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DIFFERENTIAL INDUCTION OF RAT HEPATIC GLUTATHIONE *S*-TRANSFERASE ISOENZYMES BY HEXACHLOROBENZENE AND BENZYL ISOTHIOCYANATE

COMPARISON WITH INDUCTION BY PHENOBARBITAL AND 3-METHYLCHOLANTHRENE

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Abstract—Male Wistar rats were treated with hexachlorobenzene, benzyl isothiocyanate, phenobarbital or 3-methylcholanthrene. Hepatic cytosolic glutathione *S*-transferase (GST) activity was determined with the substrates 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, ethacrynic acid and *trans*-4-phenyl-3-buten-2-one. Cytosolic glutathione peroxidase activity was measured with cumene hydroperoxide.

GST activity toward 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene and ethacrynic acid was enhanced by all compounds, hexachlorobenzene and 3-methylcholanthrene causing the largest and the smallest increase respectively. *Trans*-4-phenyl-3-buten-2-one-conjugating activity exhibited only small changes, while peroxidase activity with cumeme hydroperoxide was not changed by any of the inducing agents.

GST isoenzymes were purified on *S*-hexylglutathione Sepharose 6B and separated by means of FPLC-chromatofocusing, to evaluate effects on the GST isoenzyme pattern.

Hexachlorobenzene and phenobarbital both caused an increase in the relative amounts of subunits 1 and 3 when compared with subunits 2 and 4 respectively. For 3-methylcholanthrene only induction of subunit 1 was observed, possibly due to the relatively low induction levels of total GST activity.

In benzyl isothiocyanate-treated animals, an induction of subunit 3 was found as well as an increase in the relative amount of subunit 2. Thus, benzyl isothiocyanate behaves differently from hexachlorobenzene, phenobarbital and 3-methylcholanthrene as an inducing agent of rat hepatic glutathione *S*-transferases.

The glutathione *S*-transferases (EC 2.5.1.18) are a family of isoenzymes catalyzing the conjugation of a wide variety of hydrophobic electrophilic compounds with glutathione (GSH). At least ten different isoenzymes occur in rat liver cytosol of which six have been well documented [1–4]. They are dimeric proteins formed by binary combinations of four subunits (1 to 4) and exhibit a broad but overlapping substrate specificity.

Three classes of cytosolic glutathione *S*-transferases (alpha, mu and pi), common to several mammalian species, have recently been identified [5]. Each class comprises isoenzymes with similar structural and enzymatic properties. Rat liver isoenzymes containing subunits 1 and/or 2 belong to class alpha, while glutathione *S*-transferases formed by combinations of subunits 3 and/or 4 are members of class mu [5].

Glutathione *S*-transferase (GST) activity in rat liver can be induced by several classes of compounds [6–9]. Not only total GST activity may be enhanced but GST isoenzyme patterns may be changed as well [10–13]. In view of the differences in substrate specificity between the GST isoenzymes, such changes may have a considerable influence on the toxic effects of various xenobiotics, both with regard to detoxification [14] as well as activation [15].

Phenobarbital and 3-methylcholanthrene have been well described as inducers of rat hepatic glutathione *S*-transferases [6, 7, 11–13, 16–19]. A number of these studies were concerned with total GST activity toward several second substrates [7, 19]. From studies using immunoprecipitation and from studies comparing hepatic concentrations of translationally active GST mRNAs, it is clear that both compounds cause elevated levels of subunits 1 and 3, resulting in an increase of isoenzymes containing those subunits [12, 13, 16–18]. In rat testis, however, subunit 4 is induced by phenobarbital [20].

The present study describes a comparison of the effects of hexachlorobenzene and benzyl isothiocyanate on rat hepatic glutathione *S*-transferase isoenzymes with the effects of phenobarbital and 3-methylcholanthrene. Isoenzymes were separated by means of FPLC-chromatofocusing, as described by Ålin *et al.* [2], a technique which allows evaluation

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of relative changes for six isoenzymes in a single run.

