Biochemical Pharmacology, Vol. 36, No. 24, pp. 4269–4276, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
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CONJUGATION OF MODEL SUBSTRATES OR MICROSOMALLY-ACTIVATED AFLATOXIN B_1 WITH REDUCED GLUTATHIONE, CATALYSED BY CYTOSOLIC GLUTATHIONE-*S*-TRANSFERASES IN LIVERS OF RATS, MICE AND GUINEA PIGS

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(*Received* 27 *March* 1987; *accepted* 22 *June* 1987)

Abstract—Glutathione-*S*-transferase (GST) activity has been examined in liver cytosol fractions from guinea pigs, mice, control fed rats or rats with pre-neoplastic nodular liver lesions. The levels of activity in unfractionated cytosols have been assayed using the model substrates 1-chloro-2,4-dinitrobenzene (CDNB), 3,4-dichloronitrobenzene (DCNB) and monobromobimane (mBrB) with reduced glutathione (GSH). The order of activities in the various liver fractions using CDNB as substrate were: mouse > pre-neoplastic nodular rat > guinea pig > control rat and paralleled the capacities of the cytosols to catalyse the formation of aflatoxin B_1-GSH from microsomally-activated aflatoxin B_1 (AFB_1) and GSH. Quantitative differences between the activities of the cytosols using the three model substrates were observed. In the mouse fractionation of GST activity by isoelectric focusing (I.E.F.) on preparative granular gels showed that the most basic component (isoelectric point pH 9.4) with the highest conjugating activity with respect to microsomally-activated AFB_1 did not correspond with the peak of most activity for conjugating CDNB. In the pre-neoplastic nodular rat liver the CDNB conjugating activities of all fractions separated on granular I.E.F. gels, were higher than the corresponding fractions isolated from control rat liver, with particular enhancement of the peak containing the 3:3 isoenzyme. In contrast to control rat liver the 7:7 isoenzyme was detected in pre-neoplastic nodular liver preparations. These isoenzymes (3:3 and 7:7) did not contribute significantly to the enhanced level of AFB_1-GSH formation catalysed by cytosol fractions prepared from pre-neoplastic nodular rat liver. The microsomally-activated AFB_1-conjugating activity of unfractionated rat liver cytosols was increased to a relatively greater extent than CDNB conjugating activity during the induction of pre-neoplastic nodular liver lesions, and the elevated level of the activated AFB_1-conjugating activity was found to be associated with the most basic fraction (isoelectric point pH 9.0). Analytical isoelectric focusing gels using mBrB as substrate demonstrated the presence of a basic GST isoenzyme in the pre-neoplastic nodular rat liver, not detected in preparations from the livers of control rats. The low level of activated AFB_1-conjugating activity present in unfractionated guinea-pig cytosol was found to correspond with the fraction containing the peak of CDNB conjugating activity on preparative isoelectric focusing (isoelectric point pH 7.5).

The lack of correlation between the conjugation of model substrates and the conjugation of xenobiotics could be of importance in studies on drug resistance.

The glutathione *S*-transferases (GST)§ (EC 2.5.1.18) are a group of isoenzymes catalysing the conjugation of reduced glutathione (GSH) with a wide spectrum of compounds possessing an electrophilic centre [1, 2]. This reaction is the first step in the mercapturic acid pathway, which leads to the excretion of many xenobiotic substances. Because an alternative fate for many electrophilic substances (often formed as a result of hepatic mixed function oxidase activity) to that of conjugation with GSH can be binding to nucleophilic centres in cellular macromolecules, the liver GST enzymes are believed to play a key role in the protection of tissues from toxic reactions [3]. The potent hepatotoxin and hepatocarcinogen, aflatoxin B_1 (AFB_1) is metabolically activated in the liver to form a reactive electrophile believed to be a labile epoxide. This metabolite can bind to cellular macromolecules which results in cytotoxicity. Alternatively it is capable of GST mediated conjugation with GSH [4, 5]. Species vary greatly in their sensitivity to aflatoxin toxicity, which is in part dependent on their capacities to activate the toxin, but also is related to their abilities to detoxify the reactive metabolite, by conjugation with GSH [6]. There have been reports of studies in which the ability of the different GST isoenzymes present in rat liver to catalyse the conjugation of activated AFB_1 with GSH have been examined and the results indicate that only isoenzymes containing sub-units 1 or 2 have significant activity [7, 8]. In the present study we have compared the capacities of hepatic cytosolic GST isolated from some animal species, representing a range of susceptibilities to AFB_1 toxicity, to catalyse the conjugation of GSH with several model substrates and also with microsomally-acti-

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§ Abbreviations used: GST, glutathione-*S*-transferase; AFB_1, aflatoxin B_1; AFG_2, aflatoxin G_2; GSH, reduced glutathione.

vated AFB_1 to ascertain which model substrates most closely parallel AFB_1. The distribution of these activities amongst the individual isoenzymes has also been examined using isoelectric focusing separations.

