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HALOTHANE: INHIBITION AND ACTIVATION OF RAT HEPATIC GLUTATHIONE *S*-TRANSFERASES

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Abstract—Multiple halothane anesthesias (1.25 MAC for 1 hr on 3 alternate days) of male Long–Evans rats initially decreased by up to 30% and subsequently increased to up to 185% liver cytosolic glutathione *S*-transferase activity toward 1-chloro-2,4-dinitrobenzene, 3,4-dichloro-1-nitrobenzene and *trans*-4-phenyl-3-buten-2-one and glutathione peroxidase activity. Halothane rapidly and reversibly activated hepatic cytosolic glutathione *S*-transferases and purified isoenzyme 1-2 but not isoenzymes 1-1 and 3-3. At high concentrations of halothane (*ca.* 22 mM), maximal activation was *ca.* 25%. Halothane, enflurane, isoflurane and methoxyflurane, but not the halothane metabolite 1-chloro-2,2-difluoroethylene, inhibited a mixture of liver cytosolic glutathione *S*-transferases with time (*ca.* 30% inhibition/15 min). The inhibition exhibited pseudo-first order kinetics ($k_{obs} = 0.13\ min^{-1}$) and an I_{50} for halothane of ≥ 15 mM. Halothane inhibited glutathione *S*-transferases 3-3, 3-4, and 4-4 by 50–60%, but did not affect isoenzymes 1-1 and 1-2. The ability of halothane to diminish hepatic glutatione *S*-transferase activity *in vivo* may in part reflect the time-dependent inhibition of glutathione *S*-transferase isoenzymes containing the 3- and 4-subunits.

The GSH* *S*-transferases (EC 2.5.1.18) are a family of ubiquitous, multi-functional proteins found primarily in mammalian liver. The GSH *S*-transferases catalyze the conjugation of a wide range of hydrophobic electrophiles with GSH. CDNB is a universal substrate for all GSH *S*-transferase isoenzymes whereas the activity of isoenzymes toward other substrates is more limited. In rat liver, GSH *S*-transferase activity toward DCNB and BSP is characteristic of the 3-subunit, while activity toward *trans*-4-phenyl-3-buten-2-one is limited to the 4-subunit [1]. Several isoenzymes also function as non-selenium-dependent GSH peroxidases [1, 2]. The GSH *S*-transferases can bind covalently to strong electrophiles and non-covalently to a number of non-substrate ligands, such as BSP and bilirubin [1, 3, 4]. The cytosolic GSH *S*-transferases are dimers, with rat liver containing the 1-1, 1-2, 2-2, 3-3, 3-4, 4-4 and 5-5 isoenzymes [1].

Halothane ($CF_3CHBrCl$) is a widely used volatile anesthetic agent. The oxidative and/or reductive metabolism of halothane by cytochrome P-450 may mediate the hepatic damage occasionally observed following halothane anesthesia [5, 6].

Neither halothane nor the majority of its metabolites appear to be metabolized by the GSH *S*-transferases; one exception is the reductive metabolite 1-chloro-2,2-difluorothylene which may undergo GSH conjugation *in vivo* [7, 8]. However, repeated exposure of rats to halothane vapor reduced the clearance of BSP by isolated perfused liver, GSH dependent conjugation of BSP in liver homogenates, and cytosolic GSH *S*-transferase activity toward CDNB [9, 10]. The structural analogue 1,2-dibromoethane also diminished hepatic GSH *S*-transferase activity *in vivo* perhaps in part via the time-dependent, irreversible inhibition of selected GSH *S*-transferase isoenzymes [11, 12].

The effects of halothane anesthesia of rats on hepatic GSH *S*-transferase activities *in vivo* are reported, as are reversible and irreversible interactions of halothane with rat hepatic GSH *S*-transferases *in vitro*.

