromatography (see Scheme 1). The flow-through fractions from the anion-exchanger were collected, concentrated by $(NH_4)_2SO_4$ precipitation and, after dialysis against 10 mM-sodium phosphate/1 mM-dithiothreitol, pH 6.7, the material was applied to a column (3.2 cm × 90 cm) of CM-cellulose. This column was eluted at 36.4 ml/h with 10 mM-sodium phosphate/1 mM-dithiothreitol, pH 6.7, and was developed with a 0–120 mM-NaCl gradient, formed in the running buffer. Fractions of 9.1 ml were collected and GST activity towards both 1-chloro-2,4-dinitrobenzene (CDNB) (▲) (*b*) and AFB_1-8,9-epoxide (●) (*a*) was measured as was peroxidase activity with cumene hydroperoxide (CuOOH) (■) (*b*). Sodium concentration in the fractions was determined by flame photometry and the gradient obtained is depicted by the straight line. The horizontal bars shown in (*a*) represent the fractions with high activity for AFB_1-8,9-epoxide which were combined (pool a, fractions 115–122; pool b, fractions 129–136; pool c, fractions 154–158). The horizontal bars shown in (*b*) indicate the fractions that were used for purification of Alpha-class GST F, L and AA during the present study; GST B was purified from pool b (fractions 129–136).

Isolation of GSTs in pools a, b and c that metabolize AFB_1-8,9-epoxide

Pools a, b and c were each combined as shown in Fig. 1 and, after dialysis against 10 mM-sodium phosphate buffer, pH 6.7, containing 1 mM-dithiothreitol (four changes, each of 2 l), they were reapplied to CM-cellulose to help remove contaminating GSTs C, B and AA from the three preparations. The second CM-cellulose step was performed using 1.6 cm × 45 cm columns equilibrated with 10 mM-sodium phosphate/1-mM-dithiothreitol buffer, pH 6.7, and developed with a linear 0–100 mM-NaCl gradient formed in the running buffer. Fig. 2 shows the elution profile of pool a from CM-cellulose and demonstrates that

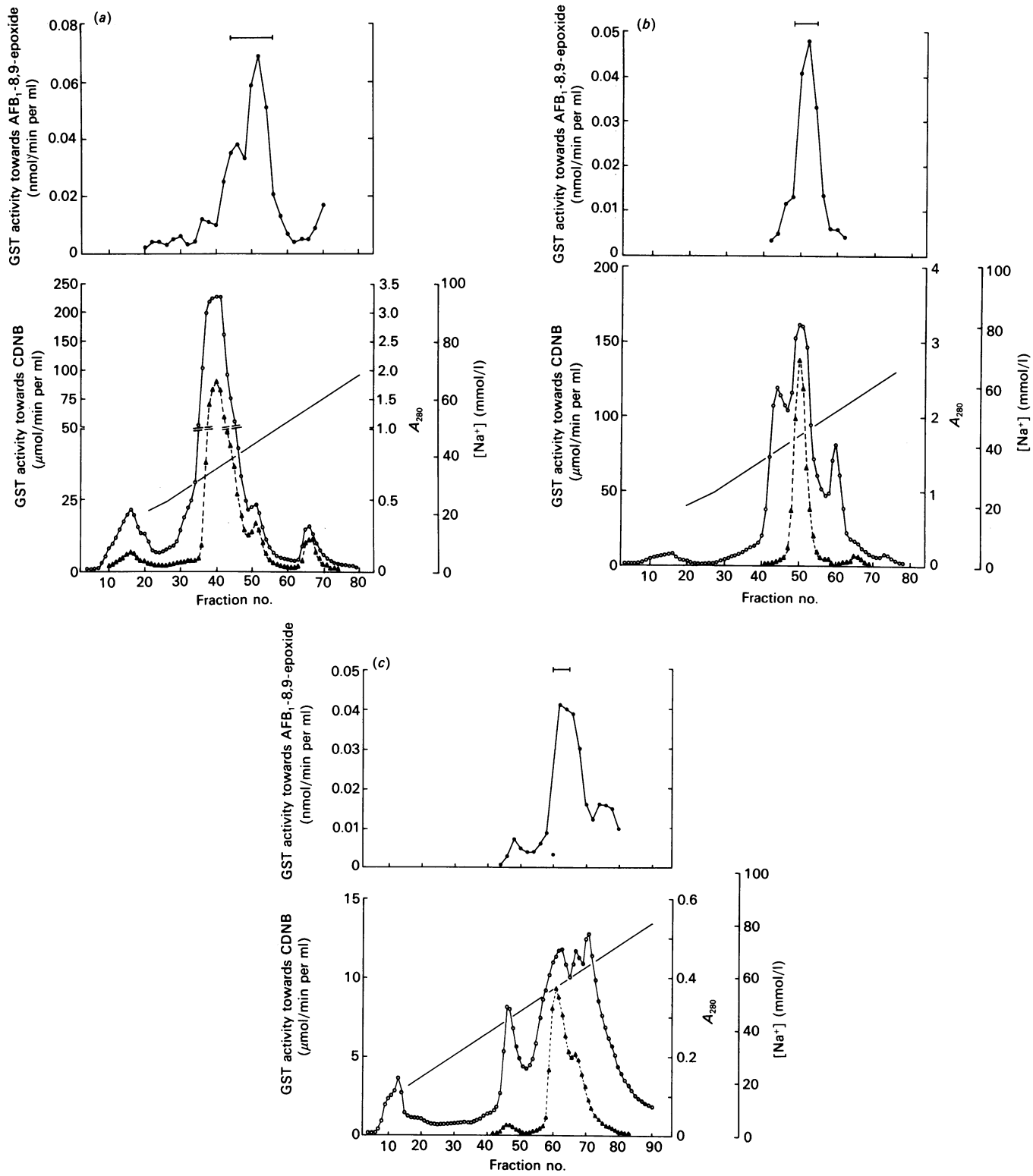


Fig. 2. Elution of AFB_1-metabolizing GSTs from CM-cellulose

The three pools of GST with high activity for AFB_1-8,9-epoxide, which were combined as shown in Fig. 1, were dialysed against 10 mM-sodium phosphate/1 mM-dithiothreitol, pH 6.7, before being applied to columns (1.6 cm × 45 cm) of CM-cellulose. These columns were eluted at 19.8 ml/h with the same buffer used for dialysis and were developed with a 0–100 mM-NaCl gradient. Fractions of 6.6 ml were collected and the absorbance at 280 nm (○) and the enzyme activities towards 1-chloro-2,4-dinitrobenzene (CDNB) (▲) and AFB_1-8,9-epoxide (●) measured. The elution profiles obtained from pools a, b and c are presented in (*a*), (*b*) and (*c*) respectively. The horizontal bars represent those fractions that were combined.

this repetitive step removed a substantial amount of GST C (eluted in fractions 37–43) from the AFB_1-8,9-epoxide–GSH-conjugating activity which was eluted in fractions 44–56. During this second CM-cellulose step the GST activity towards AFB_1-8,9-epoxide in pools a and b was eluted from the cation exchanger at closely similar positions suggesting that these two

preparations are not distinct but represent interconvertible forms. Whereas the AFB_1-metabolizing GSTs in both pools a and b were eluted from the second CM-cellulose column at a Na^+ concentration of 43 mM (suggesting that pool a had acquired the chromatographic properties of pool b), the AFB_1-metabolizing GST in pool c retained its unique chromatographic properties and was eluted from this second CM-cellulose column at a Na^+ concentration of 57 mM (Fig. 2).