MATERIALS AND METHODS

Animals

Rats used were male Fischer F344 bred on site. Pre-neoplastic nodular livers were induced in the rats by feeding a diet composed of 25% powdered MRC 41B and 75% ground nut meal containing AFB_1. Arachis oil (20 ml/kg diet) was included in all diets. In the control diet the ground nut meal used did not contain detectable levels of AFB_1, but in the induction of pre-neoplastic nodular liver lesions, a ground nut meal, naturally contaminated with AFB_1, was used, blended with the uncontaminated meal to yield a final concentration of 1 ppm AFB_1 in the diet (assayed by TLC). Feeding the experimental or control diets commenced at the weanling stage (3 weeks of age) and was continued until the animals were used at the age of 18 weeks. At this stage small nodular lesions are macroscopically detectable and gamma glutamyl transferase positive foci present in the livers of the AFB_1-fed animals [9, 10].

Mice used were C57/B1 10 males obtained from N.I.M.R. (Mill Hill, U.K.), and maintained on MRC 41B pellets *ad lib*. Mice were used experimentally at approx. 10 weeks old. Guinea pigs (Duncan–Hartley strain) were young adult females obtained from N.I.M.R. (Mill Hill, U.K.), and were maintained on pelleted guinea-pig diet *ad lib*. Japanese quail were young adult hens obtained from Lincolnshire Pheasantries (Tenby, U.K.), maintained on layers pellets and mixed corn poultry diets (Labsure Ltd., Cambridge, U.K.).

Preparation of tissue fractions

Rats and quail were killed by decapitation, mice and guinea pigs by cervical fracture. Cytosolic fractions from the rats, mice and guinea pigs were prepared by homogenising samples of liver in 4 vol. 0.25 M sucrose, 10 mM potassium phosphate (pH 7.4). Whole livers were used in all cases, no attempts were made to separate nodules from the pre-neoplastic nodular rat livers. Cytosol fractions were obtained as the supernatants from 105,000 *g*/60 min centrifugations and were stored at −70° until used. Pooled livers were used for preparing individual samples of cytosol (rats 2, mice 4 and guinea pigs 2) and duplicate samples were prepared from each species.

Microsomal fractions used to activate AFB_1 in *in vitro* incubations were prepared from quail liver by homogenising in 150 mM KCl as previously described [11, 12]. Washed microsomal pellets were finally suspended in 150 mM KCl (5 g original weight liver/8 ml suspension) and were stored in aliquots at −70° until required. No loss of AFB_1-activating capacity was observed over a storage period of 6 months of the microsomal suspensions in KCL.

Chemicals

1-Chloro-2,4-dinitrobenzene (CDNB) and Kenacid blue^R were obtained from BDH Chemicals Ltd. (Poole, U.K.), 3,4-dichloronitrobenzene (DCNB) from Fluka AG (Buchs, Switzerland), monobromobimane (mBrB) (3-bromomethyl-2,5,6-trimethyl-1*H*,7*H*-pyrazole [1,2-*a*] pyrazole-1,7-dione) from Biosciences Ltd. (Cambridge, U.K.), and AFB_1, from Makor Chemical Co. (Jerusalem, Israel). Aflatoxin G_2 (AFG_2) was separated by column chromatography from a sample of mixed aflatoxins obtained from the Microbiological Research Establishment (Porton, U.K.). Ampholines pH range 3.5–9.5 and Ultrodex were obtained from LKB (Croydon, U.K.). Other chemicals were obtained from Sigma Ltd. (Poole, U.K.), and were of the highest purity available.

Assays of GST activity

(a) *CDNB as substrate.* The activity was assayed at ambient temperature by the method of Habig *et al.* [13]. Cytosols were diluted to limit the reaction rate to $<\Delta 0.05$ AU/min. Activities were calculated using an extinction coefficient of 9.6 mmole/l/cm.

(b) *DCNB as substrate.* Activity was assayed essentially as described by Habig *et al.* [13]. The concentrations of cytosols were adjusted to give reaction rates $<\Delta 0.05$ AU/min. Enzyme activities were calculated using an extinction coefficient of 8.6 mmol/l/cm.

(c) *mBrB as substrate.* Activity was assayed according to the method of Hulbert and Yakubu [14]. The assay mixture contained 100 μM GSH and 50 mM potassium phosphate pH 6.5. The reaction was started by the addition of an aliquot of a solution of mBrB in acetonitrile (10 μM final conc.). The reaction was monitored using a Perkin-Elmer LS5 fluorimeter (400 nm excitation, 475 nm emission) for 3 min, during which period the rate of reaction was linear and proportional to the amount of cytosolic protein added. In preliminary experiments in which the reaction was allowed to proceed to completion, it was observed that the reaction was limited by mBrB and could be restarted by addition of a further aliquot of this substrate. The level of the fluorescence at the stage of completion of the reaction was therefore used to calculate a molar fluorescence and to quantitate the specific activities of the cytosol-catalysed reactions.

