Increased bioactivation of dihaloalkanes in rat liver due to induction of class Theta glutathione S-transferase T1-1

Philip J. SHERRATT*[1], Margaret M. MANSON†, Anne M. THOMSON*, Erna A. M. HISSINK‡, Gordon E. NEAL†, Peter J. VAN BLADEREN‡, Trevor GREEN§ and John D. HAYES*

*Biomedical Research Centre, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, Scotland, U.K., †Medical Research Council Toxicology Unit, Hodgkin Building, University of Leicester, P.O. Box 138, Lancaster Road, Leicester LE1 9HN, U.K., ‡Department of Biological Toxicology, TNO Nutrition and Food Research, P.O. Box 360, 3700 AJ Zeist, The Netherlands, and §Zeneca Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire SK10 4TJ, U.K.

A characteristic feature of the class Theta glutathione S-transferase (GST) T1-1 is its ability to activate dichloromethane and dibromoethane by catalysing the formation of mutagenic conjugates. The level of the GSTT1 subunit within tissues is an important determinant of susceptibility to the carcinogenic effects of these dihaloalkanes. In the present study it is demonstrated that hepatic GST activity towards these compounds can be elevated significantly in female and male Fischer-344 rats by feeding these animals on diets supplemented with cancer chemopreventive agents. Immunoblotting experiments showed that increased activity towards the dihaloalkanes is associated with elevated levels of the GSTT1 subunit in rat liver. Sex-specific effects were observed in the induction of GSTT1 protein. Amongst the chemopreventive agents tested, indole-3-carbinol proved to be the most potent inducer of hepatic GSTT1 in male rats (6.2-fold), whereas coumarin was the most potent inducer of this subunit in the livers of female rats (3.5-fold). Phenobarbital showed significant induction of GSTT1 only in male rat liver and had little effect in female rat liver. Western blotting showed that class Alpha, Mu and Pi GST subunits are not co-ordinately induced with GSTT1, indicating that the expression of *GSTT1* is determined, at least in part, by mechanisms distinct from those that regulate levels of other transferases. The increase in amount of hepatic GSTT1 protein was also reflected by an increase in the steady-state level of mRNA in response to treatment with chemopreventive agents and model inducers. Immunohistochemical detection of GSTT1 in rat liver supported the Western blotting data, but showed, in addition to cytoplasmic staining, significant nuclear localization of the enzyme in hepatocytes from some treated animals, including those fed on an oltipraz-containing diet. Significantly, the hepatic level of cytochrome *P*-450 2E1, an enzyme which offers a detoxification pathway for dihaloalkanes, was unchanged by the various inducing agents studied. It is concluded that the induction of GSTT1 by dietary components and its localization within cells are important factors that should be considered when assessing the risk dihaloalkanes pose to human health.

INTRODUCTION

The dihaloalkanes are a group of chemicals that have a wide range of applications for both the consumer and industry. These compounds are employed because of a combination of useful properties that include low boiling-point, high solvency power, chemical inertness, low toxicity and non-inflammability. Two dihaloalkanes which have attracted particular interest are dichloromethane (DCM) and dibromoethane (DBE). The former compound is mass-produced and is widely used in paint and varnish strippers, the synthesis of plastics, the manufacture of film and in the synthesis of pharmaceutical drugs. The latter compound is also produced on a large scale, primarily for use as a lead-scavenging agent in anti-knock mixtures added to gasoline [1]

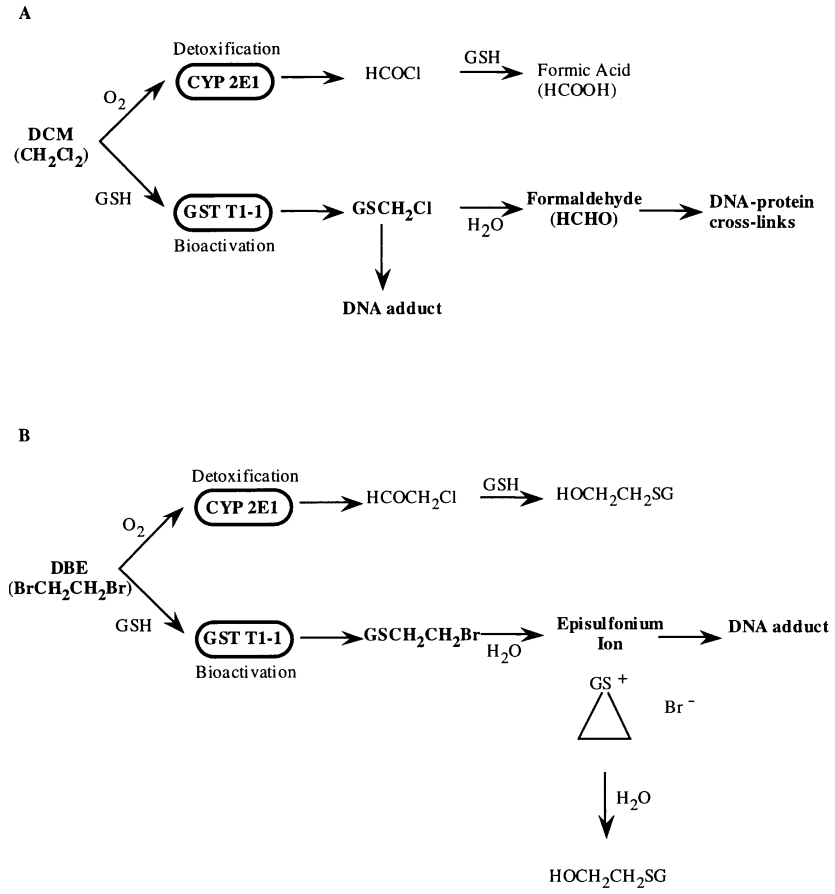
DCM and DBE are currently anticipated to be human carcinogens following toxicology and carcinogenicity tests carried out as part of the American National Toxicology Program [2,3]. The tests conducted have shown that DCM increases the incidence of pulmonary and hepatic neoplasms in female and male mice. The data obtained also suggested that DCM exerts some carcinogenic effects in rats, particularly female rats, although to a lesser extent than in the mouse. DBE was found in carcinogenicity tests to give positive results in mice and rats of both sexes. As a consequence, restrictions have been enforced upon the use of both DCM and DBE.

Two major metabolic pathways have been elucidated for the biotransformation of dihaloalkanes (Scheme 1). The first pathway is a detoxification process beginning with oxidation of the dihaloalkane by cytochrome *P*-450 2E1 (CYP 2E1) [4]. The second pathway is a toxification route where a nucleophilic substitution of thiolate for halide occurs in a reaction catalysed by glutathione S-transferase (GST) [5]. This reaction produces *S*-haloalkylglutathione conjugates which are more reactive than the parent compound. The DCM intermediate, *S*-chloromethylglutathione, has the capacity to undergo a further nucleophilic substitution with cellular macromolecules such as DNA. This intermediate, however, is short lived and rapidly hydrolyses to give formaldehyde as a product, which itself is a potent cross-linking agent. The DBE intermediate, *S*-2-bromoethylglutathione, spontaneously rearranges to eliminate the remaining halogen atom, creating an episulphonium ion, a powerful electrophile. The class Theta enzyme, GST T1-1, is almost exclusively responsible for the activation of DCM and is also the most efficient transferase for activating DBE [6–10]

Notable species differences occur in sensitivity to DCM. This correlates with the ability to conjugate dihaloalkanes with GSH. In carcinogenicity tests, the mouse has been shown to be

Abbreviations used: Ah, arylhydrocarbon; ARE, antioxidant responsive element; BHA, butylated hydroxyanisole; CDNB, 1-chloro-2,4-dinitrobenzene; CYP 2E1, cytochrome *P*-450 2E1; DBE, dibromoethane; DCM, dichloromethane; DEM, diethylmaleate; ECL, enhanced chemiluminescence; ENPP, 1,2-epoxy-3-(4′-nitrophenoxy)propane; GST, glutathione S-transferase; MS, 1-menaphthyl sulphate; NBDC, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; β-NF, β-naphthoflavone; PB, phenobarbital; Pefabloc, 4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride; tSO, *trans*-stilbene oxide.

[1] To whom correspondence should be addressed (e-mail P.J.Sherratt@dundee.ac.uk).



Scheme 1 Metabolism of dihaloalkanes

(**A**) Two metabolic routes for DCM. The first is a saturable detoxification pathway proceeding via oxidation by CYP 2E1 ; the second is a higher-capacity bioactivation pathway where the dihaloalkane is directly metabolized by GST T1-1 to produce the reactive *S*-chloromethylglutathione intermediate. (**B**) The metabolism of DBE, which utilizes the same two metabolic routes as in (**A**). In this instance, the resulting intermediate of GSH conjugation, *S*-2-bromoethylglutathione, rearranges spontaneously to eliminate the remaining halogen to yield an episulphonium ion, which is a highly potent DNA alkylating agent.

considerably more sensitive to exposure to DCM than the rat and hamster [11–13]. The selective toxicity of DCM in the mouse is attributed to the fact that this species constitutively expresses GST T1-1 in liver and lung at a higher level than is found in either rat or hamster. As these rodent species all share a similar metabolic detoxification capacity via the CYP 2E1 pathway, the sensitivity of the mouse to DCM appears to be due to its greater capacity to activate DCM by a GST-dependent mechanism. In addition to differences in basal expression, the sensitivity of the mouse may also be influenced by the localization of the activating transferase in cell nuclei [14]. It appears likely that the ability of the unstable *S*-chloromethylglutathione intermediate to cause genotoxic damage will be increased if it is generated in close proximity to DNA [15]. Consistent with this proposal, Thier et al. [8] found that during mutagenicity testing of dihaloalkanes, marked differences were obtained when GST T1-1 was added exogenously as opposed to being expressed within the *Salmonella* tester strain. In contrast with DCM, DBE does not display a marked species-specific toxicity, possibly because other transferases which play a role in its activation, notably the class Alpha and Mu GST, are more uniformly distributed than GST T1-1.