The pool a, b and c fractions from CM-cellulose with the highest activity for AFB_1-8,9-epoxide were each combined and applied immediately to hydroxyapatite; 1.6 cm × 18.5 cm columns were employed and these were developed with linear gradients of 10–250 mM-sodium phosphate buffer, pH 6.7, which contained 1 mM-dithiothreitol throughout. The AFB_1-8,9-epoxide–GSH-conjugating activity in each of the three pools was eluted from the hydroxyapatite column in closely similar positions (i.e. between fractions 58 and 62). Fig. 3 shows the elution of pool c from hydroxyapatite.

SDS/PAGE examination of the fractions from hydroxyapatite that possessed greatest activity for AFB_1-8,9-epoxide revealed the presence of an electrophoretic band in all three preparations, pools a, b and c, that had a marginally slower mobility than the Ya_1 and Ya_2 subunits (both of M_r 25500). This common band has an estimated M_r of 25800. Pool a was found to comprise equimolar amounts of M_r-25500 and M_r-25800 subunits. Pool b contained polypeptides of M_r 25500, M_r 25800 and M_r 27500. Pool c comprised equimolar amounts of M_r-25800 and M_r-27500 subunits. SDS/PAGE also revealed that, whereas the preparation of pool c obtained from hydroxyapatite was essentially pure, both pool a and pool b contained contaminating protein; fractions 58–60 from hydroxyapatite chromatography of pool c were combined for further characterization (please see below).

The AFB_1-8,9-epoxide–GSH-conjugating activities in pools a and b were finally purified by cation-exchange f.p.l.c. on a Protein PAK SP-5PW column (Waters Chromatography Division). This was carried out using 10 mM-sodium phosphate buffer, pH 7.1, containing 0.5 mM-dithiothreitol, as running buffer and a 0–300 mM-NaCl gradient, formed in the running buffer, to develop the column. The elution of pools a and b from the Protein PAK SP-5PW column is shown in Fig. 4. In the case of pool a, examination of the column eluate by SDS/PAGE indicated that fractions 37–40 were essentially homogeneous (they contained equimolar amounts of two polypeptides) and these were combined for further characterization. In the case of pool b, paucity of material prevented a comprehensive characterization and it is therefore not described further.

Catalytic properties of the Alpha-class GSTs

The SDS/PAGE data suggest that the GSTs in pools a and c are members of the Alpha-class multigene family because, in addition to the common electrophoretic band of M_r 25800, they appear to contain either subunits of M_r 25500 (Ya_1 and/or Ya_2) or subunits of M_r 27500 (Yc). Indeed, amino acid sequencing experiments (see below) demonstrated that the M_r-25800 polypeptide is a Yc-type GST subunit and therefore it is referred to as Yc_2 whilst the constitutively expressed M_r-27500 subunit has been renamed Yc_1.

Table 3 shows a comparison of the substrate specificities of the GSTs in pools a and c with those of other Alpha-class GSTs. The enzymic properties of the M_r-25800 (Yc_2) subunit in pools a and c can be inferred from comparisons with the GSTs that contain only M_r-25500 or M_r-27500 subunits. As expected, pools a and c exhibit at least a 25-fold greater specific activity for AFB_1-8,9-epoxide than the Alpha-class GSTs that lacked the M_r-25800 SDS/PAGE band. Pool a, which contained subunits of M_r 25500 and M_r 25800, was found to possess 2.2-fold greater glutathione peroxidase activity than those enzymes (GST F and L) that contain only M_r-25500 subunits. Pool c, which comprises subunits of M_r 25800 and M_r 27500, exhibited slightly greater peroxidase activity than GST AA (Yc_1Yc_1), which contains only M_r-27500 subunits. These data indicate that the subunit of M_r 25800 (Yc_2) which is present in both pools a and c possesses high activity for cumene hydroperoxide. Table 3 also indicates that Yc_2 has no oxosteroid isomerase activity and, moreover, has little activity for 1-chloro-2,4-dinitrobenzene. Although these data suggest that the subunit(s) of M_r 25800 in pools a and c display similar substrate specificities, it is not possible to state that they are catalytically identical.

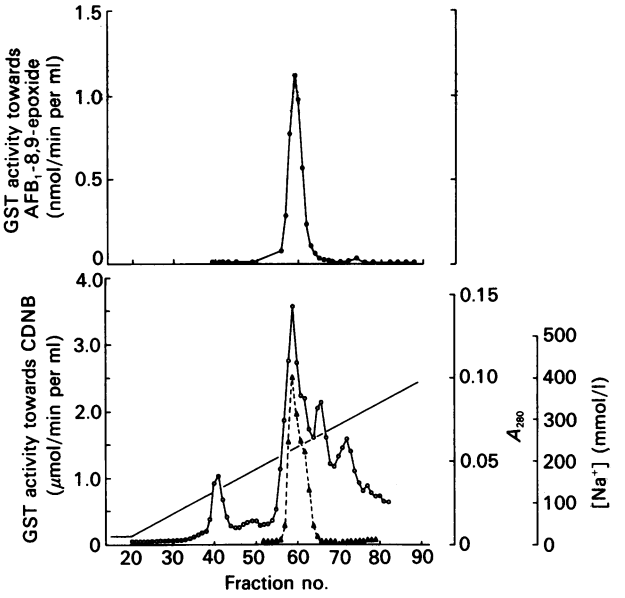


Fig. 3. Purification of AFB_1-metabolizing GSTs by hydroxyapatite chromatography

The GST-containing fractions from CM-cellulose which contained pool c were combined (see Fig. 2) and applied directly to a 1.6 cm × 18.5 cm column of hydroxyapatite. The column was eluted at 15 ml/h with 10 mM-sodium phosphate/1 mM-dithiothreitol, pH 6.7, and developed with a 10–250 mM-disodium phosphate gradient, pH 6.7, that contained 1 mM-dithiothreitol throughout. Fractions of 5 ml were collected and the absorbance at 280 nm (○), the GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) (▲) and the activity with AFB_1-8,9-epoxide (●) were measured. The gradient formed by disodium phosphate was monitored by determining the concentration of sodium in the fractions; the sodium gradient is depicted by the straight line. Fractions 58, 59 and 60 were considered homogeneous and were not subjected to further purification steps; these fractions represent purified GST Yc_1Yc_2.

Although the M_r 25800 subunit was not isolated as a homodimer, the data in Table 3 indicate that the catalytic activity of this subunit more closely resembles the Yc_1 subunit (i.e. the subunit in GST AA) than the Ya_1/Ya_2 subunits (i.e. the subunits in GST F and L).