(d) *Microsomally-activated AFB_1 as substrate.* GST activity using activated AFB_1 as substrate was assayed by a modification of the previously reported method [15]. The incubation medium consisted of 72 mM potassium phosphate pH 7.4, 5 mM $MgCl_2$, 2.6 mM GSH, 0.39 mM $NADP^+$, 6.0 mM glucose-6-phosphate, 0.25 U/ml glucose 6-phosphate dehydrogenase and 64 nmole/ml AFB_1. Following a 5 min pre-incubation at 37° in a shaking water bath under O_2, 600 μl of quail hepatic microsome suspension (~12 mg protein) followed by 1.0 ml of unfractionated cytosol or cytosol fractions from isoelectric focusing (I.E.F.) separations, were added, and the incubations continued for 10 min. The AFB_1-activating capacity of each quail microsomal preparation was monitored in parallel incubations in which the potassium phosphate buffer was replaced by Tris–HCl buffer (80 mM, pH 7.4) and the cytosol component was replaced by 1.0 ml distilled water. At the

end of the incubation period the samples were placed in ice and 3.0 ml ice-cold methanol added immediately followed by 20 μl AFG_2 in DMSO (0.5 mg/ml) as an internal standard. Samples were centrifuged (30 min later) at 1000 g for 1 hr at −20°. The supernatant was removed and the pellet washed once with 2.0 ml ice-cold methanol and recentrifuged for 1 hr as before. The combined supernatants were reduced to 0.5–1.0 ml in a stream of O_2-free N_2 at 37°. The remaining solution was freeze-dried and reconstituted with 1.0 ml ice-cold methanol/water (1:1 v/v) and centrifuged at 1000 g at −20° for 60 min. Samples obtained from incubations including the cytosol fractions were assayed for the presence of AFB_1-GSH conjugate using UV absorption monitoring at 365 nm by HPLC on a 100 × 4.6 mm Magnusphere 5 ODS reverse phase column, with a linear 15–40% methanol/water gradient containing 0.01% phosphoric acid and 8% acetonitrile at a flow rate of 1.2 ml/min. Recoveries of AFB_1-GSH were corrected using the AFG_2 internal standard. In those samples from which the cytosol fractions were omitted, and the phosphate buffer was replaced with Tris–HCl, activation of AFB_1 was determined by the formation of the Tris–AFB_1 dihydrodiol complex [16]. Typical chromatograms obtained in the HPLC assays used in this study either to assess the capacity of the microsomes to activate AFB_1 (plus Tris) or determine the formation of AFB_1-GSH (plus cytosol) are illustrated in Fig. 1.

(e) *Protein assays.* Protein levels were assayed by the method of Lowry *et al.* [17] using bovine serum albumin as standard.

Separations of GST isoenzymes by isoelectric focusing on polyacrylamide gels

(a) *Analytical gels.* Separation of the GST isoenzymes by isoelectric focusing on polyacrylamide gels was followed by detection of the GST activity using mBrB as described by Hulbert and Troughton [18]. Isoelectric focusing was carried out on an LKB 2217 Ultraphor electrofocusing unit using an LKB 2197 power supply, the temperature being maintained at 10° using an LKB 2209 Multitemp unit. Aliquots (20 μl containing approx. 100 μg protein) of the cytosol fractions were applied, in duplicate, on filter paper applicators to each half of an LKB polyacrylamide plate (1 mm, pH 3.5–9.5) 3 cm from the anode. The gel was focused for 90 min (initial settings 1500 V, 150 mA). pI markers were included on the plate to monitor the pH gradient. On completion of the focusing the plate was cut into halves, one piece being stained for protein and the other for GST activity using mBrB. The half used for protein visualisation was placed immediately in fixing solution (300 ml containing 34.5 g TCA and 10.4 g sulphosalicylic acid) for 30 min. The gel was then stained for 30 min in Kenacid blue R solution, followed by destaining.

The GST activity was detected on the other half of the gel by placing it for 2 min in 1 M potassium phosphate (pH 6.5) containing 0.5 mM GSH. This solution was replaced for 30 sec with 1 M potassium phosphate (pH 6.5) containing 0.5 mM GSH and 0.5 mM mBrB (the mBrB, dissolved in acetonitrile, was added to the solution immediately prior to use) just sufficient to cover the gel. The gel was then rapidly rinsed in 1 M potassium phosphate pH 6.5, placed on a TM40 Transilluminator (Ultraviolet Products Inc., San Gabriel, CA) and photographed, at frequent intervals, as the fluorescent bands developed, using a Polaroid MP-3 Land camera and a Polaroid type 55 positive/negative film. A Kodak Wrattan gelatin (No. 58) filter was placed in front of the camera lens. Standard preparations of rat liver cytosol GST isoenzymes, partially purified by isoelectric focusing on columns were included on the gels as markers. Control gels, from which GSH was omitted from the mBrB development schedule, demonstrated that the presence of the fluorescent bands was dependent on GST activity.