EXPERIMENTAL

Materials and animals. Materials were obtained as follows: halothane, Maybaker, R.S.A.; isoflurane, enflurane and methoxyflurane, Abbott Laboratories, Kent, U.K.; GSH, Sigma Chemicals, St Louis, MO; *trans*-4-phenyl-3-buten-2-one, Aldrich Chemicals, Gillingham, Dorset, U.K.; cumene hydroperoxide (70% in cumene), Fluka AG, Buchs, Switzerland; bovine serum albumin, Miles Research Products, Cape Town, R.S.A. 1-Chloro-2,2-difluoroethylene was from PCR Research Chemicals, Gainsville, FL.

All experiments utilized male Long–Evans rats (200 ± 10 g) or preparations derived therefrom. Hepatic cytosol was prepared by differential centrifugation, the mixture of rat liver cytosolic GSH *S*-transferases essentially free of other proteins was isolated by *S*-hexyl GSH affinity chromatography, and rat liver GSH *S*-transferase isoenzymes were purified by chromotofocusing and characterized, as described earlier [12].

Halothane anesthesia. Rats (groups of 9–18) were anesthetized with halothane (1.25 MAC in medical air) or exposed to medical air alone, once for 3 hr or thrice for 1 hr on three alternate days, in a manner previously detailed [13]. Rats were fasted for 24 hr

* Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 3,4-dichloro-1-nitrobenzene; GSH, reduced glutathione; BSP, bromsulfophthalein.

prior to each exposure to halothane or medical air. GSH *S*-transferase and GSH peroxidase activities were measured with liver cytosol from anesthetized or control rats.

Assays. Protein concentration was determined by the method of Lowry *et al.* [14] as modified by Chaykin [15] using bovine serum albumin as standard. The purified isoenzymes were precipitated with ammonium sulfate (90% saturation), centrifuged at 2000 *g* for 10 min, and redissolved in water prior to assay.

Initial rates of GSH *S*-transferase activity toward CDNB, DCNB and *trans*-4-phenyl-3-buten-2-one and of GSH peroxidase toward cumene hydroperoxide activity were measured spectrally for one minute at 25° [16–19] on a Beckman 5230 or Unicam SP1800 spectrophotometer. All other experimental details are as given earlier [12, 13].

Metabolism of halothane by GSH S-*transferases.* The metabolism of halothane by hepatic cytosolic GSH *S*-transferases was assessed by GSH depletion in reaction mixtures containing an ethanolic solution of halothane or 1,2-dibromoethane (20 mM) vortex mixed into Tris–HCl (35 mM), pH 8.2, dialyzed cytosol* (0.5 unit/ml) and GSH (0.2 mM). Incubations were at 25° with shaking at 60 cycles per min. At the end of the incubation period, 50 μl of the reaction mixture was diluted with 10 ml of 10 mM HCl. Two ml thereof was lyophilized, reconstituted with 2 ml of 10 mM HCl, and heated at 60° for 30 min. GSH concentration was analyzed spectrophotometrically with Ellman's reagent by the method of Brehe and Burch [20].

Reversible activation of GSH S-*transferases by halothane.* Halothane (50 μl of an 0.6–1.3 M solution in ethanol, initial concentration) or ethanol (50 μl) was vortex mixed into 2.60 to 2.85 ml of phosphate buffer, pH 6.5 (0.1 M). GSH (0.13–1.45 mM) and CDNB (0.028–0.27 mM) were added. The reaction was initiated with the addition of 50 μl of enzyme preparation (*ca.* 0.5 unit/ml).

Time-dependent inhibition of the GSH S-*transferases.* The effect of xenobiotics on the activity of rat liver GSH *S*-transferases *in vitro* was assessed as a function of time in reaction mixtures (total volume 500 μl) constituted as follows: Forty μl of an ethanolic solution of the anesthetic (5–40 mM) or haloethylene (16–57 mM) or an equivalent volume of ethanol was vortex mixed into Tris–HCl buffer (35 mM), pH 8.2, followed by the addition of EDTA (4 mM) and GSH *S*-transferase preparation (normally *ca.* 0.3 unit/ml). Reaction mixtures were incubated at 25° with shaking at 60 cycles per min. The reaction was terminated by chilling to 0°. GSH *S*-transferase activity toward CDNB was assessed with reaction mixtures diluted 60 fold in phosphate buffer (0.1 M), pH 6.5. Slight losses of activity (viz. 10 ± 5%) were noted on incubation of enzyme in buffer for 15 min. Ethanol (3.3%) was without effect on enzyme activity.