Subunit compositions of the major AFB_1-metabolizing GST

To help establish the subunit composition of the GST with high activity for AFB_1-8,9-epoxide, portions of the purified pools a and c were subjected to reverse-phase h.p.l.c. on a μ-Bondapak C_{18} column. Using the h.p.l.c. system described in the Materials and methods section, it was found that both preparations yielded a prominent protein-containing peak which was eluted between 40.5 and 41.5 min, immediately after the constitutively expressed Yc_1 subunit. The elution profiles obtained from pools a and c are

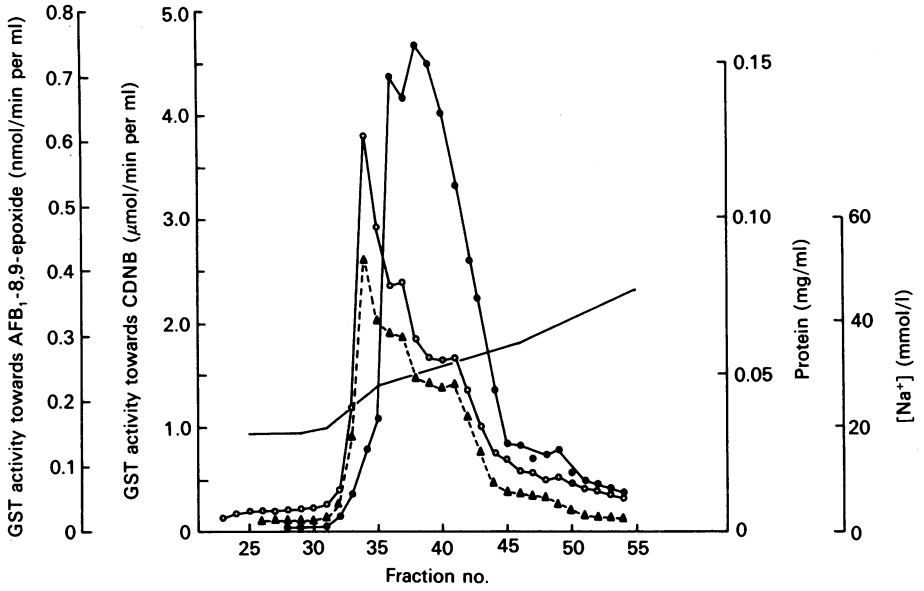


Fig. 4. H.p.l.c. purification of AFB_1-metabolizing GSTs in pool a

The fractions that were combined after hydroxyapatite chromatography of pool a were dialysed extensively against 10 mM-sodium phosphate buffer, pH 7.1, that contained 1 mM-dithiothreitol. Portions (5 ml) of the dialysed material were injected on to a Protein PAK Glass SP-5PW column (0.8 cm × 7.5 cm) equilibrated with the sodium phosphate buffer, pH 7.1. The GSTs were eluted from this cation-exchange column, which was controlled using a Waters Advanced (650 E) Protein Purification System, by a 0–60 mM-NaCl gradient formed in the running buffer; this was carried out in the presence of 1 mM-dithiothreitol. The flow rate was 60 ml/h and fractions of 1 ml were collected. Protein concentration (○) in the eluate was determined by the method of Bradford (1976) and GST activities with 1-chloro-2,4-dinitrobenzene (CDNB) (▲) and that towards AFB_1-8,9-epoxide (●) were assayed as described in the text. Fractions 36–42 were considered highly purified and contain primarily GST Ya_1Yc_2.

Table 3. Catalytic properties of the basic Alpha-class GSTs purified from Fischer 344 rats administered dietary ethoxyquin

The enzymes were prepared as described in the text. All analyses were performed at 37 °C and the results for all substrates, except the activities of GST F, L, B and AA towards AFB_1-8,9-epoxide, are expressed as means ± S.D. for four determinations; the activities of GST F, L, B and AA for AFB_1-8,9-epoxide are expressed as the mean of two results. Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; Δ^5-Add, Δ^5-androstene-3,17-dione; CuOOH, cumene hydroperoxide.

Enzyme*	Subunit content ($10^{-3} \times M_r$)	Specific activity (nmol/min per mg)			
		AFB_1-8,9-epoxide	CDNB	Δ^5-Add	CuOOH
Pool a (Ya_1Yc_2)	25.5, 25.8	10.3 ± 1.72	23600 ± 950	1090 ± 70	7420 ± 160
Pool c (Yc_1Yc_2)	25.8, 27.5	6.3 ± 0.56	13700 ± 600	< 10	15460 ± 620
GST F	25.5, 25.5	0.043	33500 ± 1200	2340 ± 110	3360 ± 110
GST L	25.5, 25.5	0.064	34400 ± 1000	1440 ± 90	3380 ± 80
GST B	25.5, 27.5	0.159	23500 ± 900	890 ± 60	8850 ± 620
GST AA	27.5, 27.5	0.216	17800 ± 700	< 10	13660 ± 530

* Subunit designations for the GSTs contained in pools a and c are given on the basis of elution from the μ-Bondapak h.p.l.c. column and amino acid sequencing experiments (see below).

shown in Fig. 5. The individual h.p.l.c. peaks were collected, and, after being freeze-dried, were subjected to SDS/PAGE (Fig. 6). This confirmed that the peak (Yc_2) eluted from the μ-Bondapak C_{18} column between 40.5 and 41.5 min, and may be common to pools a and c, contains a GST subunit of M_r 25800.

The combined h.p.l.c. and SDS/PAGE results suggest that pool a contains a mixture of two heterodimers. The predominant enzyme appears to be a hybrid between Ya_1 (M_r 25500) and Yc_2 (M_r 25800); however, the preparation also appears to contain lesser amounts of a hybrid between Ya_2 (M_r 25500) and the Yc_2 subunit. The data in Figs. 5 and 6 reveal that pool c comprises a hybrid of the Yc_1 and Yc_2 subunits.

Immunochemical properties of the M_r-25800 subunit from pool a and pool c

The subunit of M_r 25800 from pool a and that from pool c were prepared separately by h.p.l.c. as described in Fig. 5. The peaks eluted from the μ-Bondapak C_{18} column at 40.5–41.5 min were collected and subjected to Western blot analysis using polyclonal antisera against rat GST F (Ya_1/Ya_2 subunits) and rat GST AA (a Yc_1Yc_1 dimer). In addition to these antibodies against rat GST, the two rat M_r-25800 subunit preparations were probed with antibodies raised against the mouse Ya_3Ya_3 enzyme because it was thought that this GST is related to the