(b) *Preparative gels.* Aliquots (5 ml) of the unfractionated liver cytosols (equivalent to 1.25 g fresh weight of tissue) were desalted on a Sephadex G25 column (5.7 × 1.7 cm). The protein-containing fractions were pooled and the volume reduced to 1.5 ml using an immersible Millipore CX-10 ultrafiltration unit (M_r cut off 10,000, Millipore Corporation, Harrow, U.K.) 1.5 ml of ampholine (pH 3.5–9.5, 2% w/v) was added to the concentrated, desalted cytosol fractions. Granular gels were prepared and isoelectric focusing (I.E.F.) carried out essentially as described in LKB Application Note 198. Aliquots (3 ml) of the cytosol/ampholine mixture were applied to the gel 6 cm from the anode and electrophoresed at 8 W (constant power) for 14 hr at 10°. The gel was then cut into 30 sections, and each eluted with distilled water (3 ml). The pH of each fraction was measured before adding 1.0 M potassium phosphate pH 6.5 (0.6 ml). Aliquots of the fractions were assayed for GST activity using CDNB as substrate. Results are expressed as activities present in 1 ml of each eluted fraction which allows the relative

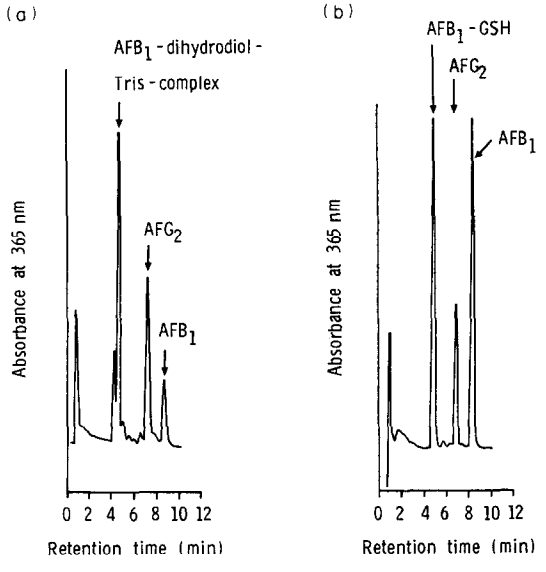

Fig. 1. HPLC assay of AFB_1-GST activity using quail microsomal activation. (a) Activation of AFB_1 by quail microsomes assayed by the formation of AFB_1-dihydrodiol–Tris complex. (b) Formation of AFB_1-GSH conjugate catalysed by cytosolic GST. For details of assays see Materials and Methods.

contribution of the separated fractions to the total CDNB-conjugating activity to be assessed. The mBrB conjugating activities of the eluted fractions were also assayed and essentially similar distributions of GST activity to those observed using CDNB as substrate were obtained. Recovery of the total CDNB activity applied to the gels was in the range 60–75%. Fractions were pooled on the basis of peaks of CDNB-conjugating activity, but due to the wide pH range gels used in these studies, this did not permit the separation of isoenzymes having similar isoelectric points. The pooled fractions were dialysed against 3 changes of 2 1. 0.1 M phosphate buffer pH 7.4. The dialysed solutions were concentrated using the immersible Millipore CX-10 ultrafiltration unit and finally made up to a volume of 5 ml with 0.1 M phosphate buffer pH 7.4. Aliquots (1 ml) of these fractions were used to assay for the presence of AFB_1-conjugating activity by the method described above. It was essential to carry out extensive dialysis of the eluted fractions prior to AFB_1-GSH conjugation assays. Although the presence of the ampholine did not affect the CDNB-GSH conjugation assay it was strongly inhibitory to the activation of AFB_1 by the quail microsomal preparation. The results of these assays are also expressed in terms of the distribution of the total activity observed between the individual fractions. Total recoveries of activated AFB_1-conjugating capacity were in the range 25–40%.

RESULTS

Conjugation of model substrates and microsomally activated AFB_1 with GSH

The abilities of the unfractionated cytosols to catalyse the conjugation of GSH with CDNB, DCNB, mBrB and activated AFB_1 are given in Table 1. Considerable differences were observed between the activities of cytosols from different species. The conjugating activity for all the substrates was considerably enhanced in pre-neoplastic nodular liver as compared with the control rat liver. The closest parallel between the substrates in order of activities was seen between CDNB and AFB_1, the order in both cases being mouse > pre-neoplastic nodular rat liver > guinea pig > control rat.