Statistical analysis and calculations. Results are given as means ± SD. For *in vivo* studies, N represents the number of determinations each on the pooled cytosols from control or anesthetized rats. Reported N values for *in vitro* experiments assessing GSH *S*-transferase activity as a function of time, reflect the number of determinations each at zero time or following 15 min incubation. For kinetic studies of GSH *S*-transferase activity in the presence or absence of halothane, curves were generated by non-linear regression analysis of the Henri–Michaelis–Menten equation. The apparent K_m and V_{max} values generated by non-linear regression analysis for the rat hepatic cytosolic GSH *S*-transferases and purified isoenzymes in the absence of halothane were consistent with previously reported values [3]. Statistical analyses were performed using Student's *t*-test. A significant difference between means was taken as $P < 0.01$.

* Dialyzed versus 10 mM Tris–HCl, pH 7.8, for 16 hr at 4°.

RESULTS

Effect of halothane anesthesia on hepatic GSH S-*transferase activity* in vivo

A single halothane anesthesia (1.25 MAC for 3 hr) did not significantly affect the specific activity of hepatic cytosolic GSH *S*-transferase toward CDNB at 1, 5, 10, 15, 25 or 30 days after anesthesia (data not shown). In contrast, repeated halothane anesthesia initially decreased and subsequently increased the specific activity of rat hepatic cytosolic GSH *S*-transferase and of GSH peroxidase (Table 1). The specific activity of hepatic GSH *S*-transferase toward CDNB and DCNB and GSH peroxidase toward cumene hydroperoxide reached a nadir on day 10 and a maximum on day 25 or 30 (Table 1). The specific activity of GSH *S*-transferase toward *trans*-4-phenyl-3-buten-2-one was not strikingly affected by multiple halothane anesthesias. The effect of multiple halothane anesthesias on the GSH *S*-transferase activities per gram of wet weight liver showed patterns similar to the specific activities per mg protein, except for *trans*-4-phenyl-3-buten-2-one (Table 1).

Halothane metabolism by GSH S-*transferases* in vitro

Halothane was not measurably metabolized (≤0.2 nmol/mg protein/min) by hepatic cytosolic GSH *S*-transferases. Under identical reaction conditions, 1,2-dibromoethane exhibited significant GSH *S*-transferase dependent conjugation (3.8 nmol/min/mg protein).

Reversible activation of hepatic GSH S-*transferase by halothane* in vitro

Halothane produced a rapid activation of moderate magnitude of certain GSH *S*-transferase preparations under standard assay conditions (1 mM CDNB, 1 mM GSH). The activation was apparent with liver cytosolic GSH *S*-transferases and one pure isoenzyme, and was complete before spectral tracings were begun (i.e. $t_{\frac{1}{2}} \leq 5$ sec). The rapid activation of the cytosolic GSH *S*-transferases by halothane was readily reversible. Dilution of halothane in reaction mixtures from 22.4 mM to 11.2 mM and 7.8 mM, reduced the percentage activity in the presence of halothane relative to controls from 117 ± 1%* to 112 ± 1%* and 104 ± 1%, respectively (N = 4–8).