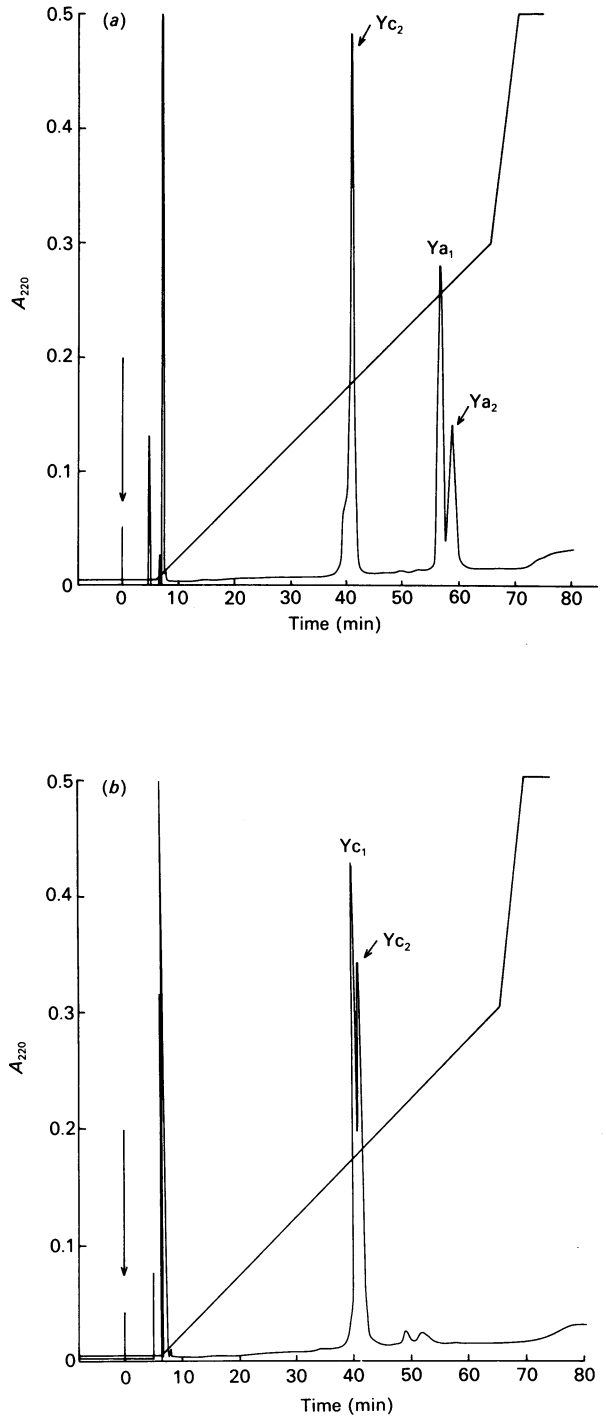


Fig. 5. Preparation of the individual GST subunits in AFB_1-metabolizing pools a and c

Portions (between 100 and 115 μg) of purified GST from pool a (*a*) and pool c (*b*) were applied to a Waters μ-Bondapak C_{18} column (10 μm particle size; column size 0.39 cm × 30.0 cm). The samples were loaded on to the column isocratically over the first 5 min of each run and during this time the flow rate from pump A was increased linearly from 0.1 ml/min to 1.0 ml/min. The column was subsequently developed at 1 ml/min by a 40–58% acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid, formed over 60 min, which was followed by a 58–70% acetonitrile gradient in aq. 0.1% trifluoroacetic acid, formed over 5 min. The eluate was monitored continuously at 220 nm. The relative output of pump B is shown by the continuous line; pump A delivered 40% acetonitrile and pump B delivered 70% acetonitrile. The Yc-type subunit of M_r 27500 eluted at about 39 min is designated Yc_1 whilst the Yc-type subunit of M_r 25800 eluted between 40.5 and 41.5 min is designated Yc_2.

inducible rat M_r 25800 subunit; the murine Alpha-class GST Ya_3Ya_3 not only has a subunit M_r of 25800 (McLellan & Hayes, 1989) but it also possesses a high specific activity towards AFB_1-8,9-epoxide (G. E. Neal, D. J. Judah, L. I. McLellan & J. D. Hayes, unpublished work).

The results of the blotting experiments, which are presented in Table 4, show that the two M_r-25800 subunit preparations are immunochemically indistinguishable. The M_r-25800 subunit was found to cross-react with antibodies raised against the rat Yc_1 (M_r-27500) subunit but reacted only weakly with antibodies against the rat Ya_1/Ya_2 (M_r-25500) subunits. Both rat M_r-25800 subunit preparations showed strong cross-reactivity with antibodies raised against the mouse Ya_3 (M_r-25800) Alpha-class GST subunit. As expected, the rat Yc_2 subunit (M_r 25800) did not cross-react with antibodies raised against either Mu-class or Pi-class GSTs.

Structural examination of the M_r-25800 subunit from pool a and pool c

To allow a structural comparison between the M_r-25800 subunit from pool a with that from pool c, the two h.p.l.c.-purified polypeptides were subjected, in parallel, to CNBr cleavage. The peptides obtained from these digests were resolved using reverse-phase h.p.l.c. Fig. 7 shows that the CNBr 'maps' of the M_r-25800 subunit from pool a and the M_r-25800 subunit from pool c are closely similar; the major peptides eluted at 37.5 min, 43.5 min, 45 min, 58 min and 67.5 min appeared to be common to pools a and c but differences in the two chromatograms were noted in the recoveries of peptides eluted between 63 min and 66 min as well as those eluted between 70 min and 75 min. By contrast, the CNBr 'map' of the Yc_1 (M_r-27500) subunit is readily distinguishable from that of the two M_r-25800 subunit preparations; it should be noted that the CNBr peptide a is common to Yc_1 and the M_r-25800 subunit(s). The Ya_1 and Ya_2 subunits were also subjected to CNBr cleavage in parallel with these other Alpha-class GSTs and the peptide 'maps' yielded by Ya_1 and Ya_2 were distinct from those of the M_r-25800 subunit(s) (results not shown).

Automated amino acid sequencing of the CNBr peptides shown in Fig. 7 was undertaken to establish the molecular relationship not only between the M_r-25800 subunit isolated from pool a and the M_r-25800 subunit from pool c but also to determine the relationship between these two subunit preparations and other rat GSTs. The sequence data in Fig. 8, obtained from the M_r-25800 subunit in pool a, confirm that this polypeptide is a member of the Alpha-class gene family. Moreover, it is more closely related to the Yc_1 (M_r-27500) subunit than to either of the Ya_1/Ya_2 (M_r-25500) subunits. The two M_r-25800 subunit preparations appear to be identical and when residues 112–142 and residues 170–185 in the M_r-25800 subunit from pool c were sequenced, no differences between the primary structure of the Yc_2 subunit from pools a and c were observed.

As mentioned above, we have chosen to designate the rat M_r-25800 subunit Yc_2 because it is an Alpha-class GST that has not been described previously but is closely related to the Yc subunit encoded by pGTB42 (Telakowski-Hopkins *et al.*, 1985); we have renamed this latter polypeptide Yc_1.