Fractionation of GST activity on I.E.F. granular gels

Cytosols were fractionated on I.E.F. granular gels and the CDNB-conjugating activities of eluted fractions determined. mBrB-conjugating activities were also determined and yielded essentially similar results to those obtained using CDNB (data not shown). Control rat liver cytosol showed 5 peaks of CDNB-conjugating activity at pH 9.0, 8.2, 7.7, 6.8 and 6.3 (Fig. 2a). The fractions in each of the peaks were pooled and their capacities to catalyse the conjugation to AFB_1 was determined. Although this activity was low in all the pooled peak fractions, peak A contained the most activity and peak B the least. In the pre-neoplastic nodular rat liver fractionations it was found that the CDNB-conjugating activities of all of the peak fractions observed in the control rat liver extracts were elevated. In addition a new peak of activity at pH 7.1 was detected (Fig. 2b). The increased capacity of the pre-neoplastic nodular rat liver to catalyse the conjugation of AFB_1 observed using the unfractionated cytosol (Table 1) was found to be due to the high activity present in the basic Fraction A (Fig. 2b).

The rat cytosol peak fractions were concentrated by freeze drying and run on I.E.F. analytical gels with GST markers. The isoenzymes present in the various fractions were tentatively identified Fraction A isoenzymes 1:1, 1:2, 2:2, Fraction B isoenzyme 3:3, Fraction C isoenzyme 3:4, Fraction D (only detected in pre-neoplastic nodular liver) isoenzyme 7:7, Fraction E isoenzyme 4:4 and Fraction F isoenzyme 4:6.

Fractionation of mouse liver cytosol demonstrated the presence of 5 peaks of CDNB-conjugating activity at pH 9.4, 7.9, 7.5, 6.5 and 5.5 (Fig. 2c). The AFB_1-conjugating activity was predominantly (>80%) present in the basic Fraction A (pH 9.4) whereas the CDNB-conjugating activity was predominantly located in Fraction B (pH 7.9).

Fractionation of guinea-pig cytosol revealed the presence of three peaks catalysing the conjugation of CDNB at pH 9.0, 7.5 and 6.3 (Fig. 2d). The

Table 1. Glutathione *S*-transferase activity in unfractionated liver cytosols using CDNB, DCNB, mBrB and AFB_1 as substrates

Source of cytosol	Conjugation substrate CDNB*	DCNB*	mBrB†	Microsomally-activated AFB_1†
Rat				
Control	0.525 ± 0.071 (14)	0.033 ± 0.001 (63)	0.236 ± 0.077 (23)	0.022 ± 0.002 (8)
Pre-neoplastic	1.910 ± 0.480 (53)	0.247 ± 0.008 (475)	3.123 ± 1.101 (305)	0.244 ± 0.023 (90)
Guinea pig	1.260 ± 0.196 (38)	0.044 ± 0.005 (85)	1.745 ± 0.294 (170)	0.040 ± 0.003 (15)
Mouse	3.630 ± 0.473 (100)	0.052 ± 0.009 (100)	1.025 ± 0.017 (100)	0.270 ± 0.042 (100)

Means of 3 replicate samples ± SD.
* μmole/min/mg protein.
† nmole/min/mg protein % of mouse activity in parentheses.