In common with other drug-metabolizing enzymes, the level of GST expression can be influenced by natural dietary compounds, synthetic antioxidants, GSH-depleting agents and cancer chemopreventive drugs [6,16,17]. These changes in expression are most apparent in the liver and gastrointestinal tract [6]. Exposure to such inducing agents usually results in transcriptional activation of multiple class Alpha, Mu and Pi GST genes, and causes an enhanced capacity to metabolize noxious chemicals [6,18]. It is not known whether *GSTT1* is inducible by xenobiotics. However, it was noted recently that GST T1-1 is over-expressed in the livers of selenium-deficient rats [19], suggesting that the level of this enzyme might be responsive to various stimuli, including phytochemicals and drugs.

In the present study, female and male rats were treated with various inducers of drug-metabolizing enzymes to determine whether dietary factors or the administration of xenobiotics might increase the capacity to activate DCM or DBE. Enzyme assays and immunoblotting experiments have shown that many compounds can increase the ability of rat liver extracts to catalyse the activation of dihaloalkanes and that this is associated with elevated levels of the GSTT1 subunit. Immunohistochemistry has demonstrated an increase in staining for GST T1-1 in treated rat liver, and revealed its presence in the nucleus of many hepatocytes. Together, these effects of xenobiotics on the expression and location of GSTT1 in rat liver are likely to result in treated animals having an increased sensitivity to the toxic effects of DCM and DBE.

MATERIALS AND METHODS

Chemicals and reagents

Chemicals for enzyme assays were all obtained from Sigma Chemical Co. (Poole, Dorset, U.K.), except 1-menaphthyl sulphate (MS) which was custom synthesized by Ultrafine Chemicals (Manchester, U.K.) and DCM which was purchased from BDH/Merck (Thornliebank, Glasgow, Scotland, U.K.). Pefabloc [4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride], a serine protease inhibitor, was obtained from Pentapharm AG (Basel, Switzerland). The DBE was purchased from Aldrich (Milwaukee, WI, U.S.A.) whereas [^{14}C]DBE and *S*-hexylglutathione were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals used were from commonly available sources. Immobilon-P transfer membrane was purchased from Millipore (Watford, Herts., U.K.). Hybond-N transfer membrane and Rapid-hyb rate-enhancing hybridization buffer for use with radiolabelled nucleic acid probes were obtained from Amersham Life Science (Little Chalfont, Buckinghamshire, U.K.). Chroma Spin™ columns for purification of radiolabelled nucleic acid probes were purchased from Clontech Laboratories Inc. (Palo Alto, CA, U.S.A.). The Brownlee C_{18} reversed-phase HPLC column was from Anachem (Luton, Bedfordshire, U.K.) and the C_{18} Hypersil ODS 5 reversed-phase column was from Chrompack (Middelburg, The Netherlands).

Animals

Female and male Fischer-344 rats were used throughout the study and were purchased from Harlan Olac Ltd. (Bicester, Oxon., U.K.). The animals were obtained at 10 weeks of age and were allowed to acclimatize for 2 weeks on standard powdered RM1 diet (SDS Ltd., Witham, Essex, U.K.) containing 2% (v/v) arachis oil before being treated with xenobiotics as outlined in Table 1. Throughout the acclimatization period and during the experiment, the rats were housed in Moredun isolators purchased from Moredun Animal Health Ltd. (Edinburgh, Midlothian, Scotland, U.K.) under negative pressure with 12 h light/dark cycles. Food and water were provided *ad libitum*.

Preparation of rat liver extract for biochemical analysis

All enzyme assays were carried out at 37 °C. Replicate assays were performed using hepatic extracts prepared from a pool of three livers from similarly treated rats. Two types of hepatic extract were used to measure GST activity. A 15000 ***g*** supernatant fraction was prepared from portions of liver (approx. 100 mg) that were allowed to thaw in 4 vol. of ice-cold 0.1 M sodium phosphate buffer, pH 6.9, containing 0.05% (v/v) Nonidet P-40, 2.5 mg/ml Pefabloc protease inhibitor and 1 mM dithiothreitol (buffer A). Soluble liver extracts from this material were obtained after homogenization using an Omni EZ Connect Homogeniser (Omni International, Gainsville, VA, U.S.A.) and centrifugation to remove cellular debris 15000 ***g*** (for 10 min, 4 °C). These extracts were used to measure activity towards DBE and 1,2-epoxy-3-(4′-nitrophenoxy)propane (ENPP). A 100000 ***g*** supernatant fraction was prepared from a total of 1 g of rat liver taken from the three available specimens and thawed in 4 vol. of buffer A. After homogenization, the cytosol was prepared by collection of the supernatants following two centrifugation steps, 17000 ***g*** (for 45 min, 4 °C) and 100000 ***g*** (for 90 min, 4 °C); the 100000 ***g*** pellet is referred to as microsomes and was retained to measure levels of CYP 2E1 (see below). These cytosols were used to measure GST activity towards DCM, MS, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBDC) and 1-chloro-2,4-dinitrobenzene (CDNB).

Measurement of enzyme activities

Transferase activity with ENPP was measured at 360 nm in 100 mM sodium phosphate buffer, pH 6.5, containing 10 mM GSH and 0.5 mM ENPP [20,21]. *S*-Hexylglutathione (2.5 mM) was included to inhibit the activity of class Alpha, Mu and Pi GST subunits present in the reaction mixture [10]. GST activity towards MS was monitored spectrophotometrically at 298 nm in 100 mM sodium phosphate buffer, pH 7.5, by previously described methods [20]. The NBDC–GSH-conjugating and CDNB–GSH-conjugating activities were measured at 419 nm and 340 nm respectively [21,22] using the Cobas Fara II Chemistry System (Hoffmann-La Roche Ltd., Basel, Switzerland) [19]. Enzymic activity towards DCM was measured by pre-incubating a mixture containing 10 mM GSH and aliquots of rat liver cytosol in 100 mM sodium phosphate buffer, pH 7.4. The reaction was started by addition of DCM to a final concentration of 40 mM, giving a final volume of 1.3 ml [23]. The reaction was allowed to continue for 20 min before quenching by addition of 20% (w/v) trichloroacetic acid (100 μl) and incubation on ice. After centrifugation to remove precipitated protein (15000 ***g*** for 10 min at 4 °C), the supernatant was assayed for the production of formaldehyde during the reaction by the method of Nash [24].

GST activity towards DBE was determined radiochemically by measuring the amount of glutathione conjugate formed. Incubations were performed in glass vials with caps treated with Teflon in a reaction mixture of 1 ml containing 0.1 M potassium phosphate buffer, pH 7.4, and 30 mM GSH, 2.5 mM *S*-hexylglutathione, 1.8 mM [^{14}C]DBE (with a specific activity of

Table 1 Dietary and drug treatments employed in the study

Inducing agent	Source and type of compound	Treatment of animals
Control	–	Powdered RM1 diet supplemented with 2% arachis oil for 14 days
Benzyl isothiocyanate	Naturally occurring (in garden cress) chemopreventive agent	0.5% in control diet for 14 days
Coumarin	Naturally occurring (in leguminous vegetables) chemopreventive agent	0.5% in control diet for 14 days
Indole-3-carbinol	Naturally occurring (in cruciferous vegetables) chemopreventive agent	0.5% in control diet for 14 days
BHA	Synthetic antioxidant and chemopreventive agent	0.75% in control diet for 14 days
Ethoxyquin	Synthetic antioxidant and chemopreventive agent	0.5% in control diet for 14 days
Oltipraz	Antischistosomal drug and chemopreventive agent	0.075% in control diet for 14 days
DEM	Synthetic GSH-depleting agent and model inducer of drug-metabolizing enzymes	0.5% in control diet for 14 days
β-NF	Synthetic flavonoid and model inducer of drug-metabolizing enzymes	200 mg/kg daily intraperitoneally for 7 days
PB	Barbiturate, model inducer of drug-metabolizing enzymes	0.1% in drinking water for 7 days
tSO	Synthetic model inducer	400 mg/kg daily intraperitoneally for 3 days

Table 2 Hepatic GST activities in female and male rats after treatment with xenobiotics

Transferase activities were measured on hepatic extracts prepared from female (A) and male (B) rats as described in the Materials and methods section. The numbers in parenthesis represent the mean activity value as a percentage of control. Abbreviations: BITC, benzyl isothiocyanate; I3C, indole-3-carbinol.