Hexachlorobenzene is a waste product in the manufacture of various chlorinated compounds. Prolonged exposure leads to the development of porphyria cutanea tarda, resulting from a defect in the heme biosynthesis pathway [21]. *In vivo* studies of its biotransformation have demonstrated the occurrence of oxygen- and sulphur-containing metabolites, indicating that both cytochrome P-450 and glutathione *S*-transferases are involved in its metabolism [22–24]. Elevated levels of GST activity following hexachlorobenzene-treatment have been found in rat liver [25, 26] and in the liver of the Japanese quail, a species very susceptible to the development of porphyria [27].

Benzyl isothiocyanate is a naturally occurring, anti-carcinogenic agent, which is also metabolized by glutathione *S*-transferases [28]. Benzyl isothiocyanate-treatment causes a substantial induction of GST activity in several organs of the mouse [9]. No studies on effects of benzyl isothiocyanate on rat glutathione *S*-transferases are as yet available.

MATERIALS AND METHODS

Materials. Hexachlorobenzene (BDH Chemicals Ltd., Poole, U.K.), phenobarbital (Interpharm B.V., Rotterdam, The Netherlands), 1-chloro-2,4-dinitrobenzene (Aldrich Chemical Co., Milwaukee, WI), ethacrynic acid (Sigma Chemical Co., St. Louis, MO), GSH (Merck, Darmstadt, F.R.G.), Servalyt 4-9T and Servalyt 9-11T (Serva Feinbiochemica, Heidelberg, F.R.G.) were purchased from the companies indicated. Benzyl isothiocyanate and 3-methylcholanthrene were obtained from Fluka AG (Buchs, Switzerland). *Trans*-4-phenyl-3-buten-2-one and 1,2-dichloro-4-nitrobenzene were from Janssen Chimica (Beerse, Belgium). All other chemicals used were standard commercial products of analytical purity.

Epoxy-activated Sepharose 6B and Bio-Gel P-6 DG were from Pharmacia Fine Chemicals (Uppsala, Sweden) and from Bio-Rad Laboratories (Richmond, CA) respectively.

S-hexylglutathione was synthesized according to Vince *et al.* [29] and coupled to epoxy-activated Sepharose 6B as described by Mannervik and Guthenberg [30].

Treatment of animals. Male Wistar rats (260–310 g) received one of the following treatments: 1 g hexachlorobenzene/kg food, prepared from a 1% (w/v) solution in olive oil, *ad libitum*, for 14 days; 5 g benzyl isothiocyanate/kg food, prepared from a 5% (w/v) solution in olive oil, *ad libitum*, for 14 days; 0.1% (w/v) phenobarbital in the drinking-water, *ad libitum*, for 7 days; three intraperitoneal injections of 3-methylcholanthrene (30 mg/kg, in olive oil) on three consecutive days.

Control rats received similar treatments, i.e. olive oil in food, plain drinking water or three intraperitoneal injections of olive oil.

Purification of glutathione S-transferase isoenzymes. Glutathione *S*-transferase isoenzymes were purified essentially as described by Ålin *et al.* [2]. Livers were perfused with 25 mM Tris–HCl pH 7.4, containing 0.25 M sucrose, and homogenized in the same buffer. The homogenates were centrifuged at 16,000 *g* for 20 min to remove cell debris. The cytosol fraction was obtained by centrifugation of the resulting supernatant at 90,000 *g* for 90 min.