Inducibility of Yc_2, the M_r-25800 subunit

To address the question of whether the hepatic Yc_2 subunit is expressed constitutively or whether it is only expressed in rats fed on ethoxyquin-containing diets, Western blotting experiments were undertaken. Fig. 9 shows that Yc_2 is not expressed in the livers of adult Fischer 344 rats fed on control diets. However, this subunit appears to be expressed neonatally and is not only found

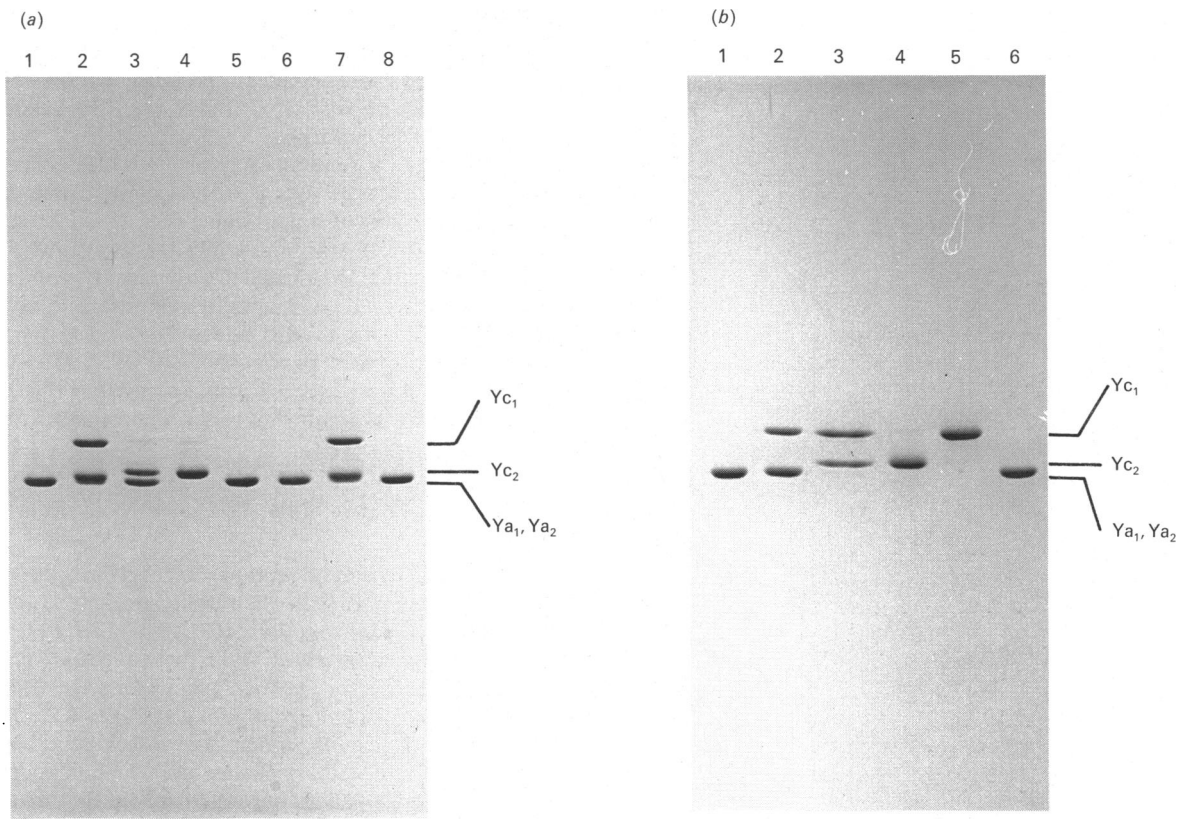


Fig. 6. SDS/PAGE of purified GST subunits obtained from AFB_1-metabolizing enzymes

The purified enzyme preparations from pool a and pool c were subjected to reverse-phase h.p.l.c. as shown in Fig. 5. The protein peaks eluted from the μ-Bondapak C_{18} column were each collected and freeze-dried. The freeze-dried material was resuspended in a solution containing 1% (w/v) SDS, 10% (v/v) glycerol and 1% (v/v) 2-mercaptoethanol and analysed by SDS/PAGE as described in the text. (*a*) and (*b*) show the electrophoretic patterns of the pool-a- and pool-c-derived proteins respectively. The tracks shown in (*a*) are as follows: 1 and 8, GST F (a preparation containing both Ya_1 and Ya_2); 2 and 7, fraction 34 from the f.p.l.c. Protein PAK SP-5PW column shown in Fig. 4; 3, purified pool a (fractions 36–42 from the f.p.l.c. Protein PAK SP-5PW column); 4, Yc_2 from the μ-Bondapak h.p.l.c. column; 5, Ya_1 from μ-Bondapak; 6, Ya_2 from μ-Bondapak. The tracks shown in (*b*) are as follows: 1 and 6, GST F (Ya_1/Ya_2); 2, GST B (a preparation containing Ya_1, Ya_2 and Yc_1); 3, purified pool c (fractions 58–60 from hydroxyapatite); 4, Yc_2 from the μ-Bondapak h.p.l.c. column; 5, Yc_1 from the μ-Bondapak column.

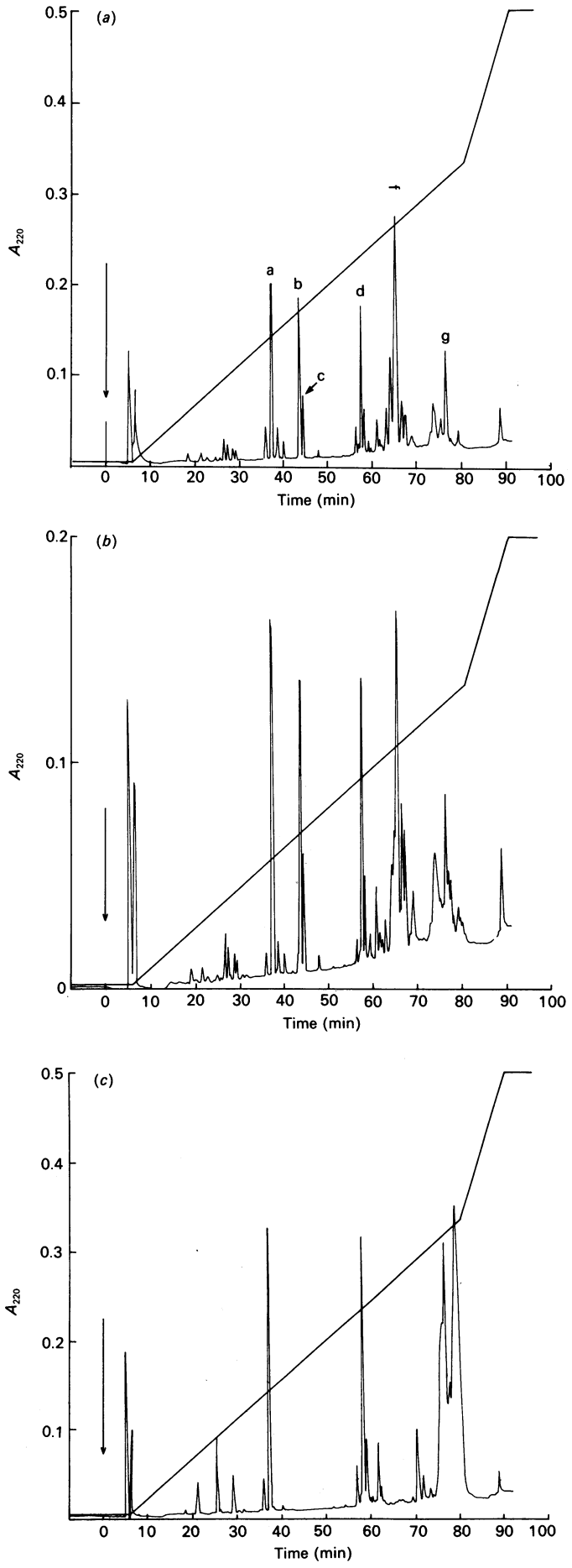
Table 4. Immunochemical properties of the GST subunits from pool a and pool c

The GSTs were purified as described in the text and individual subunits were isolated by reverse-phase h.p.l.c. on a μ-Bondapak C_{18} column (Fig. 5). Antibodies against rat GST F (Ya_1 and Ya_2 subunits), GST AA (Yc_1 subunits), GST A (Yb_1 subunits) and GST H (Yf subunits) were raised as described by Hayes & Mantle (1986*b*). Antibodies against mouse GST Ya_3Ya_3 were obtained as described by McLellan & Hayes (1989).