(A) Female rat hepatic activity

Treatment	DCM (nmol · min^{-1} · mg of cytosol^{-1})	DBE (nmol · min^{-1} · mg of extract^{-1})	ENPP (nmol · min^{-1} · mg of extract^{-1})	MS (nmol · min^{-1} · mg of cytosol^{-1})	NBDC (μmol · min^{-1} · mg of cytosol^{-1})	CDNB (μmol · min^{-1} · mg of cytosol^{-1})
Control	0.80 ± 0.01 (100)	0.16 ± 0.01 (100)	21.8 ± 1.3 (100)	4.13 ± 0.13 (100)	2.31 ± 0.04 (100)	2.84 ± 0.01 (100)
Ethoxyquin	1.23 ± 0.10[a] (154)	0.27 ± 0.01[a] (172)	72.6 ± 1.2[a] (330)	4.36 ± 0.27 (106)	4.50 ± 0.01[a] (195)	11.46 ± 0.04[a] (404)
BHA	1.29 ± 0.02[a] (161)	0.23 ± 0.02[a] (149)	42.7 ± 1.0[a] (196)	3.66 ± 0.09 (87)	4.13 ± 0.06[a] (179)	7.45 ± 0.04[a] (265)
Oltipraz	1.44 ± 0.05[a] (180)	0.29 ± 0.09 (182)	42.0 ± 3.9[a] (193)	4.31 ± 0.23 (104)	3.71 ± 0.04[a] (161)	5.50 ± 0.02[a] (194)
Coumarin	1.57 ± 0.03[a] (196)	0.55 ± 0.02[a] (352)	76.6 ± 0.3[a] (351)	4.33 ± 0.09 (105)	4.26 ± 0.02[a] (184)	10.05 ± 0.01[a] (354)
PB	1.13 ± 0.07[a] (141)	0.32 ± 0.04[a] (201)	51.6 ± 5.6[a] (237)	3.86 ± 0.30 (93)	4.29 ± 0.03[a] (186)	5.74 ± 0.03[a] (202)
DEM	0.94 ± 0.01[a] (118)	0.26 ± 0.02 (166)	56.7 ± 0.1[a] (260)	3.22 ± 0.08[b] (78)	2.60 ± 0.03[a] (113)	4.95 ± 0.03[a] (174)
BITC	1.40 ± 0.08[a] (175)	0.19 ± 0.01 (123)	40.5 ± 2.4[a] (186)	4.22 ± 0.22 (102)	3.63 ± 0.01[a] (157)	6.42 ± 0.01[a] (226)
β-NF	1.28 ± 0.05[a] (160)	0.17 ± 0.02 (106)	46.8 ± 7.3[a] (215)	3.78 ± 0.12 (92)	6.87 ± 0.05[a] (297)	9.68 ± 0.03[a] (341)
I3C	0.98 ± 0.04[a] (123)	0.24 ± 0.03 (155)	58.8 ± 2.6[a] (270)	3.43 ± 0.07 (83)	4.26 ± 0.02[a] (184)	6.80 ± 0.04[a] (239)
tSO	0.92 ± 0.01[a] (115)	0.17 ± 0.05 (110)	41.4 ± 1.4[a] (190)	4.37 ± 0.17 (106)	5.54 ± 0.05[a] (240)	7.42 ± 0.08[a] (261)

(B) Male rat hepatic activity

Treatment	DCM (nmol · min^{-1} · mg of cytosol^{-1})	DBE (nmol · min^{-1} · mg of extract^{-1})	ENPP (nmol · min^{-1} · mg of extract^{-1})	MS (nmol · min^{-1} · mg of cytosol^{-1})	NBDC (μmol · min^{-1} · mg of cytosol^{-1})	CDNB (μmol · min^{-1} · mg of cytosol^{-1})
Control	0.91 ± 0.02 (100)	0.12 ± 0.01 (100)	21.5 ± 2.7 (100)	3.56 ± 0.08 (100)	2.03 ± 0.03 (100)	3.59 ± 0.04 (100)
Ethoxyquin	1.34 ± 0.01[a] (147)	0.21 ± 0.03[a] (172)	39.7 ± 8.4 (185)	3.71 ± 0.17 (104)	4.93 ± 0.01[a] (243)	9.89 ± 0.03[a] (275)
BHA	1.20 ± 0.04[a] (132)	0.21 ± 0.03[a] (172)	72.6 ± 2.2[a] (338)	3.28 ± 0.08 (92)	3.37 ± 0.01[a] (166)	5.78 ± 0.04[a] (161)
Oltipraz	1.59 ± 0.20[a] (175)	0.24 ± 0.01[a] (200)	68.7 ± 4.9[a] (320)	4.16 ± 0.16[a] (117)	4.31 ± 0.10[a] (212)	7.94 ± 0.02[a] (221)
Coumarin	1.74 ± 0.10[a] (191)	0.34 ± 0.09 (281)	62.6 ± 2.3[a] (291)	3.67 ± 0.17 (103)	5.69 ± 0.01[a] (280)	10.01 ± 0.08[a] (279)
PB	1.47 ± 0.01[a] (162)	0.32 ± 0.03[a] (260)	77.6 ± 0.3[a] (361)	3.66 ± 0.14 (103)	4.79 ± 0.03[a] (236)	7.17 ± 0.04[a] (200)
DEM	1.31 ± 0.07[a] (144)	0.20 ± 0.04 (165)	53.9 ± 3.5[a] (251)	2.82 ± 0.07[b] (79)	3.02 ± 0.02[a] (149)	5.38 ± 0.02[a] (150)
BITC	1.24 ± 0.04[a] (136)	0.26 ± 0.01[a] (214)	62.0 ± 2.2[a] (288)	3.10 ± 0.23 (87)	3.67 ± 0.05[a] (181)	5.23 ± 0.13[a] (146)
β-NF	1.42 ± 0.04[a] (156)	0.18 ± 0.01[a] (145)	82.2 ± 8.0[a] (382)	4.30 ± 0.30 (121)	5.63 ± 0.04[a] (277)	7.85 ± 0.02[a] (219)
I3C	1.88 ± 0.01[a] (207)	0.37 ± 0.10 (302)	93.6 ± 9.9[a] (435)	3.90 ± 0.39 (110)	5.21 ± 0.07[a] (257)	9.55 ± 0.03[a] (266)
tSO	1.34 ± 0.06[a] (147)	0.17 ± 0.03 (140)	45.2 ± 5.6[a] (210)	3.38 ± 0.17 (95)	6.45 ± 0.05[a] (318)	9.50 ± 0.03[a] (265)

[a] Values are significantly greater than control ($P < 0.01$).
[b] Values are significantly lower than control ($P < 0.01$).

4200 dpm/nmol) and 1 mg of hepatic protein. The [^{14}C]DBE was dissolved in DMSO/ethanol (2:3, v/v), resulting in a solvent concentration in the final reaction mixture of 10% (v/v), which is sufficient to inhibit completely CYP 2E1, the enzyme responsible for oxidation of DBE [10]. Mixtures were incubated at 37 °C for 30 min before the reactions were stopped by the addition of 0.3 M HCl and by being placed on ice. The reaction vials were centrifuged and the supernatants analysed by HPLC using a Chrompack C_{18} Hypersil ODS 5 reversed-phase column (250 × 4.6 mm) and a gradient elution (at 1 ml/min) with solvent A consisting of 0.2% (v/v) trifluoroacetic acid in water and solvent B containing 0.2% (v/v) trifluoroacetic acid in acetonitrile. The following gradient programme was used: 0–3 min, 0–1% B; from 3–11 min, 1–15% B; from 11–15 min, 15–95% B; from 15–20 min, 95–100% B. The HPLC system used consisted of a Pharmacia LKB Autosampler 2157 injector (temperature controlled at 4 °C), a Pharmacia LKB HPLC 2248 gradient pump, a Pharmacia VWM 2141 UV detector and an on-line Canberra Packard Radiomatic Detector (type A500, Flo-one beta). A liquid flow cell of 500 μl was used and radioactivity was measured using Flo Scint A, purchased from Packard Instrument Company (Reading, U.K.), at a flow rate of 2 ml/min.

The mean enzyme activities and S.D.s of control and treated rat livers to GST substrates were compared for statistical analysis in unpaired *t*-tests using Instat 2.01 from GraphPad Software (San Diego, CA, U.S.A.).

Protein determination

Protein concentrations were determined using the method of Bradford [25] with reagent obtained from Bio-Rad Laboratories (Hemel Hempstead, Hertfordshire, U.K.).

Western blotting

Soluble hepatic extracts (15000 ***g*** supernatants) were prepared as described above and portions (4 μg of protein) were subjected to SDS/PAGE in 12% (w/v) polyacrylamide resolving gels [26]. Microsomes were also prepared from the treated rat livers by resuspending the pellet from the 100000 ***g*** centrifugation step described above in buffer A, and aliquots (10 μg of protein) were then subjected to SDS/PAGE, also in 12% (w/v) polyacrylamide gels. The resolved soluble and microsomal liver proteins were transferred to Immobilon-P by previously described methods [26,27]. The blotted soluble proteins were probed with polyclonal antiserum raised against rat (r) and human (h) transferases as follows: class Theta (diluted 1:10000 for hGSTT1 and 1:5000 for rGSTT2), class Alpha (diluted 1:20000 for rGSTA1/2, 1:5000 for rGSTA3 and 1:1000 for rGSTA4), class Mu (diluted 1:1000 for hGSTM1a and 1:3000 for hGSTM3) and class Pi (1:1000 dilution for rGSTP1) transferases. Monoclonal antibodies generated in the mouse NSO cell line [19] were also used to detect the class Alpha rGSTA5 transferase (1:500 dilution). As a loading control, the samples were also probed with antibodies for rat lactate dehydrogenase (diluted 1:1000). These