The activation of the cytosolic GSH *S*-transferases as a function of GSH and CDNB concentration is

Table 1. Effect of multiple halothane anesthesias on hepatic GSH *S*-transferase and GSH peroxidase activity

Assay	Activity per	Percentage activity relative to air exposed controls Time after first halothane anesthesia (days) 5	10	16	20	25	30
CDNB	mg protein	94 ± 3	64 ± 2†	93 ± 3	108 ± 4	153 ± 2†	111 ± 2†
	g liver	94 ± 3	70 ± 2†	89 ± 3*	92 ± 3	114 ± 2†	125 ± 2†
	(N)	(6)	(4)	(6)	(6)	(6)	(6)
DCNB	mg protein	89 ± 2†	66 ± 1†	90 ± 1†	103 ± 2	133 ± 1†	102 ± 2
	g liver	90 ± 2*	72 ± 1†	85 ± 1†	87 ± 2†	99 ± 1	115 ± 3†
	(N)	(4)	(6)	(6)	(6)	(6)	(6)
Trans-4-phenyl-3-buten-2-one	mg protein	95 ± 7	90 ± 1†	115 ± 6*	104 ± 2	109 ± 4*	95 ± 3*
	g liver	95 ± 7	98 ± 2	110 ± 6	88 ± 1†	82 ± 4†	107 ± 3*
	(N)	(6)	(6)	(6)	(8)	(7)	(8)
Cumene hydroperoxide	mg protein	101 ± 3	84 ± 2†	99 ± 1	122 ± 3†	185 ± 7†	107 ± 1†
	g liver	102 ± 3	92 ± 3*	95 ± 1*	103 ± 3	138 ± 5†	121 ± 1†
	(N)	(6)	(6)	(6)	(6)	(6)	(6)

Groups of 9–15 rats were exposed to 1.25 MAC halothane or medical air for 1 hr on days 1, 3 and 5. Results are from one experiment, with each time point representing n determinations each on the pooled cytosols from either 3 contol or 3 anesthetised rats. Similar results for days 5, 10 and 16 were obtained in a separate experiment. Specific activities for control rats in μmol/mg protein/min were as follows: CDNB, 1.0; DCNB, 0.055; *trans*-4-phenyl-3-buten-2-one, 0.013; cumene hydroperoxide, 0.17.

* Differs significantly from corresponding control, $P < 0.01$.

† Differs significantly from corresponding control, $P < 0.001$.

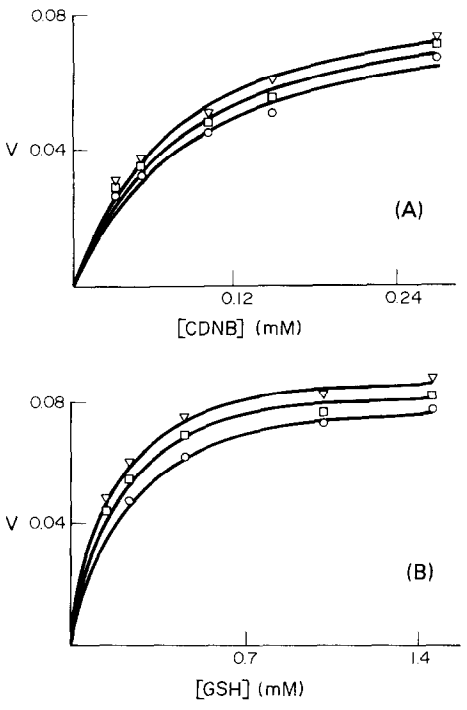

Fig. 1. Reversible activation by halothane of cytosolic GSH *S*-transferases as a function of the concentration of (A) CDNB and (B) GSH. v is given as the change in absorbance at 340 nm per min. For (A), GSH concentration was 1 mM; for (B), CDNB concentration was 0.27 mM. ○, ethanol; □, 11.2 mM halothane; ▽, 22.4 mM halothane.

shown in Fig. 1. Halothane significantly activated purified isoenzyme 1-2 (Fig. 2), but did not produce significant, reproducible activation of isoenzymes 1-1 and 3-3 as a function of CDNB concentration (data not shown).

Time-dependent inhibition of hepatic GSH S-*transferases* in vitro

Four volatile anesthetic agents produced a significant, time-dependent inhibition of the activity toward CDNB of the mixture of rat hepatic GSH *S*-transferases in the absence of GSH (Table 2). The metabolite of halothane 1-chloro-2,2-difluoroethylene (16 mM–57 mM), did not produce significant, dose-dependent inhibition of the mixture of rat liver isoenzymes with time; percent activities remaining at 15 min varied from 80 to 104% relative to controls (data not shown).