			Cross-reactivity with antibodies raised against:				
Enzyme preparation	Subunit M_r	Subunit* designation	Rat Ya_1/Ya_2 (Alpha)	Rat Yc_1 (Alpha)	Rat Yb_1 (Mu)	Rat Yf (Pi)	Mouse Ya_3 (Alpha)
Pool a	25500	Ya_1	+++	±	−	−	±
Pool a	25500	Ya_2	+++	±	−	−	±
Pool a	25800	Yc_2	±	+	−	−	+++
Pool c	25800	Yc_2	±	+	−	−	+++
Pool c	27500	Yc_1	±	+++	−	−	+
Rat GST AA	27500	Yc_1	±	+++	−	−	+
Mouse GST Ya_3Ya_3	25800	Ya_3	±	±	−	−	+++

* The rat GST subunits of M_r 27500 and M_r 25800 have been designated Yc_1 and Yc_2 respectively because they are members of the same Alpha-class subfamily.

in the livers of 5-day-old Fischer 344 rats but is also found in the livers of 6-week-old rats of both sexes. Also included in the blots shown in Fig. 9 are cytosols prepared from the livers of adult male Fischer 344 rats which contained preneoplastic nodules; the preneoplastic nodules were produced by feeding AFB_1-containing diets. The blots indicated that Yc_2 is expressed in both the hepatic nodules and the surrounding 'normal' liver tissue obtained from these animals.



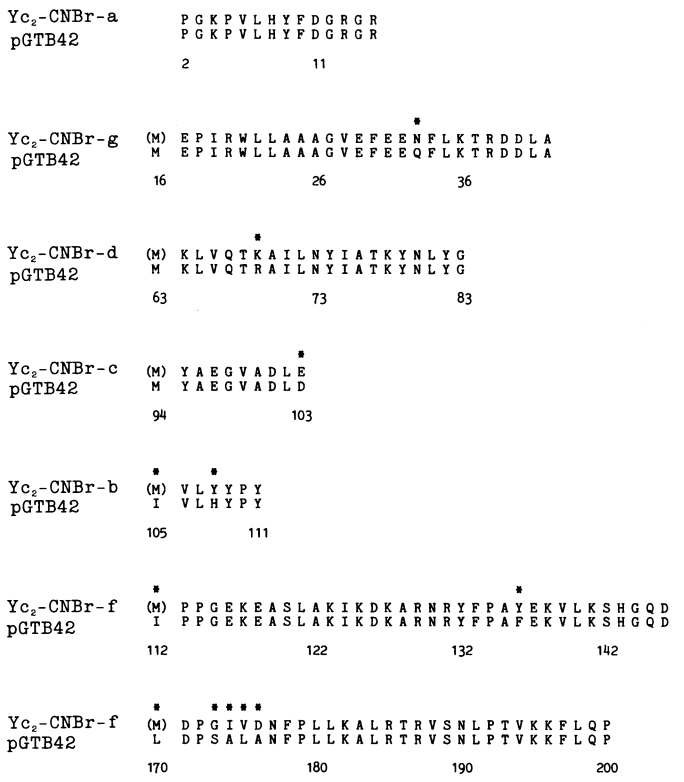


Fig. 8. Primary structure of Yc_2

The sequence of the CNBr-derived peptides from the Yc_2 subunit is aligned for comparison with the sequence deduced from the cDNA pGTB42 (Telakowski-Hopkins *et al.*, 1985) which encodes the Yc_1 subunit. The system of numbering the residues includes the initiator methionine and therefore the *N*-terminal amino acid in the mature enzyme is residue 2. Residues marked by an asterisk are those that differ between Yc_1 and Yc_2.

Induction of hepatic GST by ethoxyquin

It is apparent from the SDS/PAGE analysis shown in Fig. 9 that, besides induction of Yc_2, feeding rats ethoxyquin-containing diets also results in the over-expression of other GSTs. To enable the changes in GST expression produced by ethoxyquin to be studied, hepatic cytosols from treated and untreated animals were applied to columns of glutathione-Sepharose and the affinity-purified material was subsequently subjected to reverse-phase h.p.l.c. to enable the individual GST subunits to be resolved and their levels quantified (Ostlund Farrants *et al.*, 1987; Hayes *et al.*, 1990). Using this analytical approach, the Alpha-class Ya_1, Ya_2, Yc_1 and Yk subunits were found to be over-expressed 2.2-, 10.9-, 2.7- and 2.3-fold respectively (Table

Fig. 7. Analytical and preparative peptide maps of Yc_1 and Yc_2 subunits

The two preparations of Yc_2, isolated from the AFB_1-metabolizing GST pools a and c, were digested simultaneously with CNBr as described elsewhere (Hayes *et al.*, 1989); CNBr digests of h.p.l.c.-purified Yc_1 were carried out in parallel. The resulting CNBr fragments were dissolved in 3 ml of aq. 0.1% trifluoroacetic acid and portions (1 ml) were applied to a Waters μ-Bondapak C_{18} column. The column was developed at 1 ml/min by a 10–50% acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid, formed over 75 min, which was followed by a 50–70% acetonitrile gradient in aq. 0.1% trifluoroacetic acid, formed over 10 min. The h.p.l.c. analysis of the CNBr digests of the Yc_2 subunit from pools a and c are shown in (*a*) and (*b*) respectively, and the profile obtained from h.p.l.c. analysis of the Yc_1 CNBr digest is shown in (*c*). The peptides from Yc_2 that were subjected to automated amino acid sequencing are indicated.