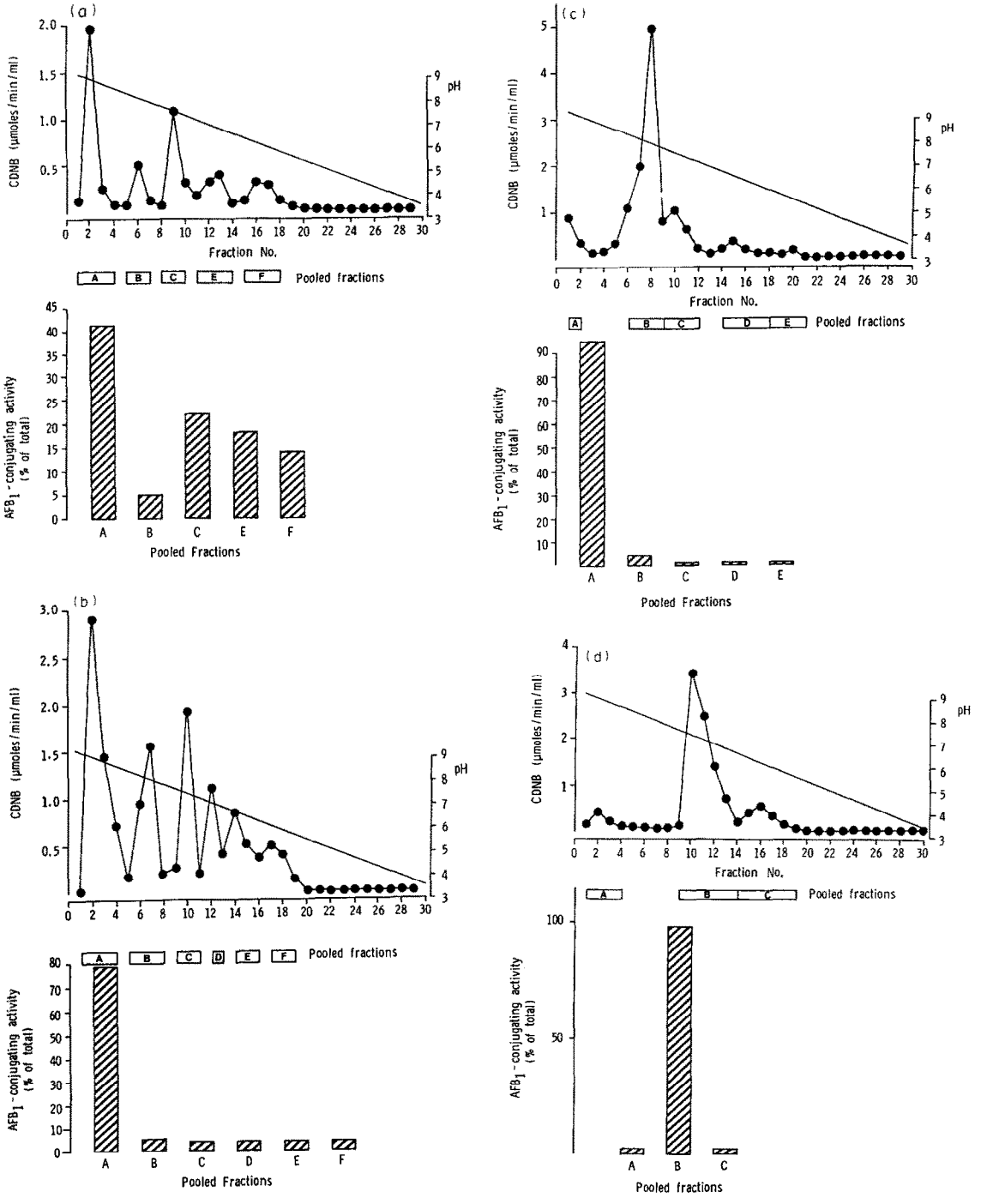

Fig. 2. Fractionation of cytosolic GST activity by isoelectric focusing on granular gels: (a) control rat liver cytosol; (b) pre-neoplastic rat liver cytosol; (c) mouse liver cytosol; (d) guinea-pig liver cytosol. For details of assays see Materials and Methods.

predominant AFB_1-conjugating activity (>95%) was associated with the highest activity towards CDNB (pH 7.5).

Isoelectric focusing on analytical gels

Total cytosols were focused on analytical polyacrylamide gels and the protein stained with Kenacid blue R and GST activity visualised with mBrB. The results for mouse and pre-neoplastic nodular rat liver are given in Fig. 3. The areas of the gels corresponding to the pooled fractions used in the granular gel studies (Figs. 2b and c) are indicated in Fig. 3. The presence of a very basic isomer catalysing GST activity was detected in the mouse cytosol fractions (arrowed Fig. 3) but no band was present at this pH in the control rat or guinea-pig cytosols (data not