The inhibition of the partially purified mixture of rat liver cytosolic GSH *S*-transferase isoenzymes by halothane in the absence of GSH followed pseudo-first order kinetics (Fig. 3). The inhibition was characterized by a pseudo-first order rate constant of

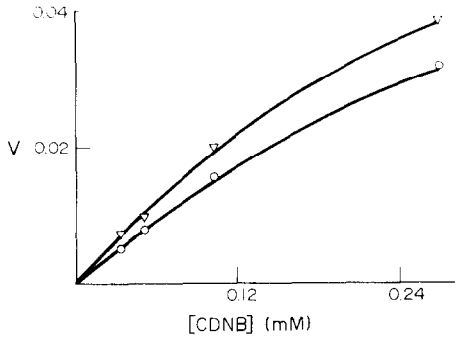

Fig. 2. Reversible activation by halothane of GSH *S*-transferase 1-2. v is given as the change in absorbance at 340 nm per min. The concentration of GSH was 1 mM. ○, Ethanol; ▽, 22.4 mM halothane.

0.13 min^{-1} (half-life of 5.4 min). Half-maximal inhibition of the activity of the mixture of isoenzymes occurred at concentrations of halothane greater than or equal to 15 mM (Fig. 4).† The addition of albumin (400 mg/ml) or liver cytosol (0.04 mg protein/ml)‡ to incubations containing halothane and the mixture of GSH *S*-transferase isoenzymes eliminated the halothane dependent inhibition (data not shown). With rat liver cytosol as the source of GSH *S*-transferase activity, halothane failed to produce statistically significant time-dependent inhibition. Neither the *in situ* perfusion of the rat liver to blanching with ice cold 10 mM Tris–HCl, pH 7.8, prior to the isolation of cytosol, nor the dialysis of the liver cytosol against the same buffer for 1 to 24 hr at 4° subsequent to isolation altered this result (data not shown).

Halothane produced significant time-dependent inhibition of purified rat liver GSH *S*-transferase isoenzymes 3-3, 3-4 and 4-4 but not of isoenzymes 1-1 and 1-2 as a function of time (Table 3).

DISCUSSION

Multiple halothane anesthesias decreased GSH *S*-transferase activity as reported earlier [9], and produced a previously unreported, subsequent rebound in activity (Table 1). The rebound of GSH *S*-transferase activity may be analogous to the superinduction phenomenon seen following suicide inhibition [21, 22] or may represent enzyme activation. In our study and elsewhere [9], a single halothane anesthesia did not affect the binding or catalytic function of the hepatic GSH *S*-transferases.

Information on the hepatic GSH *S*-transferase isoenzymes affected by multiple halothane anesthesias is available from a comparison of the data in Table 1 with the substrate specificities of GSH *S*-transferase isoenzymes. The loss of DCNB activity indicates that isoenzymes containing the 3-subunit were affected by halothane. Consistent with this observation, multiple halothane anesthesias have been reported to decrease hepatic BSP metabolism [9], a process which is characteristic of GSH *S*-transferases containing the 3-subunit [1].

The slight loss of *trans*-4-phenyl-3-buten-2-one activity following multiple halothane anesthesias

* Differs significantly from activity in the absence of halothane, $P < 0.001$.

† These assays were not performed under conditions where the loss of enzyme activity was directly proportional to time (Fig. 3).

‡ This level of cytosolic protein contributed less than 10% of the total GSH *S*-transferase activity in the incubation mixture.

Table 2. The time-dependent inhibition of GSH S-transferases by anesthetic agents *in vitro*

Anesthetic (mM)	% CDNB Activity after 15 min (N)	
	−Anesthetic	+Anesthetic
Halothane (40)	91 ± 5 (18)	70 ± 1 (18)*
Isoflurane (40)	93 ± 7 (12)	76 ± 4 (12)*
Enflurane (40)	93 ± 7 (12)	70 ± 10 (12)*
Methoxyflurane (40)	93 ± 7 (12)	65 ± 5 (12)*

Incubation mixtures contained the mixture of rat liver GSH S-transferases (*ca.* 0.3 Unit/ml), and an ethanolic solution of anesthetic or ethanol in 35 mM Tris–HCl, pH 8.2, at 25°. Percentage activity is relative to zero time samples constituted exactly as were those incubated for 15 min.