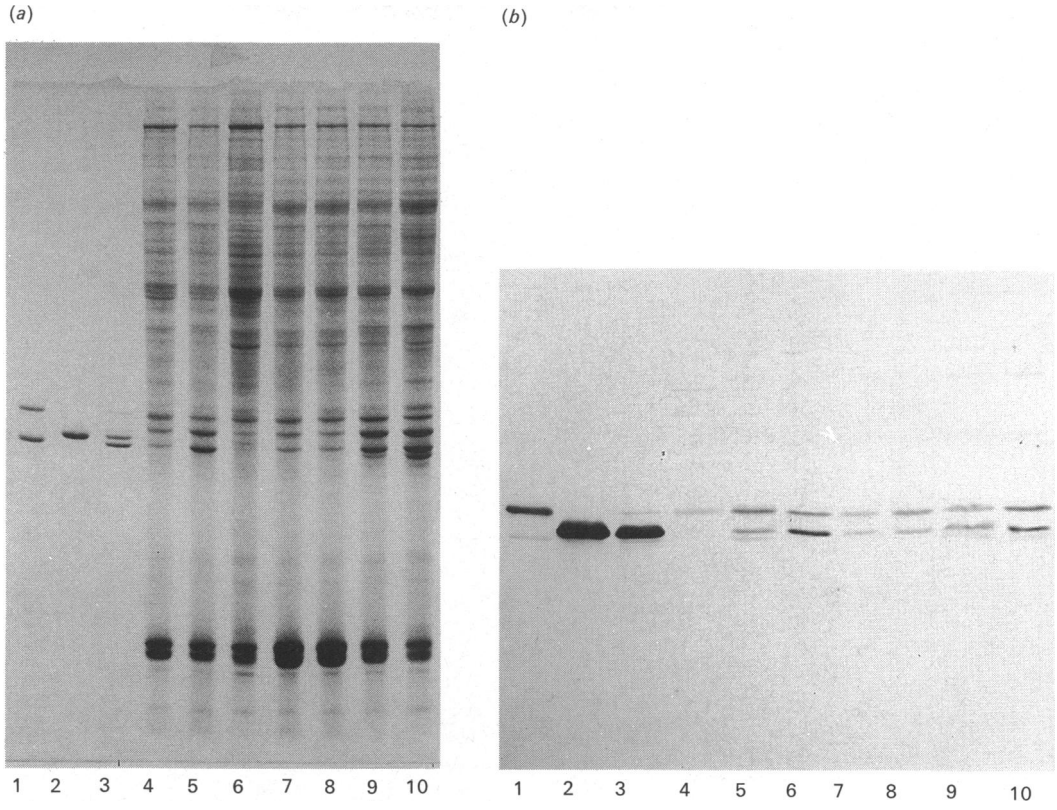


Fig. 9. Developmental control of Yc_2 and its expression in livers containing preneoplastic nodules

Cytosols were prepared from the livers of Fischer 344 rats of various ages (5 days–10 weeks) and from the livers of Fischer 344 rats (1 year old) bearing AFB_1-induced preneoplastic nodules. (*a*) shows SDS/PAGE analysis of hepatic samples which were applied as follows: track 1, 1 μg of rat GST B (a preparation containing Ya_1, Ya_2 and Yc_1); track 2, 1 μg of murine GST Ya_3Ya_3; track 3, 1 μg of rat GST Ya_1Yc_2; track 4, 30 μg of hepatic cytosol from 175 g male rats fed on a normal diet; track 5, 30 μg of hepatic cytosol from 175 g male rats administered dietary ethoxyquin; track 6, 30 μg of hepatic cytosol from 5-day-old rats; track 7, 30 μg of hepatic cytosol from 6-week-old male rats; track 8, 30 μg of hepatic cytosol from 6-week-old female rats; track 9, 30 μg of cytosol from livers bearing preneoplastic nodules; track 10, 30 μg of cytosol from an individual hepatic nodule. (*b*) shows an immunoblot analysis of Yc_2 expression using antibodies raised against mouse GST Ya_3Ya_3, to probe the same samples that were analysed in (*a*); the faster-migrating band in the immunoblot represents Yc_2 (M_r 25800).

Table 5. Induction of hepatic GST by dietary ethoxyquin and comparison with changes in GST expression during preneoplasia

The liver GSTs were purified by affinity chromatography and individual subunits were resolved using the μ-Bondapak C_{18} reverse-phase h.p.l.c. column as described elsewhere (Hayes *et al.*, 1990). The samples were analysed in duplicate and these gave identical results.

Subunit	Class	M_r (by SDS/PAGE)	Ethoxyquin (fold increase)	Preneoplasia* (fold increase)
Ya_1	Alpha	25500	2.2	2.8
Ya_2	Alpha	25500	10.9	7.5
Yc_1	Alpha	27500	2.7	4.7
Yc_2	Alpha	25800	> 15	Not estimated†
Yk	Alpha	25000	2.3	2.0
Yb_1	Mu	26300	4.5	3.7
Yb_2	Mu	26300	1.7	Not estimated
Yn_1	Mu	26000	0.6	1.2

* Data from Hayes *et al.* (1990).
† See Fig. 9 for evidence of over-expression of the Yc_2 subunit.

5). It is interesting to note that of the constitutively expressed GSTs in rat liver, ethoxyquin induces the Ya_2 subunit to the greatest extent. Our previous work has shown that this subunit is also preferentially elevated in the livers of rats bearing preneoplastic nodules, produced by feeding AFB_1-containing diets (Hayes *et al.*, 1990).

DISCUSSION

Purification of Yc_2-containing GSTs

The present study, which was undertaken to identify the GSTs that confer resistance to AFB_1, has led to the isolation of two inducible enzymes which have not been isolated previously from adult rat liver. Although the purification of the two enzymes with high activity for AFB_1-8,9-epoxide was achieved using conventional methods their existence only became apparent when the specific h.p.l.c.-based assay for AFB_1 metabolites (Moss *et al.*, 1983) was employed to examine column fractions; assay for GST activity with more commonly used substrates, such as 1-chloro-2,4-dinitrobenzene and cumene hydroperoxide, did not prove helpful in identifying these two enzymes. The purification scheme devised resulted in approximately a 200-fold increase in specific activity towards AFB_1-8,9-epoxide (Table 2). Three pools (a, b and c) of GST which could detoxify activated AFB_1 were isolated. Of these, pools a and b contained enzymes which were chromatographically interconvertible and are therefore believed to represent alternative forms of a single GST; relatively little GST with high activity for AFB_1-8,9-epoxide was isolated from pool b and therefore the majority of the characterization of this enzyme was carried out with GST from pool a. By contrast with

pools a and b, pool c contained a chromatographically distinct isoenzyme. The GSTs isolated from pools a, b and c were all heterodimers. The enzyme recovered in either pool a or pool b was found to comprise subunits of M_r 25500 and M_r 25800. The enzyme recovered in pool c comprised subunits of M_r 25800 and M_r 27500. Our data suggest that the electrophoretic band of M_r 25800, found in all these pools, represents a common polypeptide.

Comments about GST nomenclature

The M_r-25800 subunit has been designated Yc_2. Although the Ya/Yb/Yc nomenclature was originally proposed by Bass *et al.* (1977) to describe the mobility of GST subunits during SDS/PAGE, and we have shown that the M_r-25800 subunit has electrophoretic properties distinct from those of the constitutive Yc subunit, we have nonetheless chosen to retain the term Yc when referring to the M_r-25800 subunit to emphasize that this polypeptide is a member of the same Alpha class subfamily as the constitutively expressed M_r-27500 subunit; Southern blot analysis has revealed the existence of at least two Yc-type genes in the rat (Rothkopf *et al.*, 1986). The GST subunit of apparent M_r 27500 (Hayes & Mantle, 1986*c*) which was designated Yc by Bass *et al.* (1977) is now called Yc_1 and the inducible M_r-25800 subunit is called Yc_2. Using this terminology, pool a contains GST Ya_1Yc_2, with lesser amounts of GST Ya_2Yc_2 being present, whereas pool c represents GST Yc_1Yc_2 (see Fig. 5 and Fig. 6).

Structural characterization of rat Yc_2 and its relationship with other GSTs

The Yc_2 subunits from pools a and c were purified by reverse-phase h.p.l.c. Peptide 'mapping' experiments demonstrated that the two Yc_2 preparations comprised essentially identical proteins (Fig. 7) and this conclusion is supported by automated amino acid sequencing.