The cytosol fraction from one liver was filtered over glass-wool and loaded onto an affinity column of *S*-hexyl GSH Sepharose 6B (2.5 × 8 cm), equilibrated with 100 mM Tris–HCl pH 7.8/1 mM EDTA/0.2 mM dithiothreitol (DTT) (buffer A). The column was washed with approx. 400 ml of buffer A, containing 200 mM NaCl, until no more protein could be detected in the effluent. The bound glutathione *S*-transferases were eluted with a 200 ml gradient of 0–2.5 mM *S*-hexyl GSH in the salt-fortified buffer. The GST-containing fractions (approx. 90 ml) were pooled, concentrated by ultrafiltration (Amicon Diaflo YM-10 filter) to approx. 6 ml and desalted on a Bio-Gel P-6 DG column (2.5 × 11 cm), equilibrated with 5 mM Tris–HCl pH 8.0/1 mM EDTA/0.2 mM DTT. The resulting GST-pool was again concentrated by ultrafiltration to approx. 6 ml. GST isoenzymes were subsequently separated by chromatofocusing on a Mono P™ HR 5/20 column (Pharmacia FPLC system), equilibrated with 25 mM triethylamine–HCl, pH 10. One-millilitre fractions of the GST concentrate, containing approx. 5 mg of protein, were adjusted to pH 9 with 25 mM triethylamine immediately before chromatofocusing. The eluant consisted of a 1 : 200 dilution of a mixture of 1 ml Servalyt 4-9T and 1 ml Servalyt 9-11T, adjusted to pH 7 with HCl.

A prerun of 2 ml of the eluant was carried out before injection of the above 1 ml sample onto the column. A flow rate of 1 ml/min was used throughout the run, and the effluent was monitored continuously at 280 nm. Individual isoenzymes were collected in separate fractions and samples from each fraction were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli [31], using 17.5% (w/v) polyacrylamide gels. Finally, ampholytes were removed on a Bio-Gel P-6 DG column (1.5 × 16 cm), equilibrated with 25 mM potassium phosphate buffer pH 7.4/1 mM EDTA/0.2 mM DTT. All steps up to chromatofocusing were performed at 4°. Chromatofocusing and subsequent removal of ampholytes were carried out at room temperature.

Enzyme assays. Glutathione *S*-transferase activity toward 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, ethacrynic acid and *trans*-4-phenyl-3-buten-2-one was measured spectrophotometrically at 25°, according to Habig *et al.* [32]. Peroxidase activity was measured with cumene hydroperoxide as described by Lawrence and Burk [33].

Microsomal GST activity was measured with 1-chloro-2,4-dinitrobenzene as the second substrate, using microsomes washed twice in 0.15 M Tris–HCl, pH 8.0, to remove contaminating cytoplasm. The extent of removal was assessed by measuring lactate dehydrogenase activity according to Mitchell *et al.* [34]. Less than 3% of the lactate dehydrogenase activity in unwashed microsomes remained after two washes.

Protein. Protein concentrations were determined by the method of Lowry *et al.* [35], with bovine serum albumin as a standard. For the isoenzyme

Table 1. Effects of hexachlorobenzene (HCB), benzyl isothiocyanate (BITC), phenobarbital (PB) and 3-methylcholanthrene (3MC) on rat hepatic cytosolic glutathione *S*-transferase activities toward several substrates

Treatment	N	Enzyme activity with: CDNB* (nmol min^{-1} mg^{-1})	DCNB* (nmol min^{-1} mg^{-1})	EA* (nmol min^{-1} mg^{-1})	TPBO* (nmol min^{-1} mg^{-1})	Cumene hydroperoxide (nmol min^{-1} mg^{-1})
None (HCB/BITC control)	4	1590 ± 50	54 ± 4	34 ± 2	28 ± 3	530 ± 50
HCB	4	5570 ± 170 (350)‖	157 ± 12 (291)‖	160 ± 14 (471)‖	37 ± 3 (132)	490 ± 80 (92)
BITC	4	3160 ± 400 (199)‖	118 ± 11 (219)‖	60 ± 4 (176)‖	38 ± 4 (136)†	540 ± 60 (102)
None (PB control)	2	2220 ± 100	69 ± 1	38 ± 4	33 ± 7	570 ± 40
PB	2	4860 ± 390 (219)§	149 ± 9 (216)§	68 ± 4 (179)‡	39 ± 1 (118)	640 ± 20 (112)
None (3MC control)	2	1780 ± 10	56 ± 2	32 ± 3	21 ± 8	540 ± 40
3MC	4	2980 ± 180 (167)‡	79 ± 3 (141)§	44 ± 2 (138)‡	28 ± 1 (133)	500 ± 50 (93)

