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In Vitro Inhibition of Rat and Human Glutathione S-Transferase Isoenzymes by Disulfiram and Diethyldithiocarbamate

Jan-Peter H. T. M. Ploemen,§ Marlou L. P. S. van Iersel,† Lars W. Wormhoudt,‡ Jan N. M. Commandeur,‡ Nico P. E. Vermeulen‡ and Peter J. van Bladeren*†*

*TNO Toxicology, Utrechtseweg 48, P.O. Box 360, 3700 AJ Zeist, The Netherlands; †Department of Toxicology, Wageningen Agricultural University, The Netherlands; and ‡LACDR-Department of Pharmacochemistry, Free University, Amsterdam, The Netherlands

ABSTRACT. The drug disulfiram (DSF, Antabuse®) has been used in the therapy of alcohol abuse. It is a potent inhibitor of aldehyde dehydrogenase. Its reduced form, diethyldithiocarbamate (DDTC), and further metabolites show similar activities. DSF and DDTC have also been widely used to inhibit mixed-function oxidases. In this study, the reversible inhibition and time-dependent inactivation of the major rat and human glutathione S-transferase (GST) isoenzymes by DSF and DDTC was investigated. Reversible inhibition, using 1-chloro-2,4-dinitrobenzene as substrate for the GST alpha-, mu-, and pi-class, expressed as I_{50} (in μM), ranged from 5–18 (human A1-1), 43–57 (rat 4-4) and 66–83 (rat 1-1), for both DSF and DDTC. The I_{50} for rat GST theta, using 1,2-epoxy-3-(p-nitrophenoxy)-propane as substrate, was 350 μM for DDTC. The other GSTs were significantly less sensitive to inhibition. The major part of reversible inhibition by DSF was shown to be due to DDTC, formed rapidly upon reduction of DSF by the glutathione (GSH) present in the assay to measure GST activity. The oxidized GSH formed upon reduction of DSF might also have made a minor contribution to reversible inhibition. The rat and human pi-class was, by far, the most sensitive class for time-dependent inactivation by DSF, but no such inactivation was observed for any of the GSTs by DDTC. Moderate susceptibility to inactivation by DSF of all the other GSTs was observed, except for human A2-2, which does not possess a cysteine residue. Consistent with the assumption that a thiol residue is involved in this inactivation, a significant part of the activity could be restored by treatment of the inactivated GST with GSH or dithiotreitol. BIOCHEM PHARMACOL 52;2:197–204, 1996.

KEY WORDS. glutathione S-transferases; disulfiram; inhibition; diethyldithiocarbamate; glutathione; *in vitro*

Disulfiram (Antabuse®) has been used in the treatment of alcohol abuse for more than 40 years [1]. The combined intake of disulfiram and ethanol provokes an unpleasant reaction (associated with nausea and vomiting), which is the basis of its therapeutic use. After oral ingestion, disulfiram is rapidly absorbed from the human gastrointestinal tract [2]. Although a small part of the drug is already reduced in the stomach, the major fraction of disulfiram is rapidly reduced to its monomer DDTC[‖] after absorption into the blood [3–6]. Next, DDTC undergoes further metabolism that involves S-methylated compounds [7], S-glucuronides [8], some minor glutathione conjugates [9], and the formation of carbon disulfides [2]. Moreover, disulfiram has been shown to form mixed disulfides with proteins [2]. The potent inhibition of the liver mitochondrial low-K_m form of aldehyde dehydrogenase by disulfiram and its metabolites results in a dramatic rise in the concentration of acetaldehyde after alcohol ingestion, which is considered to account for most of the symptoms observed during treatment with disulfiram [1, 2, 10]. In particular, the methyl-N,N-diethylthiocarbamoyl sulfoxide and its corresponding sulfone metabolites of disulfiram have been identified as the most potent inhibitors of the low-K_m form of aldehyde dehydrogenase [9, 11].

Disulfiram and DDTC have also been used as inhibitors of cytochrome-P450 enzymes for more than 20 years [12, 13]. The biotransformation reactions undergone by xenobiotics can be conveniently divided into two major classes: phase-I reactions, in which oxidation, reduction, and hydrolysis reactions often serve to provide a chemical handle on the xenobiotic molecule, to which a hydrophilic group can be conjugated in the phase-II reaction [14]. Cytochrome-P450 enzymes are an integral part of the enzyme system catalyzing these phase-I reactions [15]. Because of the crucial role played by P450s in biotransformation reac-

§ Corresponding author. Tel. +31 30 69 44418; FAX +31 30 69 60264.

‖ *Abbreviations:* CDNB, 1-chloro-2,4-dinitrobenzene; DDTC, diethyldithiocarbamate; DTT, dithiotreitol; EPNP, 1,2-epoxy-3-(p-nitrophenoxy)-propane; GSSG, oxidized glutathione; GSH, reduced glutathione; GST, glutathione S-transferase

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tions, inhibitors such as disulfiram and DDTC have been useful in studying its function in the metabolism of xenobiotics *in vivo* [16, 17] and *in vitro* [18].