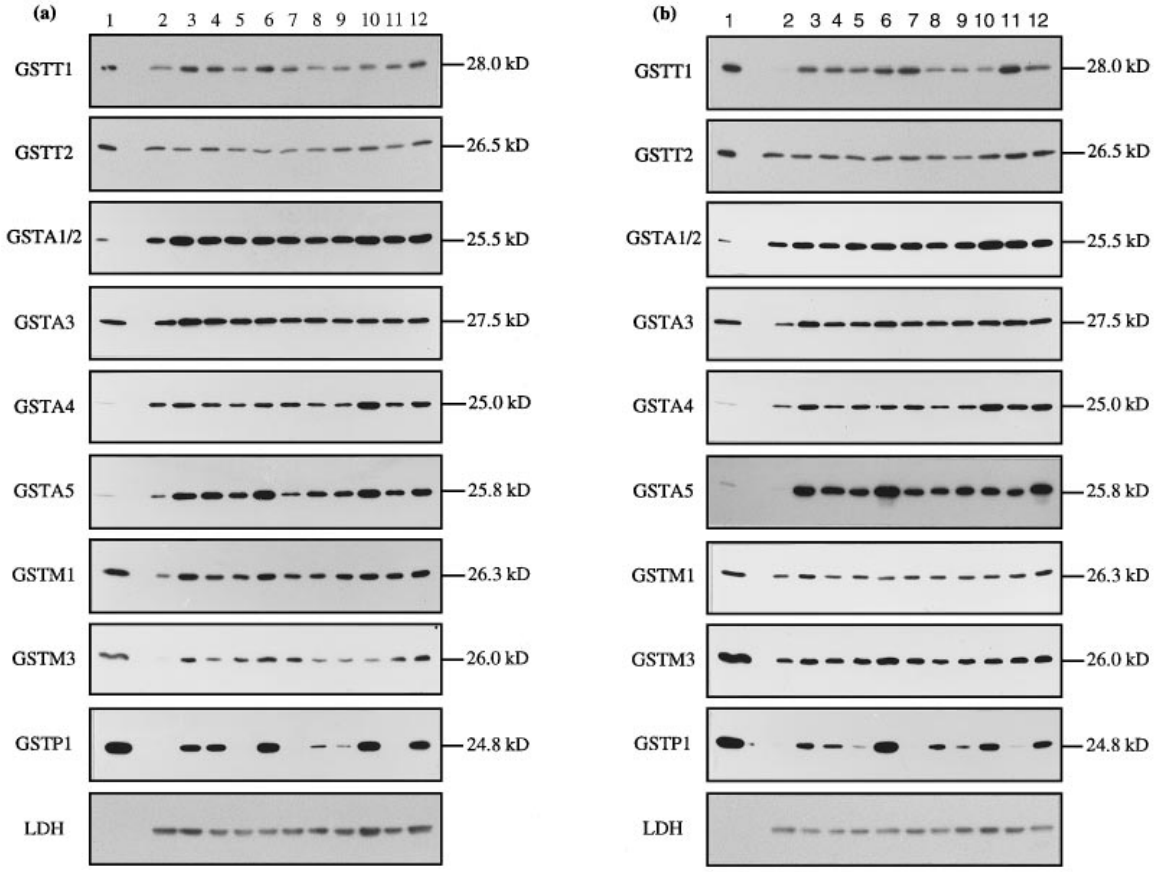


Figure 1 Levels of GST subunits in livers of female and male rats treated with cancer chemopreventive agents and xenobiotics

Portions of soluble hepatic extract (4 μg of protein) from treated female (**a**) and male (**b**) rats were subjected to SDS/PAGE before the resolved polypeptides were transferred on to Immobilon-P. The lanes in both cases were loaded as follows: 1, standard (bacterially expressed native human class Theta cDNAs or affinity purified GST from rat spleen); 2, control liver; 3, ethoxyquin-treated rat liver; 4, BHA-treated rat liver; 5, oltipraz-treated rat liver; 6, coumarin-treated rat liver; 7, PB-treated rat liver; 8, DEM-treated rat liver; 9, benzyl isothiocyanate-treated rat liver; 10, β-NF-treated rat liver; 11, indole-3-carbinol-treated rat liver; and 12, tSO-treated rat liver. The blots were probed with antibodies raised against the subunits indicated. As a loading control, the samples were probed with antisera raised against lactate dehydrogenase.

antibodies have all been described previously [23,28,29]. The blotted microsomal proteins were probed with polyclonal serum raised against CYP 2E1 provided by Professor C. S. Yang (Department of Pharmacology and Toxicology, Rutgers University, Piscataway, NJ, U.S.A.). Horseradish peroxidase-labelled secondary goat (anti-rabbit IgG) antibody, obtained from Bio-Rad Laboratories, was used to locate the primary antibody, and detection of the complex was achieved by enhanced chemiluminescence (ECL) with reagents from Amersham Life Sciences [27].

Estimation of GST subunit levels in hepatic extracts

The relative amount of GST subunits was estimated by phosphorimage analysis of Western blots. All equipment and software used for phosphorimaging was obtained from Bio-Rad Laboratories. A GS-250 imaging screen-CH was exposed to immunoblots treated with ECL reagents for detection of GST subunits in a GS-250 sample-loading dock. After exposure of the screen for approx. 45 min, the relative levels of enzyme were measured using the Bio-Rad model GS-525 molecular imager® system and molecular analyst software. In the case of class Alpha, Mu and Pi subunits, HPLC was also carried out to determine the basal levels of the various GST polypeptides. The class Alpha, Mu and Pi transferases were affinity purified on glutathione-agarose beads and individual subunits were quantified by reversed-phase HPLC on a 250×4.6 mm Brownlee C_{18} column (7 mm particle size and 300 Å pore size) using published specific absorption coefficients [30] to estimate the amount of different polypeptides resolved by the acetonitrile gradient [19].

Northern blotting

Total RNA was isolated from rat liver using the method of Chomczynski and Sacchi [31]. From each sample, 20 μg of total RNA was separated by denaturing electrophoresis in formaldehyde–agarose gels. The RNA was transferred to Hybond-N (Amersham Life Science) and detection of *GSTT1* message was achieved using a random-primer labelled probe which was generated using the Prime-It® II random-primer labelling kit from Stratagene (Cambridge, U.K.). The coding sequence for rat *GSTT1* was used as template for the priming reaction. A probe generated from the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as the loading control. Non-specifically bound radioactive probe was removed from the filters by washing once in $2 \times$ SSC/0.1 % SDS at room temperature ($1 \times$ SSC = 0.15 M NaCl/0.015 M sodium citrate), and then twice in $0.5 \times$ SSC/0.1 % SDS at 65 °C, before the blots were subjected to

autoradiography. An estimate of relative levels of message was made using the phosphorimager by exposing a high-sensitivity imaging screen to the probed Hybond-N filters for 1–3 h.

Immunohistochemistry

Immunohistochemistry was performed on acetone-fixed rat liver sections as described previously [32,33], using the same polyclonal antibodies against either human GST T1-1 or rat GST T2-2 (diluted 1:250) that were employed in the Western blotting experiments, and alkaline phosphatase-conjugated secondary antibodies obtained from Bio-Rad Laboratories (diluted 1:100).

RESULTS

Modulation of GST activity in rat liver by chemical inducers

Rats fed on diets containing the cancer chemopreventive agents benzyl isothiocyanate, butylated hydroxyanisole (BHA), coumarin, ethoxyquin, indole-3-carbinol and oltipraz, or rats treated with inducing agents diethylmaleate (DEM), phenobarbital (PB), β-naphthoflavone (β-NF) and *trans*-stilbene oxide (tSO), all possessed elevated hepatic GST activity towards the dihaloalkanes DCM and DBE.

As shown in Table 2, transferase activity towards DCM was found to be essentially doubled in the livers of female and male rats fed on a coumarin-containing diet, and was increased 1.8-fold in livers from female and male rats that had been administered oltipraz. Other treatments were found to be relatively sex-specific in their effects on GST activity towards dihaloalkanes. Thus, indole-3-carbinol increased DCM–GSH-conjugating activity 2.1-fold in male rat liver, but only 1.2-fold in female rat liver. Activity for DCM was also higher in livers from male PB-treated rats than in female PB-treated rat liver.

Transferase activity towards DBE was found to be 3.5-fold higher in the livers of female rats fed coumarin-containing diets. Similar to DCM, the indole-3-carbinol activity was increased 3.0-fold in male rat liver but only 1.6-fold in female rat liver. Activity for DBE was also increased more in male rat liver than in female rat liver.

In order to determine whether inducible GST activity for dihaloalkanes might be attributed to an increase in the amount of the T1-1 or another transferase, hepatic ENPP–GSH-conjugating activity was examined. Activity towards ENPP was measured in the presence of 2.5 mM *S*-hexylglutathione in order to inhibit class Alpha, Mu and Pi transferases (primarily GST M2-2) that can utilize this substrate; inhibition is effective at the substrate concentrations used with a negligible level of activity from other isoenzymes [10]. Comparison between GST activity for the dihaloalkanes with that for ENPP showed broad similarity. For example, among the treatments, livers from female rats fed on coumarin-containing diets exhibited highest transferase activity for DCM, DBE and ENPP. Similarly, livers from male rats fed on indole-3-carbinol-containing diets possessed highest activity for the three compounds (Table 2). These results therefore suggest that the increased hepatic activity towards dihaloalkanes is due to induction of GST T1-1.

In contrast with GST activity towards DCM, DBE and ENPP, activity towards MS was not significantly increased in liver extracts prepared from control and treated rats, except in oltipraz-treated male liver extract. As this substrate is specific for the class Theta family member GST T2-2 [6,20], it appears that the T2 subunit is not inducible in rat liver.

The NBDC–GSH- and CDNB–GSH-conjugating activities were determined in the hepatic cytosols from all animals as an independent means of assessing the effectiveness of the inducing agents. The class Theta transferases, T1-1 and T2-2, have negligible activity towards CDNB [6,34], thus this activity provides a broad indication of the combined effect of treatment

Table 3 Estimation of GST subunit levels in female rat liver

The amount of class Theta GSTT1 and T2 subunits shown in (A) was calculated from the results of Hiratsuka et al. [34]. The amounts of class Alpha, Mu and Pi GST were calculated during the present study using affinity chromatography and reversed-phase HPLC [19]. The nomenclature used for GST is defined in [6]. In (B), the relative increase in GST was determined by phosphorimaging of the blots shown in Figure 1(a) and the value shown has been rounded up to give two significant figures. Each blot was repeated several times and the data presented represent typical results.