Male Wistar rats (260–310 g) were treated with 0.1% (w/w) hexachlorobenzene in food for 14 days, 0.5% (w/w) benzyl isothiocyanate in food for 14 days, 0.1% (w/v) phenobarbital in drinking water for 7 days, or 3 i.p. injections of 3-methylcholanthrene (30 mg/kg/day) on 3 successive days. Controls received olive oil in food, plain drinking water, or i.p. injections of olive oil. GST activities toward 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, ethacrynic acid and *trans*-4-phenyl-3-buten-2-one were measured at 25° according to Habig *et al.* [31]. Activity with cumene hydroperoxide was measured at 25° by the method of Lawrence and Burk [32]. Values are expressed as means ± SEM. Percentage of control is given in parentheses.

* CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; EA, ethacrynic acid; TPBO, *trans*-4-phenyl-3-buten-2-one.

† $P < 0.05$ (one-sided Student's *t*-test).

‡ $P < 0.025$ (one sided Student's *t*-test).

§ $P < 0.01$ (one-sided Student's *t*-test).

‖ $P < 0.005$ (one-sided Student's *t*-test).

fractions, a modified procedure, described by Peterson [36], was followed.

RESULTS

Effects on body weights and liver weights

Initial body weights, final body weights and liver weights were recorded during the experiments. Hexachlorobenzene-treated animals exhibited lower weight gain (+13 ± 13 g) than their controls (+49 ± 11 g), while benzyl isothiocyanate-treatment resulted in a loss of body weight (−35 ± 6 g). All compounds, with the exception of benzyl isothiocyanate, caused an increase in liver weight expressed as a percentage of body weight. No change was observed for benzyl isothiocyanate; actual liver weights of benzyl isothiocyanate-treated rats were lower than those of their respective controls, due to the overall loss of body weight.

Effects on glutathione S-transferase activities

Cytosolic hepatic GST activities were determined using several second substrates (Table 1). Hexachlorobenzene increased GST activity with 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene to 3 to 4 times control levels, while in benzyl isothiocyanate-, phenobarbital-, and 3-methylcholanthrene-treated animals an approx. 2-fold stimulation was found. GST activity toward ethacrynic acid was enhanced by all compounds, hexachlorobenzene and 3-methylcholanthrene again causing the largest and the smallest increase respectively.

Changes in the formation of the conjugate of *trans*-4-phenyl-3-buten-2-one were relatively small. Peroxidase activity towards cumene hydroperoxide was unchanged in all groups.

Microsomal GST activity with 1-chloro-2,4-dinitrobenzene was determined in microsomes washed twice in 0.15 M Tris–HCl pH 8.0 and was not enhanced by any of the inducing agents (results not shown).

Effects on glutathione S-transferase isoenzyme patterns

Glutathione *S*-transferases were purified by use of affinity chromatography. Approximately 5% of the activity loaded onto the affinity column was not retained. This percentage was similar for control and for treated samples. The total amount of GST protein recovered from the affinity column followed by the Bio-Gel P-6 DG desalting column was increased from 1.7 ± 0.3 mg/g liver in control rats to 3.9 ± 0.3 mg/g liver in hexachlorobenzene-treated and 3.0 ± 0.5 mg/g liver in benzyl isothiocyanate-treated animals. Following phenobarbital- and 3-methylcholanthrene-treatment, GST protein amounted to 2.8 ± 0.2 mg/g liver and 2.1 ± 0.0 mg/g liver respectively.