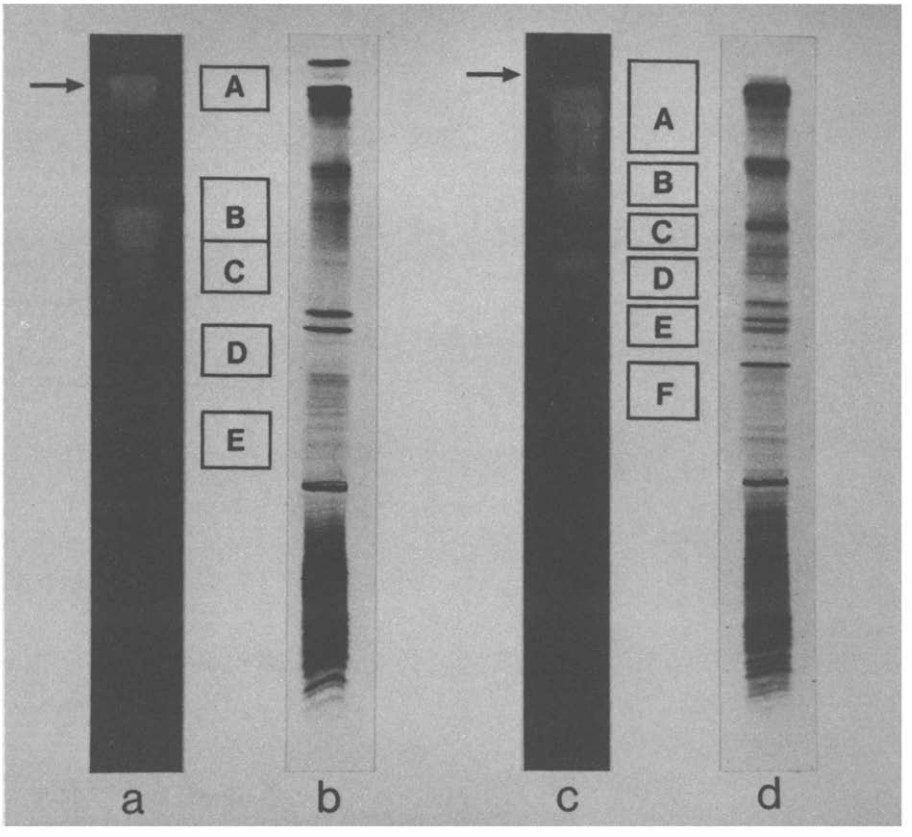

Fig. 3. Separation of GST activity by isoelectric focusing on polyacrylamide gels: (a and b) mouse liver cytosol; (c and d) pre-neoplastic rat liver cytosol; (a and c) GST activity revealed by mBrB as substrate; (b and d) proteins visualised by staining with Kenacid blue^R. Positions of pooled fractions used in AFB_1 conjugating assays (Fig. 2) indicated between (a and b)—mouse pooled fraction— and (c and d) pre-neoplastic rat pooled fraction. Arrowed a—most basic mBrB +ve mouse GST isoenzyme; c—basic mBrB +ve rat isoenzyme not detected in control rat cytosol. For other details see Materials and Methods.

shown). However, in the pre-neoplastic nodular rat liver cytosol, mBrB conjugating activity was detected in the same pH range as the fraction present in mouse cytosol (arrowed Fig. 3). The presumed 7:7 isoenzyme was detected on analytical gels of pre-neoplastic nodular rat liver cytosol (Fraction D, Fig. 3).

DISCUSSION

There have been numerous studies of the GST isoenzymes present in liver cytosols [1, 2]. Comparisons between studies, however, are often difficult to make because many of them have been carried out using only one animal species and separation techniques have varied between studies. In the present work we have compared the hepatic cytosol GST of three animal species, rat, guinea pig and mouse, using several substrates, and have also examined the isoenzyme patterns using the same conditions of isoelectric focusing. The species were selected for their differing susceptibilities to a hepatotoxic substance, AFB_1, in which the capacity to conjugate the active metabolite with GSH is believed to play a major role in determining the susceptibility of the animal to the toxin [6]. The control and carcinogen-treated rat were selected because it is believed that during hepatocarcinogenesis in the rat by the administration of AFB_1, an essential feature is the development of a resistance to the toxin which is dependent on an increased capacity to detoxify the active AFB_1 metabolite by conjugation with GSH [19]. A similar mechanism of resistance develops in conjunction with prolonged usage of therapeutic drugs [20].

The levels of GST activity determined using the different model substrates or AFB_1 indicated that although there was a general pattern of relative conjugating capacity betwen the species, common to the different substrates, there were some anomalies. For example, the guinea-pig cytosol had comparable DCNB conjugating capacity to that of the mouse, a higher level of mBrB but a very much lower level of AFB_1-conjugating activity. Although the CDNB conjugating activities of pre-neoplastic nodular rat liver and guinea-pig cytosols were not very different, the AFB_1-conjugating capacity of the guinea pig was only 16% that of the pre-neoplastic nodular rat liver fraction. It was apparent that the induction of pre-neoplastic nodular in rat liver dramatically induced

GST activity as assayed by any of the substrates used. In comparing the model substrates with activated AFB_1-conjugating capacity, the closest parallel appeared to be with CDNB as substrate. The gel separations were carried out to determine if this correlation was observed in the substrate specificities of the isoenzymes present in the different cytosols.

In this initial study we did not attempt absolute separations of the individual isoenzymes, by using restricted pH range isoelectric focusing gels, but instead have used wide pH range conditions of focusing in order to span the possible range of isoenzymes present in the cytosols from the different species.

In the granular gel separations of the cytosols of rat liver, five peaks of CDNB conjugating activity were observed in the preparations from control animals and six in the pre-neoplastic nodular livers. As referred to in the results section, Fraction A (pH 9.0) contained isoenzymes 1:1, 1:2 and 2:2, Fraction B (pH 8.2) isoenzyme 3:3, Fraction C (pH 7.7) isoenzyme 3:4, Fraction D (pH 7.1) isoenzyme 7:7, Fraction E (pH 6.8) isoenzyme 4:4 and Fraction F (pH 6.3) isoenzyme 4:6. Isoelectric points of pH 10 for isoenzyme 1:1, pH 9.9 for 1:2, pH 9.8 for 2:2, pH 8.4 for 3:3, pH 8.1 for 3:4 and pH 6.9 for 4:4 have been reported in the literature [21]. The results in the present study are essentially in agreement with these values but a lower pH (9.0) was observed in the present study for the peak containing the 1:1, 1:2 and 2:2 isoenzymes. No evidence, however, was obtained indicating a loss of basic isoenzymes from the gel. Reports in the literature have suggested that rat liver cytosolic transferases containing the 1 or 2 subunit are more active in conjugating AFB_1 than isoenzymes containing the other subunits, although these studies have usually used prestimulated animals with enhanced levels of this activity [7, 8]. In the present study in cytosols from the livers of control rats, which possess only low total AFB_1-GSH conjugating activity (consistent with the animal's extreme sensitivity to the toxin) in contrast to the results obtained with pre-neoplastic nodular rat, guinea-pig and mouse cytosols this conjugating capacity was not found to be predominant in any peak, although the basic Fraction A contained more activity than the other fractions.