* Differs significantly from value for ethanol, $P < 0.001$.

(Table 1), suggests that GSH S-transferases containing the 4-subunit were affected, but to a lesser extent than isoenzymes containing the 3-subunit. The loss of cumene hydroperoxide activity following multiple halothane anesthesias may indicate decreased activity of the 1-, 2- or 5-subunit containing GSH S-transferase and/or decreased selenium-dependent GSH peroxidase activity.

Halothane does not appear to be a GSH S-transferase substrate: The anesthetic was not appreciably metabolized by hepatic cytosol plus GSH (see Results) while the positive control, 1,2-dibromoethane, was [23]. However, halothane bound to one or more hepatic GSH S-transferases as evidenced by the rapid, reversible activation of enzyme preparations by this drug. Halothane produced a net activation of hepatic cytosolic GSH S-transferases and purified isoenzyme 1-2, but not isoenzymes 1-1 and 3-3 (Figs 1 and 2 and Results). The physiological relevance of the reversible activation of hepatic GSH S-transferases by halothane appears questionable since the process was readily reversible, and exhibited low extents of activation (≤125% of controls) even at concentrations of halothane 20-fold greater than the blood level of halothane (*ca.* 1 mM) required for anesthesia [24].

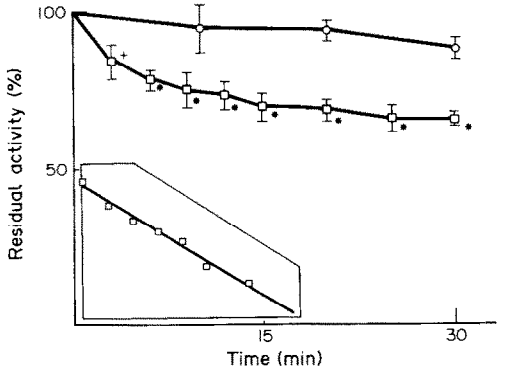

Fig. 3. Time-dependence of halothane inhibition of GSH S-transferase activity. Inset: Ln $(A_t - A_\infty)$ vs time. The mixture of rat liver isoenzymes (0.44 units/ml) was incubated at 25° with EDTA (4 mM), Tris–HCl (40 mM), pH 8.2, in the presence (□) and absence (○) of halothane (40 mM). Differs from zero time sample, * $P < 0.01$, † $P < 0.05$.

The time-dependent inhibition of GSH S-transferases by halothane *in vitro* occurred on the minute time scale, and exhibited an I_{50} approximately one order of magnitude greater than halothane blood levels [24] (Figs. 3 and 4). The reaction exhibited pseudo first-order kinetics and incomplete inhibition of purified isoenzymes at infinity (Table 3). The incomplete inhibition may reflect that only half the sites are reactive, incomplete reaction, or competing pathways (partitioning) [25]. It appears that the halogenated anesthetics isoflurane, enflurane and methoxyflurane inhibit the GSH S-transferases in a manner similar to halothane (Table 2), although the effects of these anesthetic agents were not studied further.

The isoenzyme specificity of halothane inhibition of the GSH S-transferases *in vitro* correlated with the effects of halothane anesthesias *in vivo*. Notably, the 3- and 4-subunit containing isoenzymes were affected in both systems. Although both forms appeared to be equally susceptible to inhibition *in vitro*, the activity associated with the 3-subunit was inhibited more effectively by halothane *in vivo* (Tables 1 and 3). A direct correlation of magnitudes of halothane inhibition of GSH S-transferase activity *in vitro* and *in vivo* is not possible since GSH, protein and possibly other high molecular weight cytosolic components protected against halothane inhibition *in vitro* (see Results), and the magnitude of these potential effects *in vivo* is unknown.