Isoenzymes were separated by means of FPLC-chromatofocusing, and identified by their position in the elution profile, their subunit molecular weights and the specific activities toward 1-chloro-2,4-dinitrobenzene. SDS–PAGE of FPLC isoenzyme fractions showed that isoenzyme 2-2 was always contaminated with subunit 1, while isoenzyme 3-3 contained a small amount of subunit 2 only in some separations.

FPLC isoenzyme patterns are shown in Fig. 1. Since no difference could be detected between the different controls, only the hexachlorobenzene/benzyl isothiocyanate control pattern is given (Fig. 1A). From Figs 1B and 1D it is clear that both hexachlorobenzene and phenobarbital caused an increase in the relative concentrations of isoenzymes 1-1 and 3-3 as well as a decrease in the relative amounts of isoenzymes 2-2 and 4-4, the effect of hexachlorobenzene being somewhat stronger than the effect of phenobarbital.

The induction of subunits 1 and 3 by hexachlorobenzene and phenobarbital was also demonstrated by the relative amounts of protein contained in subunits 1 to 4 (Fig. 2). Both treatments resulted in an increase of the relative amounts of protein represented by subunits 1 and 3 when compared with subunits 2 and 4 respectively.

Interestingly, the majority of protein was accounted for by subunits 1 and 2 in treated animals, while in controls the reverse situation was observed.

Benzyl isothiocyanate did not induce subunit 1, but comparing Figs 1A and 1C isoenzyme 2-2 seems to be enhanced. The induction of subunit 2 was confirmed by an increase in the relative amount of protein represented by this subunit (Fig. 2).

Although the effect of benzyl isothiocyanate on isoenzyme 3-3 was less pronounced than in the case of hexachlorobenzene and of phenobarbital, isoenzymes 3-3 and 3-4 were increased when compared with isoenzyme 4-4, suggesting that subunit 3 was in fact induced. The latter also seemed to hold for 3-methylcholanthrene (Fig. 1E). However, in this case the relative amount of protein contained in subunit 3 was not increased, indicating that no induction of subunit 3 had occurred. 3-Methylcholanthrene-treatment only resulted in an induction of isoenzyme 1-1 (Fig. 1E).

DISCUSSION

The present study demonstrates that treatment of male Wistar rats with hexachlorobenzene, benzyl isothiocyanate, phenobarbital or 3-methylcholanthrene not only results in elevated levels of hepatic cytosolic glutathione *S*-transferases, but causes changes in the GST isoenzyme pattern as well. The technique of FPLC-chromatofocusing has proved a unique tool in the evaluation of relative changes in the GST isoenzyme pattern, both on an isoenzyme and on a subunit level. Although the four compounds investigated have rather different chemical structures, three of these, hexachlorobenzene, phenobarbital and 3-methylcholanthrene demonstrate similar effects, i.e. a preferential induction of subunits 1 and 3. Benzyl isothiocyanate differs from the other inducing agents in that this compound does not enhance the relative amount of subunit 1, but causes an increase in subunit 2 instead. However, all inducers demonstrate one common effect: alpha-class subunits are induced to a greater extent than mu-class subunits, since subunits 1 and 2 represent 53–60% of the GST protein in treated animals,