(A)

		Level of GST subunit (mg/g of cytosolic protein)										
Treatment	Subunit ...	T1	T2	A1	A2	A3	A4	A5	M1	M2	M3	P1
Control		0.63	4.3	1.1	4.1	6.8	0.22	0.59	1.1	2.2	< 0.1	< 0.1

(B)

		Increase in GST subunit (fold increase relative to control)								
Treatment	Subunit ...	T1	T2	A1/2	A3	A4	A5	M1/2	M3	P1
Control		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Ethoxyquin		3.2	0.9	3.7	1.8	2.3	5.0	3.4	4.2	8.7
BHA		2.6	1.0	2.1	1.6	1.5	4.2	2.3	2.2	8.8
Oltipraz		1.4	0.8	1.7	1.0	1.1	1.8	1.8	2.5	0.9
Coumarin		3.5	0.8	2.2	1.7	1.7	6.3	2.8	3.2	17.0
PB		1.4	0.8	2.0	1.2	1.6	1.8	1.5	2.2	0.8
DEM		1.3	1.0	1.2	1.4	1.2	2.3	1.5	1.8	2.3
Benzyl isothiocyanate		1.3	1.1	1.6	1.1	1.1	2.1	2.2	2.4	1.7
β-NF		1.5	1.3	4.9	1.3	4.5	4.5	2.7	3.3	10.0
Indole-3-carbinol		1.9	1.0	2.9	1.1	1.3	2.3	2.1	2.7	1.0
tSO		3.0	1.2	2.9	1.3	2.6	3.0	3.1	2.8	7.2

Table 4 Estimation of GST subunit levels in male rat liver

The amount of class Theta GSTT1 and T2 subunits shown in (A) was calculated from the data of Hiratsuka et al. [34]. The amounts of class Alpha, Mu and Pi GST were calculated during the present study using affinity chromatography and reversed-phase HPLC [19]. The data for GSTA5 were taken from [35]. In (B), the relative increase in GST was determined by phosphorimaging of the blots shown in Figure 1(b) and the value shown has been rounded up to give two significant figures. Each blot was repeated several times and the data presented represent typical results.

(A)

		Level of GST subunit (mg/g of cytosolic protein)										
Treatment	Subunit ...	T1	T2	A1	A2	A3	A4	A5	M1	M2	M3	P1
Control		0.63	4.3	3.4	2.9	4.3	0.22	0.15	3.9	7.0	< 0.1	< 0.1

(B)

		Increase in GST subunit (fold increase relative to control)								
Treatment	Subunit ...	T1	T2	A1/2	A3	A4	A5	M1/2	M3	P1
Control		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Ethoxyquin		4.1	1.0	2.5	3.5	2.3	13.0	1.8	1.6	5.0
BHA		3.5	1.0	1.8	2.3	1.6	4.1	1.1	1.3	4.5
Oltipraz		3.5	0.9	1.8	2.9	1.6	6.9	2.0	1.4	2.5
Coumarin		4.7	1.0	2.0	4.0	1.9	20.0	2.3	1.7	26.0
PB		4.3	1.1	2.5	2.8	2.1	9.3	1.8	1.5	1.6
DEM		2.1	0.9	1.3	2.2	1.2	8.1	1.6	1.0	4.7
Benzyl isothiocyanate		2.5	0.8	2.0	2.7	1.7	11.0	1.4	1.2	2.4
β-NF		1.8	1.1	4.2	3.3	3.6	13.0	1.8	1.3	7.0
Indole-3-carbinol		6.2	1.2	3.4	3.1	2.4	8.4	1.5	1.8	1.6
tSO		3.9	1.2	3.6	3.3	2.8	17.0	1.5	1.6	6.0

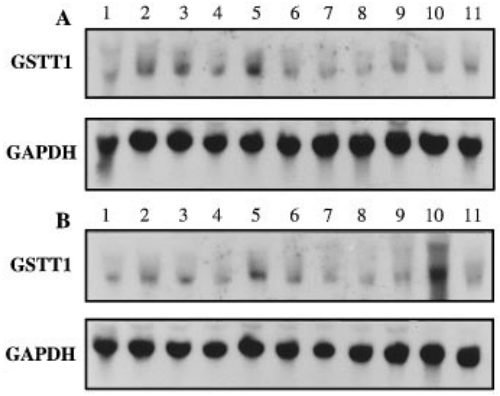


Figure 2 Estimation of GSTT1 mRNA levels in rat liver

Total RNA from livers of female (**A**) and male (**B**) treated rats (20 μg) was separated by denaturing electrophoresis before being transferred on to Hybond-N. The blot was probed with a radioactively labelled random-primed probe generated from the rat GSTT1 cDNA as template. The blot was stripped and re-probed with a probe from a reaction utilizing glyceraldehyde-3-phosphate dehydrogenase as template. The lanes were loaded with hepatic RNA from rats treated as follows: 1, control; 2, ethoxyquin; 3, BHA; 4, oltipraz; 5, coumarin; 6, PB; 7, DEM; 8, benzyl isothiocyanate; 9, β-NF; 10, indole-3-carbinol, 11, tSO.

on the levels of class Alpha, Mu and Pi transferases in rat liver. It is not known whether class Theta GSTs are active with NBDC, but this substrate appears to be relatively specific for Alpha class enzymes [22]. As expected from previous work [6], all of the drug treatments caused significant elevations in hepatic CDNB–GSH-conjugating activity, with largest increases being observed in livers from rats fed on diets containing either ethoxyquin or coumarin (Table 2). Furthermore, β-NF was found to be an excellent inducer of hepatic GST for CDNB activity in female animals, although it proved to be a less potent inducer in male rats. Indole-3-carbinol, oltipraz, PB and tSO were effective inducers of CDNB–GSH-conjugating activity in both female and male livers. Both BHA and benzyl isothiocyanate proved to be better inducers of CDNB–GSH-conjugating activity in female livers than in male livers. In male rat livers, the relative increases in GST activity observed with NBDC as substrate were similar to those obtained with CDNB. PB was a more notable inducer of NBDC activity in male rat liver cytosol than of CDNB activity. However, in female rat livers the fold increase in GST activity towards NBDC was less than that found with CDNB, suggesting that class Alpha GSTs are not greatly induced.

It is important to note, as Table 2 shows, that the relative increase in GST activity towards both CDNB and NBDC is comparable to the increase in transferase activity towards ENPP. This suggests that the putative increase in GST T1-1 is of a similar magnitude to the induction of class Alpha and Mu transferase subunits.

Elevation of hepatic GSTT1 protein by chemopreventive agents and inducers of drug-metabolizing enzymes

Immunoblot analysis of GST subunit levels in soluble hepatic extracts shows reasonable concordance with the levels of GST activity. In extracts of livers from female rats, the level of the GSTT1 polypeptide was found to be notably increased above control in response to treatment with coumarin, ethoxyquin, BHA and tSO (Figure 1a). In extracts of livers from male rats, the amount of GSTT1 was elevated most in response to treatment with coumarin, PB and indole-3-carbinol (Figure 1b). Phosphorimaging indicated that among the inducers studied, the level of GSTT1 was increased maximally 3.5-fold in female rat liver and 6.2-fold in male rat liver.

In contrast with the T1 subunit, the levels of GSTT2 were similar to control in all treated samples. Comparison of GSTT1 protein levels in female and male animals by immunoblotting shows that female and male control animals have a similar basal