Table 3. Time-dependent inhibition of GSH S-transferase isoenzymes by halothane

Isoenzyme	% CDNB activity after 15 min	
	+Ethanol	+Halothane
1-1	N.D.	94 ± 3
1-2	95 ± 3	89 ± 6
3-3	109 ± 3	43 ± 3*
3-4	85 ± 4	62 ± 4*
4-4	80 ± 1	45 ± 12*

Incubation mixtures contained pure isoenzymes (*ca.* 0.3 Units/ml), halothane (40 mM) or ethanol in 35 mM Tris–HCl, pH 8.2 at 25° and 10 mM GSH. (N = 3–4 for each analysis). N.D. Not determined.

* Differs significantly from activity in the presence of ethanol, $P < 0.01$.

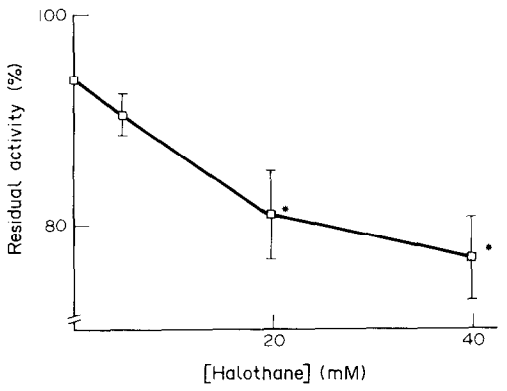

Fig. 4. Effect of halothane concentration on the time-dependent inhibition of GSH *S*-transferase activity. The mixture of rat liver isoenzymes (2.0 units/ml) was incubated with EDTA (4 mM), GSH (10 mM), Tris–HCl (40 mM) and variable concentrations of halothane at 25° for 15 min. Residual activity is given as percentage of the zero time activity of corresponding samples. * Differs significantly from controls, $P < 0.01$.

Several xenobiotics have been shown to inhibit analogous hepatic GSH *S*-transferase isoenzymes *in vitro* and *in vivo*. 1,2-Dibromoethane inhibited isoenzymes 3-3 and 3-4 *in vitro* and *in vivo* [11, 12], and chloroform diminished the activity of isoenzymes 1-2, 2-2, 3-3 and 3-4, but not 4-4 and 5-5 *in vitro* and *in vivo* [26].

The correlation between the selectivity of inhibition of GSH *S*-transferase isoenzymes by halothane and other xenobiotics *in vitro* and *in vivo* suggests that GSH *S*-transferase inhibition may provide one mechanism for the loss of transferase activity *in vivo*. An alternate mechanism may involve the release of hepatic GSH *S*-transferase activity into the serum which is stimulated by several xenobiotics [11, 26, 27]. The effect of halothane on serum GSH *S*-transferase activity was not investigated.

Since halothane did not require metabolic activation *in vitro* to produce time-dependent inhibition, the capability for transferase inactivation appears to reside in the parent molecule, in contrast to chloroform which requires metabolic activation by cytochrome P-450 to inhibit GSH *S*-transferases [26]. This proposal is supported by the relative ineffectiveness of 1-chloro-2,2-difluoroethylene to produce time-dependent inhibition of GSH *S*-transferase activity *in vitro* (see Results). This cytochrome P-450 metabolite of halothane is the only halothane metabolite thought to undergo GSH conjugation *in vivo* [7, 8]. The relative unreactivity of the haloethylene compared to the parent molecule is consistent with the reactivity toward nucleophilic addition to protein side chains. Identical order of reactivity was found for 1,1,2-trichloroethane versus trichloroethylene (unpublished results).

It is concluded that halothane rapidly and reversibly activated one or more GSH *S*-transferase isoenzymes, and by a different mechanism produced a slow inhibition of the 3- and 4-subunit containing GSH *S*-transferase isoenzymes *in vitro*. The latter effect may underlie the loss of hepatic GSH *S*-transferase activity *in vivo* following halothane anesthesias.

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