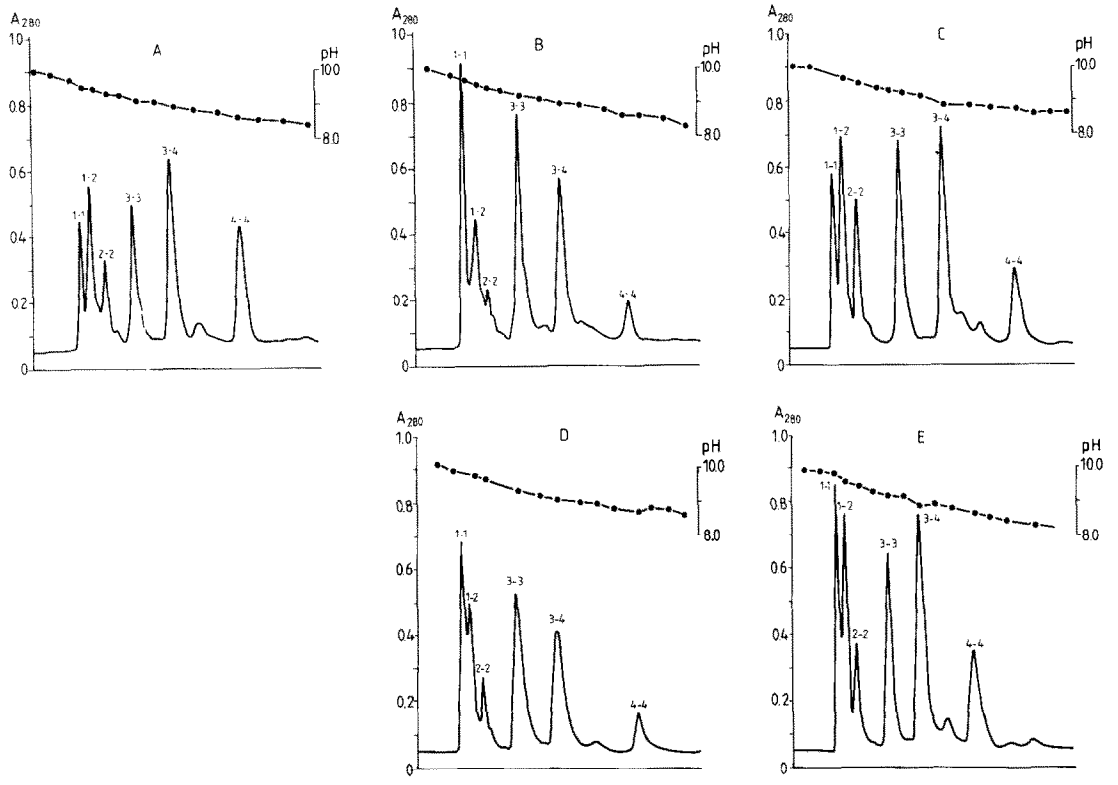

Fig. 1. Glutathione *S*-transferase isoenzyme patterns in rat liver from control (A), hexachlorobenzene (B), benzyl isothiocyanate (C), phenobarbital (D), and 3-methylcholanthrene-treated (E) rats. For treatment of animals see Materials and Methods. GST were purified on *S*-hexylglutathione Sepharose 6B and isoenzymes were separated by means of chromatofocusing on a Mono p™ HR 5/20 column. The eluant for chromatofocusing consisted of a 1:200 dilution of a mixture of 1 ml Servalyt 4-9T and 1 ml Servalyt 9-11T, adjusted to pH 7 with HCl. Individual isoenzymes were collected in separate fractions, and identified by their position in the elution profile, their subunit molecular weights and their specific activities toward 1-chloro-2,4-dinitrobenzene.

whereas only 38–45% of the protein can be accounted for by these subunits in controls.

The mechanism of induction of rat hepatic cytosolic glutathione *S*-transferases is still unknown. Alpha-class subunits 1 and 2 demonstrate approx. 68% amino acid sequence homology, but they are coded for by separate mRNAs, which are regulated independently [37–39]. Similarly, mu-class subunits 3 and 4 are closely related but distinctly different polypeptides, for which separate mRNAs exist [18, 40]. Little sequence homology is, however, shared by alpha- and mu-class subunits, which, therefore, seem to represent two distinct gene families [41].