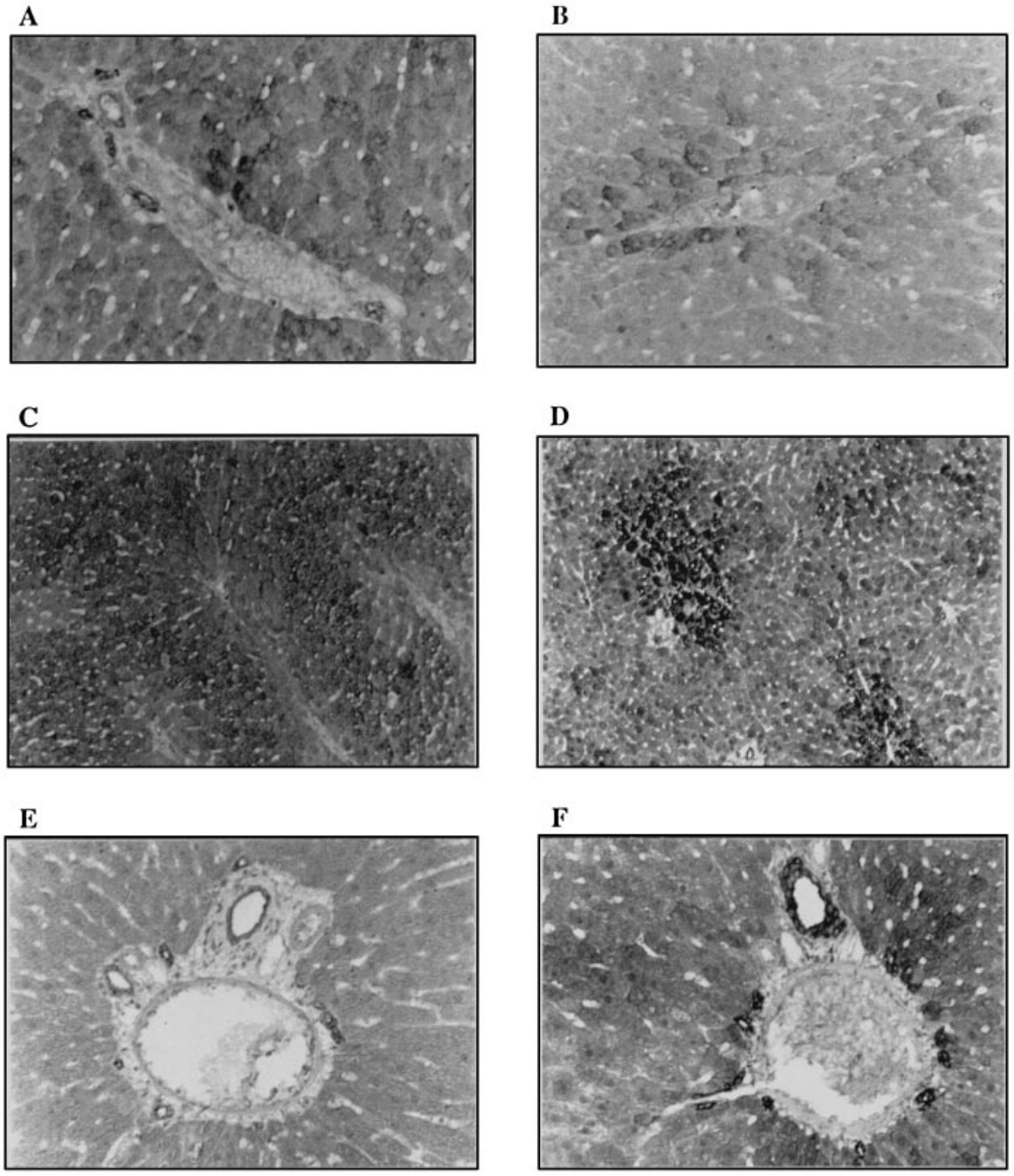


Figure 3 Immunohistochemical staining for GST T1-1 in rat liver

Livers from female control (**A**), male control (**B**), coumarin-treated female (**C**) and indole-3-carbinol-treated male (**D**) rats are shown for GST T1-1 staining with antibodies as described in the Materials and methods section. Control female liver shows some periportal staining, whereas the male exhibits light centrilobular staining. The coumarin-treated female liver displays marked induction throughout the lobule, whereas indole-3-carbinol-treated male liver displays intense centrilobular staining for the enzyme. Sections containing a portal tract are also shown for control male (**E**) and benzyl isothiocyanate-treated male (**F**) livers. The treated liver shows a marked induction of GST T1-1 levels in the bile-duct epithelial cells.

level of GSTT1 expression (P. J. Sherratt, T. Green and J. D. Hayes, unpublished work). Direct comparison between the amount of GSTT1 in livers from female and male rats fed on diets containing coumarin and indole-3-carbinol suggested that the levels of the subunit are more responsive to treatment in male rats than in female rats. Thus, these immunoblotting data are in broad agreement with the enzyme assay results.

Examination of the hepatic levels of class Alpha, Mu and Pi transferase subunits showed that ethoxyquin, coumarin and β-NF are consistently among the most potent inducers of GST in both female and male rats; the most marked variations in GST polypeptides were observed with GSTA1/2, GSTA4, GSTA5, GSTM1/2 and GSTP1. In this context it should be noted that coumarin, ethoxyquin and β-NF are not only excellent inducers of class Alpha, Mu and Pi GST but they are also good inducers of GSTT1. However, the pattern of GSTT1 induction in male animals does not correlate with the other GST. These patterns of induction were quantified using phosphorimaging (Tables 3 and 4).

Regulation of GSTT1 mRNA by inducing agents

Northern blot analysis for transcript levels of *GSTT*1 revealed a significant increase in the steady-state mRNA levels for this transferase in the livers of both female and male rats treated with cancer chemopreventive agents (Figure 2). These RNA blots also

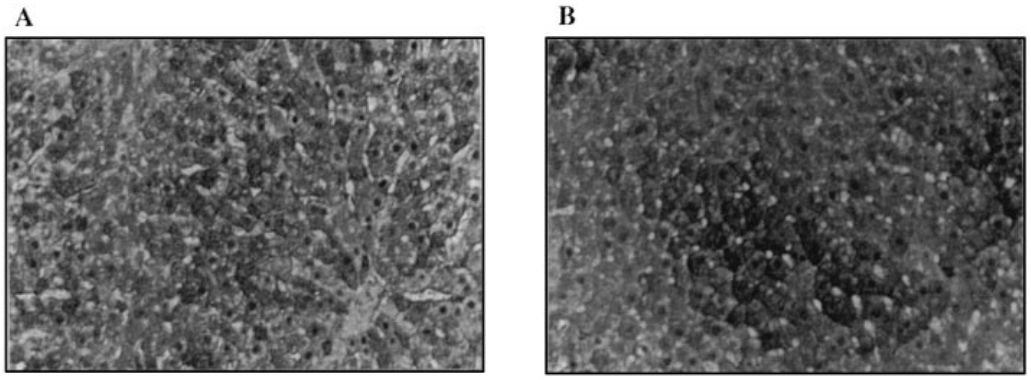


Figure 4 Detection of nuclear GST T1-1 in the liver of oltipraz-treated rats

A high proportion of hepatic nuclei in female (**A**) and male (**B**) oltipraz-treated rat livers display marked nuclear staining in the centrilobular region.

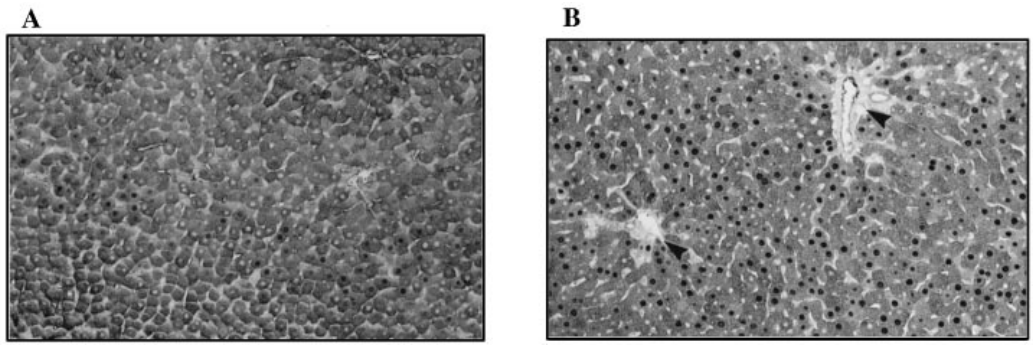


Figure 5 Translocation of hepatic GST T2-2 from cytoplasm to the nucleus of female rats treated with oltipraz

In female control rat liver (**A**), GST T2-2 is cytoplasmic in its localization. On treatment of the female rat with oltipraz (**B**) this localization is shifted to the nucleus.

show a similar trend of GSTT1 overexpression in coumarin-, ethoxyquin- and indole-3-carbinol-treated animals, as had been seen by immunoblotting. Therefore, control of GSTT1 at the mRNA level may involve transcriptional activation or stabilization of message.

Immunohistochemical localization of GSTT1 in livers of rats treated with inducing agents

Immunohistochemical staining of liver sections demonstrated that GST T1-1 is present in significant amounts throughout the periportal and midzonal areas of the female liver (Figure 3A), whereas in male rats, the enzyme is present in fewer cells mainly localized in the centrilobular (periacinar) regions of the lobule (Figure 3B). In both sexes, staining of hepatocytes occurs predominantly in the cytoplasm. Some induction of GST T1-1 was observed in livers from all the treated rats. Examination of the distribution of GST T1-1 in livers from female rats fed coumarin-containing diets showed that the increased staining is located throughout the lobule, with the strongest staining in the midzonal regions (Figure 3C). Livers from male rats fed on diets containing indole-3-carbinol demonstrated intense staining in the centrilobular areas (Figure 3D), indicating that in this case the increased expression is restricted to one area of the lobule. This pattern was reflected to a greater or lesser extent for all inducers in male rats. Also in the liver of male rats, the centrilobular region was the principle site for induction, regardless of compound, with the periportal area being least affected, but this was not the case in females. In female rat liver, the periportal region was a site for induction for all compounds, except PB, benzyl isothiocyanate and oltipraz, where strong centrilobular induction was observed. The biliary epithelium in control rats of both sexes stained positively for GST T1-1, but a characteristic of rats fed benzyl isothiocyanate in particular, was an apparent increased staining in the bile ducts (Figures 3E and 3F).

Nuclear localization of class Theta GST in male and female treated rat liver

On closer examination of the immunohistochemical data, it was apparent that there is good evidence of intense nuclear staining in treated hepatocytes. The amount of nuclear staining observed was found to vary between the treatments, although a general trend was noted that the better inducing agents gave rise to a higher intensity of nuclear staining. The most notable example of nuclear staining in both female and male animals was observed in response to oltipraz treatment (Figures 4A and 4B). The pattern of staining is unusual in that individual hepatocytes were identified which exhibited intense nuclear staining and weak cytosolic staining, whereas an adjacent cell possessed weak nuclear staining and intense cytosolic staining. Comparisons between immunostaining in female and male rat livers indicated

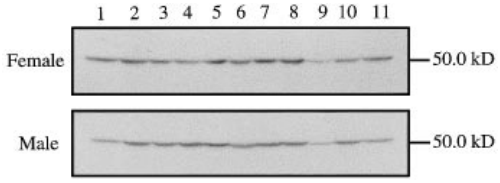


Figure 6 Comparative levels of CYP 2E1 in treated rat livers

Portions (10 μg of protein) of liver microsomes from female and male treated rats were subjected to SDS/PAGE before transfer to Immobilon-P. The transferred proteins were then probed with polyclonal antisera raised against CYP 2E1. The lanes were loaded as follows: 1, control liver; 2, ethoxyquin-treated rat liver; 3, BHA-treated rat liver; 4, oltipraz-treated rat liver; 5, coumarin-treated rat liver; 6, PB-treated rat liver; 7, DEM-treated rat liver; 8, benzyl isothiocyanate-treated rat liver; 9, β-NF-treated rat liver; 10, indole-3-carbinol-treated rat liver; 11, tSO-treated rat liver.

that the intensity of the nuclear staining appeared to be dependent upon treatment and was not sexually differentiated.