There was, however, some selective loss of AFB_1-conjugating capacity since recoveries of total CDNB conjugating activity following granular gel I.E.F. were 60–75% of the starting activities whereas the AFB_1-conjugating activities were only in the range of 25–40%. In the extracts from the pre-neoplastic nodular liver, however, where there was a general elevation of the GST activity in the unfractionated cytosol when assessed using any of the substrates, the enhanced level of activated AFB_1-conjugating activity, detected in the unfractionated cytosol, was found to be due on I.E.F. to the activity present in the basic fraction (Fraction A, Fig. 2b). It is noteworthy that the activated AFB_1-conjugating capacity of the pre-neoplastic nodular tissue was increased, compared with the control, 11-fold, whereas the CDNB-conjugating activity was increased by only 3.6 times. This suggested the induction of an isoenzyme or enzymes with a high activated AFB_1/CDNB conjugating ratio. The greatly increased activity seen using DCNB as substrate in the pre-neoplastic rat liver cytosol is consistent with the large increase in the peak containing the 3:3 isoenzyme (it is reported that isoenzymes containing the 3 subunit are more efficient at catalysing the conjugation of this particular model substrate [21]). The presence of the 7:7 isoenzyme in pre-neoplastic and neoplastic liver tissue has been the subject of many reports in the literature [22–24]. Neither the peak containing the enhanced level of the 3:3 isoenzyme (Fraction B, Fig. 2b) nor the 7:7 containing peak (Fraction D, Fig. 2b) was found to be significantly effective in catalysing conjugation of activated AFB_1 with GSH in the present study.

The I.E.F. gel fractionations of mouse cytosols indicated the presence of five peaks catalysing the conjugation of CDNB. Comparison with the results of Lee *et al.* [25] indicated the peak D pH 6.5 in the present study (Fig. 2c) as their Fraction 1 and similarly peak C pH 7.5 as Fraction 2, peak B pH 7.9 as Fraction 3 and peak A pH 9.4 as Fraction 4, although the isoelectric points of peaks B and C observed in the present study were rather lower (0.5–0.75 pH units) than those reported by Lee *et al.* [25] for their Fractions 3 and 2. The peak at pH 5.5 (Fraction E, Fig. 2c) was not observed by Lee *et al.* [25]. The activated AFB_1-conjugating activity present in the mouse cytosol was associated with the most basic fraction which was not the most active in conjugating CDNB. GST activity was visualised on analytical I.E.F. gels using mBrB as the fluorigenic substrate. This was validated by the dependence of the reaction on the presence of GSH, and the similarity between using CDNB and mBrB as substrates in *in vitro* assays with unfractionated cytosols or fractions from I.E.F. granular gels. The presence of a basic isoenzyme in the mouse cytosol, possibly that associated with the high AFB_1-conjugating activity, was observed on the analytical isoelectric focusing gels (Fig. 3).

When the cytosol of pre-neoplastic nodular rat livers was run on the I.E.F. analytical gels, the presence of a basic fraction having mBrB conjugating activity at a similar pH to the basic mouse fraction was detected, but was not observed when the control rat preparation was used. It is not known at present if this isoenzyme plays any role in the high activated AFB_1-conjugating capacity observed in unfractionated cytosols in Fraction A (Fig. 2b) in granular gel separations of pre-neoplastic nodular rat liver cytosol and its relationship with the reported AFB_1-conjugating capacity of the 1:1, 1:2 and 2:2 basic isoenzymes [7, 8]. Studies are being carried out examining this possibility.

In summary, although the activities of the individual unfractionated cytosols to conjugate CDNB paralleled their capacities to conjugate activated AFB_1 (and also the sensitivities of the species to the toxin) the separations on granular gels demonstrated that no actual correlation existed between these two activities in terms of the various isoenzymes present. It would appear therefore that in studies of the development of resistance to drugs and toxins, the use of model substrates may not give a very good understanding of the process, and could be actually misleading.

Acknowledgements—The authors are grateful to Dr. Kelvin Cain for advice on the use of electrofocusing techniques. They also wish to thank Mrs. B. Craggs for typing the manuscript.

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