The multigene family of glutathione S-transferases (GSTs; EC 2.5.1.18) is an important enzyme system involved in the conjugation of electrophilic compounds with GSH, a typical phase-II reaction [19, 20]. Supplementing the diet of mice with disulfiram induced GST activity to a slight extent in cytosols of several organs upon prolonged exposure [21–23]. On the other hand, although extensive lists of both reversible and irreversible acting inhibitors of GST isoenzymes have been published [19, 24], to our knowledge the direct-acting inhibitory potential of disulfiram and DDTC on GST has not yet been studied in detail. In this paper, we describe a series of experiments designed to determine the inhibitory capacity of disulfiram and DDTC on GSTs, using purified isoenzymes of rats and humans.

MATERIALS AND METHODS

Chemicals

Disulfiram (tetraethylthiuram disulfide, 1,2-epoxy-3-(p-nitrophenoxy)-propane (EPNP), CDNB, and GSSG were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DDTC (diethyldithiocarbamic acid, sodium salt, trihydrate, p.a.), and DTT were obtained from Acros Chimica (Geel, Belgium). GSH was purchased from Boehringer (Mannheim, Germany).

GST Purification

GST isoenzymes were purified from liver, kidney (rat GST 7-7) and placenta (human GST P1-1) using affinity chromatography (S-hexylglutathione-Sepharose 6B), as described previously [25]. The separation of the different isoenzymes was achieved by chromatofocusing on polybuffer exchanges (Pharmacia, Uppsala, Sweden), as previously described [26]. The rat recombinant GST 5-5 was purified from a culture of *E. coli* JM 105 containing the pKK233-2 plasmid with the GST 5-5 construct, as previously described [27]. Purity was confirmed by HPLC analysis [28] and isoelectric focusing [25].

Inhibition Studies

Inhibition was determined by mixing in the cuvettes of the enzymic assay to a final concentration of 10 nM GST P1-1, 20 nM Mla-1a, 25 nM 1-1, 3-3, and A1-1, 50 nN 2-2, 4-4, 5-5, 7-7, and A2-2 with at least 6 concentrations (in triplicate) in the concentration range of 5–100 μM and 5–2100 μM, for disulfiram and DDTC, respectively. Stock solutions of disulfiram were made in acetone, resulting in a constant final concentration of 2.5% acetone (v/v) in the blank and all other incubations. The maximal solubility reached for disulfiram was 100 μM. Reversible inhibition was also studied with 0.1 and 1 mM GSSG with the same enzyme solutions (in triplicate).

The K_i-values for disulfiram using the rat GST isoenzymes were measured with either GSH or CDNB held constant at 1 mM, while the concentration of the other substrate was varied. Four different inhibitor concentrations of disulfiram (range 10–100 μM) were used, resulting in at least 16 independent assays to calculate the K_i. In the case of the theta-class GST 5-5, the substrates were held at a constant concentration of 0.5 mM and 5 mM, for EPNP (see below) and GSH, respectively. The mechanism of inhibition was determined graphically from Lineweaver-Burke and Hanes-Woolf plots.

The time-dependent inactivation of the GSTs by disulfiram and DDTC was studied by the incubation of 1 μM GST isoenzyme with 50 μM disulfiram at 25°C in 0.1 M potassium phosphate buffer pH 7.4 (incubations were performed in triplicate). Samples were drawn from the incubation and assayed immediately for GST activity (see below; final concentration of 20 nM GST and 1 μM disulfiram in the assay). The catalytic activity of GST (1 μM), inactivated with disulfiram (50 μM; 133 min), was monitored after the addition of GSH (to a final concentration of 20 mM) and, finally, DTT (to a final concentration of 50 mM). For GST 5-5 and 7-7, time-dependent inactivation was also studied in the presence of 2.5 mM GSH.

Glutathione S-transferase Assay

The activity of individual alpha-, mu-, and pi-class GST isoenzymes was determined with 1 mM GSH and 1 mM CDNB as second substrate at 25°C in 0.1 M potassium phosphate buffer pH 6.5, using the spectrophotometric method of Habig *et al.* [29]. EPNP (0.5 mM) was used as a second substrate to assay the activity of the theta class GST 5-5 [29], because this enzyme has no activity toward CDNB. The final concentration of the solvent (ethanol) used for the second substrate was always 4% (v/v) in both assays.

Analysis of the Reaction of GSH with Disulfiram and DDTC by UV-spectroscopy

The UV-difference spectra after reaction of 50 μM disulfiram or DDTC with 50 μM GSH or GSSG were recorded at 25°C in 0.05 M potassium phosphate buffer pH 7.4 (range 200 to 500 nm; bandwidth 2.0 nm, scanspeed 300 nm/min) on a Cary 1E UV-Visible Spectrophotometer (Varian, Australia). The changes in the spectra were monitored by scanning several cycles (up to 15 min). Disulfiram was dissolved in ethanol (final concentration always 2.5%).