The immunohistochemical investigation was extended to include GST T2-2. This showed that T2-2, like T1-1, is expressed in highest amounts in centrilobular hepatocytes. However, T2-2 gave more intense staining than T1-1 in control liver, which is consistent with its higher level of basal expression (Figure 5A). Although the T2 subunit is not inducible by xenobiotics, it was found that, whereas immunohistochemical staining is predominantly associated with non-nuclear components of hepatocytes in control rats, staining is strongly nuclear in hepatocytes from rats treated with cancer chemopreventive agents and model inducers (Figure 5B). Therefore, these results suggest that both GST T1 and T2 subunits may translocate to the nucleus of rat hepatocytes in response to xenobiotics.

Effect of dietary treatment on CYP levels

Immunoblot analysis of microsomes taken from both female and male treated rat livers showed no indication of an increase in the levels of CYP 2E1 (Figure 6). There is a slightly lower level of the enzyme in the β-NF-treated samples.

DISCUSSION

The dihaloalkane DCM is a potent liver and lung carcinogen in B6C3F1 mice [2]. The toxicity of this compound is species specific, and the threat it poses to humans is uncertain. In contrast with the mouse, other mammals used in toxicology screening (e.g. the rat and the hamster) are considerably less susceptible to DCM carcinogenicity. Species differences in the metabolic fate of DCM appear to account for its selective toxicity. The principal enzyme responsible for the bioactivation of DCM is the class Theta transferase GST T1-1, which catalyses the formation of *S*-chloromethylglutathione, an unstable conjugate that is a reactive electrophile [11]. Two major factors contribute to the greater sensitivity of the mouse to DCM over other rodent species. Firstly, the mouse expresses high levels of GST T1-1 in the main target organ cells of dihaloalkane toxicity, and secondly this transferase has been observed in the nucleus of these cells [14]. Thus, a high level of activation in close proximity to DNA is possible. In addition to DCM, GST T1-1 is highly efficient at catalysing the activation of other dihaloalkanes such as DBE. However, this dihaloalkane does not exhibit the selective toxicity shown by DCM, because the more abundant class Alpha and Mu GST isoenzymes also metabolize this compound [10], thereby masking the species differences in GST T1-1-mediated activation.

In the present paper it is shown for the first time that the hepatic levels of GST T1-1 can be increased by inducers of drug-metabolizing enzymes. A number of the inducers included in the study are cancer chemopreventive agents, and these were chosen because it was considered important to determine whether, in addition to their beneficial effects on detoxification, they might increase toxification pathways. Furthermore, GST induction was investigated in both female and male rats because scant attention has been given to possible sexual dimorphisms in chemoprevention. Essentially all of the xenobiotics studied served to increase the hepatic levels of GST T1-1, though sex-specific differences in the extent of overexpression were observed. Measurement of GST activity towards DCM in hepatic extracts indicated that coumarin, a naturally-occurring compound in leguminous vegetables, is the most potent inducer of GST T1-1 in female rats, whereas indole-3-carbinol, a naturally occurring compound in cruciferous vegetables, is the most potent inducer in male rats. These results for enzyme activity correlate with immunoblot analysis, which indicates that the level of the transferase subunit is elevated in extracts from the livers of treated animals. Immunoblotting revealed significant induction of the GSTT1 subunit by ethoxyquin, BHA and tSO in both sexes, whereas oltipraz and PB were found to be more effective inducers of GSTT1 in male rat livers than in female rat livers. Importantly, Northern blot analysis indicated that the regulation of GSTT1 occurs at the mRNA level.

Comparison between the hepatic levels of GSTT1 and transferase subunits from other gene families revealed certain similarities in regulation. In particular, BHA, ethoxyquin, coumarin and tSO are consistently among the better inducers of the GST T1, A1/2, A5, M1/2 and P1 subunits. It is believed that these compounds all require to be converted into thiol-active agents in order to act as inducers [17]. Induction of rat *GSTA2* by BHA involves metabolism to t-butylhydroquinone and redox cycling [36,37] to cause transcriptional activation through the *cis*-acting antioxidant responsive element (ARE) [38]. Ethoxyquin possibly affects transcriptional activation of *GSTA2* through the ARE after conversion into 2,2,4-trimethyl-6-quinolone [17]. Oxidative metabolism of tSO to the carbonyl-containing compounds benzoin and/or benzil [39] may account for the strong enzyme-inducing activity of this agent, though it is not known whether it acts through the ARE. It is possible that coumarin requires to be metabolized, possibly to 3-hydroxycoumarin, via a reactive epoxide intermediate [40], in order to act as an inducer, as it is inactive in HepG2 cells [41]. All these inducers are metabolized by cytochrome *P*-450 and there is an expectation that enzyme induction may involve the generation of reactive oxygen species as a consequence of poor coupling between *P*-450 and the reductase [6]. Indeed, the requirement for the generation of the superoxide anion by oltipraz in order for it to act as an inducing agent has been discussed recently [42].

The fact that GSTT1 is inducible by antioxidants and metabolizable xenobiotics suggests that an ARE may be involved in its regulation. Our results also indicate that enhancers other than the ARE are involved in the regulation of *GSTT1*, for example in the case of the sex-specific *GSTT1* induction by indole-3-carbinol. Evidence suggests that indole-3-carbinol can bind the arylhydrocarbon (Ah) receptor and therefore its effects on gene expression may, in part, be mediated by the xenobiotic responsive element. However, if the xenobiotic responsive element were solely involved in the sex-specific induction of *GSTT1* by indole-3-carbinol, similar sex-specificity might be expected with β-NF, which is also a good ligand for the Ah receptor [43]. The observation that β-NF served as only a modest inducer of *GSTT1* in both female and male rats, while acting as a strong

inducer of other GSTs, suggests that an additional factor other than the Ah receptor is responsible for the differential induction of T1 in the two sexes. Sexual dimorphism in the induction of *GSTT1* by PB was also observed, but there is no obvious mechanism to explain this finding.

Immunohistochemistry has been undertaken in order to identify zones of the liver where induction of GST T1-1 occurs. Intense staining for this transferase was observed in livers treated with the more effective inducers. These experiments showed the sex differences of induction, with strong centrilobular staining in the livers of male rats treated with indole-3-carbinol, but less staining in livers of female rats similarly treated; interestingly the Ah receptor is expressed in centrilobular hepatocytes [44], which is consistent with the xenobiotic responsive element-mediated induction. Immunohistochemistry showed a more intense staining for GSTT1 in livers of male PB-treated rats than in female rats. Not all the inducing agents cause overexpression of T1 in the same region of the liver, and immunohistochemistry showed that certain agents were highly specific in the cell types in which they are effective. For example, Western blotting suggested that benzyl isothiocyanate is a modest inducing agent, whereas immunohistochemistry revealed that this compound not only caused induction in the centrilobular areas, but also served as a potent inducer of GST T1-1 in bile duct epithelial cells of both female and male rats. This is an interesting observation because the bile duct epithelial cells contain large amounts of GST P1-1 [45], and as this transferase has greatest capacity to conjugate benzyl isothiocyanate with glutathione [46] it is possible that the high induction of the T1 subunit in these cells could involve oxidative stress through depletion of GSH.

Immunohistochemical analysis demonstrated a significant level of nuclear staining for GSTT1 in the hepatocytes of treated rats. The nuclear staining was found to be dependent on treatment rather than sex. High levels of immunostaining were associated with an increase in the proportion of stained nuclei. The highest incidence of nuclear staining was seen in livers treated with oltipraz. The nuclear staining appeared to follow no obvious pattern, and marked variations were observed in the balance between nuclear and cytosolic staining. The mechanism responsible for the nuclear localization of GST T1-1 is not known, but it may be a form of adaptive response to stress caused by the inducing agents [17,47]. GSTs, along with other glutathione-utilizing enzymes, detoxify hydroperoxides, α,β-unsaturated carbonyls and epoxides which are generated *in vivo* by oxidative damage [6]. Since such species pose a major threat to the cell genome, it is logical that GSTs are present in the nucleus in order to protect DNA against reactive chemicals during stress. It can be postulated that mechanisms are likely to exist which allow the import of antioxidant GSH-dependent enzymes to the nucleus during, for example, inflammatory responses that involve induction of cyclo-oxygenase 2, since this latter enzyme is situated on the nuclear envelope [48] where it generates reactive oxygen species during production of prostaglandin H_2 [49]. Whether the same processes are involved in translocating GSTT1 to the nucleus during chemical stress caused by cancer chemopreventive agents, as are postulated to occur during inflammatory processes, is not known.

The finding that GST T1-1 may localize to the nucleus in the livers of treated rats, along with the observed increase in the total levels of the enzyme, suggests that these animals will be more sensitive to dihaloalkanes. For a better estimate of whether this hypothesis is correct, the detoxification pathway for dihaloalkanes must be taken into account. Immunoblot analysis in this investigation, together with results obtained in another study [45], show that the treatments used here have little or no effect on the level of CYP 2E1, and therefore the detoxification capacity for DCM is unaffected by drug treatment. Thus, upon saturation of the CYP 2E1 pathway in these rats, an indole-3-carbinol-treated male rat or a coumarin-treated female rat is likely to be more sensitive to dihaloalkane exposure.