By use of hybridization experiments with specific cDNA clones, Ding *et al.* [18] and Pickett *et al.* [17, 41] demonstrated that phenobarbital and 3-methylcholanthrene cause a 5- to 10-fold increase in

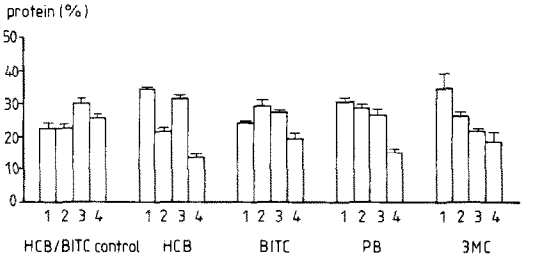

Fig. 2. Effects of hexachlorobenzene, benzyl isothiocyanate, phenobarbital and 3-methylcholanthrene on the relative amounts of protein represented by subunits 1 to 4. Relative amounts of protein were calculated from FPLC isoenzyme fractions, as follows:

$$\text{Protein}_{\text{isoenzyme}} = \frac{\text{Activity}^*_{\text{isoenzyme}}}{\text{Mean specific activity}\dagger_{\text{isoenzyme}}}$$

$$\%\ \text{Protein}_{\text{subunit}} = \frac{\text{Protein}_{\text{homodimer}} + \frac{1}{2}\,\text{Protein}_{\text{heterodimer}}}{\text{Protein}_{\text{isoenzymes 1-1 to 4-4}}} \times 100$$

Values represent means ± SEM.

* Total 1-chloro-2,4-dinitrobenzene-conjugating activity in FPLC isoenzyme fractions.

† Specific activities with 1-chloro-2,4-dinitrobenzene were determined in isoenzyme fractions from which ampholytes had been removed on Bio-Gel P-6 DG. Mean specific activities used were: isoenzyme 1-1: 60 μmol min^{-1} mg^{-1}; isoenzyme 1-2: 39 μmol min^{-1} mg^{-1}; isoenzyme 2-2: 30 μmol min^{-1} mg^{-1}; isoenzyme 3-3: 46 μmol min^{-1} mg^{-1}; isoenzyme 3-4: 39 μmol min^{-1} mg^{-1}; isoenzyme 4-4: 19 μmol min^{-1} mg^{-1}.

the level of subunit 1-mRNA and a 5- to 6-fold elevation of mRNAs coding for subunits 3 and 4, in rat liver, 16–24 hr following administration. For subunit 2-mRNA, only a 2-fold induction was found [17, 18, 41]. Activation of the transcription of glutathione *S*-transferase genes by phenobarbital and 3-methylcholanthrene appeared to account at least partly for the observed increase in mRNA-levels [41, 42].

No information is at present available on the mechanism of transcriptional activation of glutathione *S*-transferase genes by xenobiotics. There is no evidence for the involvement of specific receptors in rat liver to which xenobiotics or their metabolites could bind, in analogy to the AH-receptor-mediated induction of P450c by 3-methylcholanthrene [41, 42].

Our results clearly confirm on a protein level that the synthesis of individual subunits is regulated independently. The absolute amount of protein per subunit per gram liver can be estimated from the relative amounts of protein represented by individual subunits and the total amount of GST protein per gram liver. Following treatment with phenobarbital or 3-methylcholanthrene, no increase in subunit 4 is then found, while the increase in subunit 2 as compared with subunits 1, 3 and 4 is larger than expected on the basis of the increases in mRNA levels described above. Differences in translational efficiency and/or turnover rates between mRNAs coding for individual subunits may exist *in vivo*, indicating the importance of evaluating the induction of glutathione *S*-transferase isoenzymes on a protein level.

The differential behaviour of benzyl isothiocyanate as compared with the other compounds used, indicates that, although the exact mechanism of transcriptional activation of GST genes remains to be elucidated, some relationship may exist between chemical structure of the inducing agent and subunit induction. However, irrespective of the chemical structure of the administered xenobiotic, alpha-class subunits seem to be enhanced preferentially.

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