The amino acid sequence data indicate that the rat GST subunit shares about 92% sequence identity with Yc_1 but exhibits less than 70% sequence identity with Ya_1 and Ya_2. A comparison between the amino acid sequences of Yc_1 and Yc_2 reveals that substantial differences exist in their primary structures between residues 103 and 108 and between residues 170 and 176. As there is a large difference in the ability of Yc_1 and Yc_2 to detoxify activated AFB_1 it is tempting to suggest that either residues 103–108 or residues 170–176 may be involved in the catalytic centre responsible for metabolism of AFB_1. In this context, it is worth noting that through the use of a glutathione-based photoaffinity probe, Hoesch & Boyer (1989) provided evidence that implicated residues 91–110 and 206–218 of Alpha-class GST subunits in the active centre of the enzyme. These data would therefore favour the hypothesis that residues 103–108 may influence the catalytic properties of the active site of Yc_2.

The ethoxyquin-inducible Yc_2 subunit which we have isolated from adult rat liver has an M_r of 25800. Both Meyer *et al.* (1985) and Scott & Kirsch (1987) have described an Alpha-class GST in foetal rat liver which contains a subunit with similar electrophoretic properties to the Yc_2 subunit [for further details about the changes in the expression of hepatic GST during development, see McCusker *et al.* (1989)]. However, as it is not known whether this GST has activity towards AFB_1-8,9-epoxide, it is unclear whether the foetal GST subunit of M_r approximately 25800 represents Yc_2.

The Western blotting experiments performed during the present study (Fig. 9) show that the Yc_2 subunit, or an immunochemically related polypeptide, is expressed constitutively in the livers of neonatal rats. As the Yc_2 subunit confers resistance to AFB_1 in the adult, it is surprising that the neonatal rat, which appears to express Yc_2 constitutively, is sensitive to AFB_1. The fact that the neonatal rat is 10-fold more sensitive to AFB_1 than weanling or adult rats (Newberne & Butler, 1969) suggests that the neonate has a low detoxification capacity for AFB_1. This may be due to the neonatal subunit of M_r 25800 possessing low activity for AFB_1-8,9-epoxide or due to the neonatal rat expressing high cytochrome *P*-450 IIc levels [see Shimada & Guengerich (1989) for details about cytochrome *P*-450] or due to the neonatal rat lacking certain DNA-repair enzymes. Clearly, this area requires further investigation as it is possible that further Yc-type subunits exist.

The observation that the constitutively expressed Alpha-class GST in the livers of adult mice (GST Ya_3Ya_3) possesses high activity for AFB_1-8,9-epoxide (G. E. Neal, D. J. Judah, L. I. McLellan & J. D. Hayes, unpublished work; cf Ramsdell & Eaton, 1990; Quinn *et al.*, 1990) led us to consider its relationship with the ethoxyquin-inducible rat Yc_2 subunit. The Western blotting experiments shown in Table 4 indicated that these mouse and rat GSTs are indeed immunochemically similar. Moreover, approximately 60% of the primary structure of the constitutively expressed murine GST has been determined (McLellan *et al.*, 1991), and examination of these data indicates that, over the region sequenced, the mouse and rat GST subunits share at least 95% sequence identity. Although present evidence indicates that these two GSTs are closely similar, cDNA cloning experiments are required to establish their molecular relationship more precisely.

Induction of GST by ethoxyquin

The present study has highlighted the role of the GST Yc_2 subunit in protecting against AFB_1 and has served to document that this subunit is induced by ethoxyquin. However, ethoxyquin exerts a profound effect on all hepatic GSTs (see Table 5). Administration of dietary ethoxyquin results in an over-expression of both Alpha- and Mu-class GST subunits in the rat. Amongst the Alpha-class GSTs, the Ya_1, Ya_2 and Yc_1 subunits were found to be induced 2.2-, 10.9- and 2.7-fold respectively by ethoxyquin. As we have shown that the Ya_1-, Ya_2- and Yc_1-containing GSTs possess some activity towards AFB_1-8,9-epoxide (Table 3), it is to be expected that their induction will also provide a small level of protection against AFB_1. Notwithstanding the dramatic induction of Ya_2, it is envisaged that because of the large differences in specific activity towards AFB_1-8,9-epoxide the contribution made by these subunits to the resistant phenotype will be substantially less than that contribution made by Yc_2.

The marked over-expression of hepatic Ya_2 produced by dietary ethoxyquin is of particular interest as this subunit is also preferentially induced in rat livers bearing preneoplastic nodules produced by AFB_1-containing diets (Hayes *et al.*, 1990). Our data indicate that Ya_2, the subunit encoded by the cDNA clone pGTB38 (Pickett *et al.*, 1984), is more readily induced by xenobiotics than other constitutively expressed rat GSTs. Pickett and his colleagues have demonstrated the existence of two regulatory elements in the 5′ flanking sequence of the rat GST Ya_2 gene that respond to foreign chemicals; one element is responsive to β-naphthoflavone and 3-methylcholanthrene whilst the other is a xenobiotic-responsive element that shares identity with the xenobiotic-responsive element found in the cytochrome *P*-450 IA1 gene (Rushmore *et al.*, 1990). The β-naphthoflavone-responsive element also responds to the antioxidant t-butylhydroquinone (Rushmore & Pickett, 1990) and it therefore seems probable that the effect of ethoxyquin on the expression of Ya_2 is mediated by this element. However, it is not clear whether the over-expression of Ya_2 which results from the chronic administration of AFB_1 (i.e. in the preneoplastic nodule-bearing livers) is mediated by the xenobiotic- or the β-naphthoflavone-

responsive element. It is also possible that the preneoplastic nodule-bearing livers over-express Ya_2 either as a consequence of the carcinogenic process or through a stress-related mechanism.

It is desirable to determine the factors that mediate the control of expression of Yc_2. From the data we have presented it appears probable that, like the GST Ya_2 gene, the GST Yc_2 gene contains a functional upstream β-naphthoflavone-responsive element. Clearly, future experiments are required to establish the molecular events responsible for the induction of Yc_2 by ethoxyquin.

Examples of resistance to AFB_1

In view of the evidence we have presented suggesting that induction of Yc_2 by ethoxyquin represents the major mechanism whereby this antioxidant can produce resistance to AFB_1, it will be interesting to discover whether the Yc_2 subunit can be induced by compounds such as butylated hydroxyanisole, β-naphthoflavone, phenobarbital, oltipraz and dehydroepiandrosterone, which have also been reported to protect against the toxic effects of AFB_1 (Gurtoo *et al.*, 1985; Kensler *et al.*, 1986, 1987; Lotlikar *et al.*, 1989; Prasanna *et al.*, 1989).

Hepatic preneoplastic nodules are often quoted as an example of acquired drug resistance (Farber, 1984*a*,*b*; Fairchild *et al.*, 1987; Hayes & Wolf, 1990) and although we have demonstrated that Yc_2 is over-expressed in nodules produced by AFB_1 (Fig. 9), it remains to be seen whether Yc_2 is also over-expressed in the other models of hepatic carcinogenesis.

Note added in proof (received 23 July 1991)

Recently, Rushmore *et al.* (1991) have designated the β-naphthoflavone-responsive element, which we suggest is responsible for the induction of Yc_2 by ethoxyquin, the antioxidant-responsive element (ARE).

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