Emphasis has been placed in this paper on the induction of GST T1-1 in normal rat liver, and mention has been made of its overexpression in the selenium-deficient rat [19]. It will therefore be interesting to discover whether the T1 subunit is overexpressed in tumours, since members of this superfamily have been implicated in acquired drug resistance to cancer chemotherapeutic agents [6,50]. Should GST T1-1 be commonly overexpressed in human cancer cells, then the ability of the transferase to activate cytotoxic drugs might allow novel therapies to be devised that depend on this property.

In conclusion, the present study has shown that rats treated with either cancer chemopreventive agents or model inducers have an enhanced capacity to activate DCM and DBE. In the case of DCM, this increase is due solely to induction of GST T1-1 in the liver, whereas in the case of DBE, induction of other GST besides GST T1-1 is responsible for an enhanced capacity to activate the dihaloalkane. However, these rats with elevated GST levels do not have an increased capacity to detoxify dihaloalkanes by CYP 2E1 oxidation. Combined with the observation that GST T1-1 is present in rat liver nuclei, it is proposed that treatment of rats with cancer chemopreventive agents may increase susceptibility to the neoplastic effects of DCM and DBE. It is important to emphasize that induction of GST T1-1 and its nuclear translocation appear to be important factors which have been overlooked and should be considered when evaluating the risk to human health posed by dihaloalkanes.

We thank Professor C. S. Yang for provision of the CYP 2E1 antibody, and Dr. R. F. L. James for producing the GSTA5 antibody. Dr. Ronnie McLeod is thanked for performing the HPLC quantification of GST subunits shown in Tables 3 and 4. We also thank Dr. Richard Savoury and Miss Aileen McLaren for helpful discussions and reagents, and Ms Lynne Howells and Ms Helen Ball for performing the immunohistochemistry. This work was supported by a Medical Research Council and Zeneca collaborative studentship (awarded to P.J.S.), a Wellcome Trust Prize Studentship (awarded to A.M.T.) and Equipment Grant K/CSO/2/20/3/7 from the Scottish Office Home and Health Department to purchase the Cobas Fara II Chemistry System.

REFERENCES

1 Andersen, M. E., Clewell, III, H. J., Gargas, M. L., Smith, F. A. and Reitz, R. H. (1987) Toxicol. Appl. Pharmacol. **87**, 185–205
2 National Toxicology Program. Toxicology and Carcinogenesis Studies of Dichloromethane (methylene chloride CAS no. 75-09-2) in F344/N Rats and B6C3F$_1$ Mice (inhalation studies), NTP Technical Report no. 306, 1986
3 National Toxicology Program. Carcinogenesis Bioassay of 1,2-Dibromoethane (CAS no. 106-93-4) in F344 Rats and B6C3F$_1$ Mice (inhalation study), NTP Technical Report no. 210, 1982
4 Guengerich, F. P., Kim, D.-H. and Iwasaki, M. (1991) Chem. Res. Toxicol. **4**, 168–179
5 Ahmed, A. E. and Anders, M. W. (1976) Drug Metab. Dispos. **4**, 357–361
6 Hayes, J. D. and Pulford, D. J. (1995) Crit. Rev. Biochem. Mol. Biol. **30**, 445–600
7 Meyer, D. J., Coles, B., Pemble, S. E., Gilmore, K. S., Fraser, G. M. and Ketterer, B. (1991) Biochem. J. **274**, 409–414
8 Thier, R., Taylor, J. B., Pemble, S. E., Humphreys, W. G., Persmark, M., Ketterer, B. and Guengerich, F. P. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 8576–8580
9 van Bladeren, P. J., Breimer, D. D., Rotteveel-Smijs, G. M. T., DeJong, R. A., Buijs, W., van der Gen, A. and Mohn, G. R. (1980) Biochem. Pharmacol. **29**, 2975–2982
10 Ploemen, J. P. H. T. M., Wormhoudt, L. W., Haenen, G. R. M. M., Oudshoorn, M. J., Commandeur, J. N. M., Vermeulen, N. P. E., DeWaziers, I., Beaune, P. H., Watabe, T. and van Bladeren, P. J. (1997) Toxicol. Appl. Pharmacol. **143**, 56–69
11 Green, T. (1997) Human Exp. Toxicol. **16**, 3–13
12 Graves, R. J., Coutts, C. and Green, T. (1995) Carcinogenesis **16**, 1919–1926

13 Guengerich, F. P., Thier, R., Persmark, M., Taylor, J. B., Pemble, S. E. and Ketterer, B. (1995) Pharmacogenetics **5**, S103–S107
14 Mainwaring, G. W., Foster, J. R. and Green, T. (1998) Biochem. J. **329**, 431–432
15 Mainwaring, G. W., Williams, S. M., Foster, J. R., Tugwood, J. and Green, T. (1996) Biochem. J. **318**, 297–303
16 Wattenberg, L. W. (1985) Cancer Res. **45**, 1–8
17 Hayes, J. D., Ellis, E. M., Neal, G. E., Harrison, D. J. and Manson, M. M. (1998) Biochem. Soc. Symp. (Downes, C. P., Wolf, C. R. and Lane, D. P., eds.), **64**, 137–163
18 Primiano, T., Gastel, J. A., Kensler, T. W. and Sutter, T. R. (1996) Carcinogenesis **17**, 2297–2303
19 McLeod, R., Ellis, E. M., Arthur, J. R., Neal, G. E., Judah, D. J., Manson, M. M. and Hayes, J. D. (1997) Cancer Res. **57**, 4257–4266
20 Habig, W. H. and Jakoby, W. B. (1981) Methods Enzymol. **77**, 398–405
21 Mannervik, B. and Widersten, M. (1995) in Advances in Drug Metabolism in Man (Pacifici, G. M. and Fracchia, G. N., eds.), pp. 407–459, European Commission 15439, Directorate-General XIII-Science, Research and Development, Luxembourg
22 Ricci, G., Caccuri, A. M., Lo Bello, M., Pastore, A., Piemonte, F. and Federici, G. (1994) Anal. Biochem. **218**, 463–465
23 Sherratt, P. J., Pulford, D. J., Harrison, D. J., Green, T. and Hayes, J. D. (1997) Biochem. J. **326**, 837–846
24 Nash, T. (1953) Biochem. J. **55**, 416–421
25 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
26 Hayes, J. D. and Mantle, T. J. (1986) Biochem. J. **233**, 779–788
27 Ellis, E. M., Judah, D. J., Neal, G. E., O'Connor, T. and Hayes, J. D. (1996) Cancer Res. **56**, 2758–2766
28 Hayes, J. D., Gilligan, D., Chapman, B. J. and Beckett, G. J. (1983) Clin. Chim. Acta **134**, 107–121
29 Hussey, A. J. and Hayes, J. D. (1993) Biochim. Biophys. Acta **1203**, 131–141
30 Johnson, J. A., Finn, K. A. and Siegel, F. L. (1992) Biochem. J. **282**, 279–289
31 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
32 Green, J. A., Carthew, P., Heuillet, E., Simpson, J. L. and Manson, M. M. (1990) Carcinogenesis **11**, 1175–1182
33 Manson, M. M., Legg, R. F., Watson, J. V., Green, J. A. and Neal, G. E. (1981) Carcinogenesis **2**, 661–670
34 Hiratsuka, A., Nishijima, T., Okuda, K., Ogura, K. and Watabe, T. (1997) Anal. Biochem. **252**, 229–237
35 Meyer, D. J., Harris, J. M., Gilmore, K. S., Coles, B., Kensler, T. W. and Ketterer, B. (1993) Carcinogenesis **14**, 567–572
36 Rushmore, T. H. and Pickett, C. B. (1990) J. Biol. Chem. **265**, 14648–14653
37 Pinkus, R., Weiner, L. M. and Daniel, V. (1996) J. Biol. Chem. **271**, 13422–13429
38 Rushmore, T. H., Morton, M. R. and Pickett, C. B. (1991) J. Biol. Chem. **266**, 11632–11639
39 Watabe, T. and Akamatsu, K. (1975) Biochem. Pharmacol. **24**, 442–444
40 van Iersel, M. L. P. S., Henderson, C. J., Walters, D. G., Price, R. J., Wolf, C. R. and Lake, B. G. (1994) Xenobiotica **24**, 795–803
41 Prestera, T., Holtzclaw, W. D., Zhang, Y. and Talalay, P. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 2965–2969
42 Kim, W. and Gates, K. S. (1997) Chem. Res. Toxicol. **10**, 296–301
43 Jellinck, P. H., Forkert, P. G., Riddick, D. S., Okey, A. B., Michnovicz, J. J. and Bradlow, H. L. (1993) Biochem. Pharmacol. **45**, 1129–1136
44 Oinonen, T. and Lindros, K. O. (1998) Biochem. J. **329**, 17–35
45 Manson, M. M., Ball, H. W. L., Barrett, M. C., Clark, H. L., Judah, D. J., Williamson, G. and Neal, G. E. (1997) Carcinogenesis **18**, 1729–1738
46 Kolm, R. H., Danielson, U. H., Zhang, Y., Talalay, P. and Mannervik, B. (1995) Biochem. J. **311**, 453–459
47 Primiano, T., Kensler, T. W., Kuppusamy, P., Zweier, J. L. and Sutter, T. R. (1996) Carcinogenesis **17**, 2291–2296
48 Morita, I., Schindler, M., Regier, M. K., Otto, J. C., Hori, T., DeWitt, D. L. and Smith, W. L. (1995) J. Biol. Chem. **270**, 10902–10908
49 Marnett, L. J. (1994) Cancer Metast. Rev. **13**, 303–308
50 Tew, K. D. (1994) Cancer Res. **54**, 4313–4320

Received 1 June 1998/10 July 1998; accepted 21 August 1998