Miscellaneous

Protein was determined by the method of Lowry *et al.* [30], using bovine serum albumin as standard.

RESULTS

Analysis of the Reaction of GSH with Disulfiram and DDTC by UV-spectroscopy

Disulfiram is reduced rapidly *in vivo* [3–6]. Because the enzymic assays to assess GST activity require GSH (1 mM), a major part of disulfiram might be reduced immediately. To study this phenomenon, a UV-spectrum of disulfiram (50 μM) was recorded as baseline and verified for stability. After adding equimolar amounts of GSH to a cuvette with disulfiram, immediately after mixing (delay time about 5–10 sec), two characteristic peak maxima at 282 and 256 nm, and one characteristic minimum at 270 nm were found in the UV-difference spectra (Fig. 1). Approximately 90–95% of the spectral change was reached immediately after mixing, indicating that the reaction was rapidly completed. The recorded UV-difference spectrum was very similar to the spectrum of DDTC (in which the absorbance maxima were approximately 25% higher; result not shown), indicating that, even at an equimolar ratio, the major fraction of disulfiram is rapidly reduced by GSH. The UV-spectrum of DDTC itself was not affected by GSH nor by GSSG.

Inhibition Studies

The inhibition of GSTs by disulfiram and DDTC, expressed as I_{50}, is shown in Table 1. This inhibition (in the presence of the GSH of the enzymic assay) is characterized by a noncovalent interaction of the inhibitor with the enzyme, which is reversible (as determined by dialysis; result not shown). The human GST A1-1 was, by far, the most sensitive to reversible inhibition by disulfiram and DDTC, followed by the rat GST 4-4 and 1-1. From the UV-spectroscopy experiments described above, it was clear that significant amounts of DDTC were present in the incubations with disulfiram, despite the fact that catalytic activity was measured immediately after mixing of the enzyme, disulfiram, and the substrate. Indeed, the I_{50}-values of DDTC and disulfiram were in the same range and were correlated. In general, the I_{50}-values for disulfiram were slightly lower. Three mechanisms may be involved in this phenomenon: 1. because two molecules of DDTC were formed from disulfiram, this may result in stronger inhibition by disulfiram when DDTC is the only determinant of inhibition; 2. the presence of unreduced disulfiram, which may have a stronger inhibitory capacity than DDTC, may result in more potent inhibition; and/or 3. in principle, the GSSG, which is formed upon the reduction of disulfiram, may also add to reversible inhibition. Using 100 μM disulfiram, a maximal concentration of 100 μM GSSG is expected in these *in vitro* incubations. From Table 2, it is clear that the GSSG also contributes to the observed inhibition by disulfiram for the individual GST. A high concentration of GSSG, which might be reached using potent solvents of disulfiram (such as Tween or DMSO), will significantly inhibit all enzymes *in vitro*.

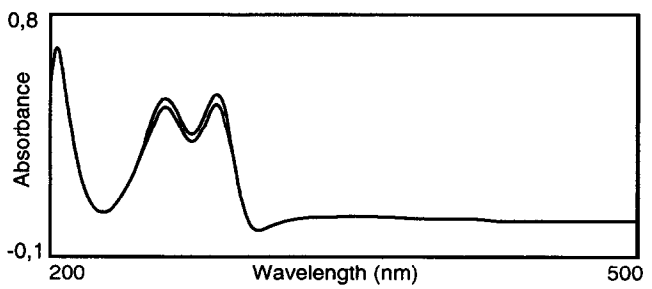

FIG. 1. Typical UV-difference spectra of GSH added to disulfiram. The disulfiram spectrum (50 μM) was recorded as baseline, then equimolar amounts of GSH were added. The spectrum shown was recorded immediately after mixing and after 10 min (after which no changes were observed).

TABLE 1. I_{50} (μM) values of rat and human GST isoenzymes inhibited by disulfiram and DDTC

Isoenzyme	Disulfiram	DDTC
Alpha-class		
1-1	66 [64. .68]*	83 [79. .89]
2-2	>100 (17 ± 0.2)**	>2100 (32 ± 1.3)
A1-1	5 [4. .6]	18 [16. .20]
A2-2	>100 (15 ± 1.1)	>2100 (22 ± 0.7)
Mu-class		
3-3	>100 (18 ± 0.4)	>2100 (14 ± 0.7)
4-4	43 [38. .48]	57 [50. .65]
M1a-1a	>100 (32 ± 2.2)	605 [555. .675]
Pi-class		
7-7	>100 (23 ± 0.7)	>2100 (31 ± 0.8)
P1-1	>100 (30 ± 3.6)	>2100 (41 ± 2.6)
Theta-class		
5-5	>100 (19 ± 5.2)	350 [295. .445]

I_{50}-values for the alpha-, mu-, and pi-class were determined with CDNB (1 mM) as second substrate, and for the theta-class, EPNP (0.5 mM) was used. GSH was held constant at 1 mM. *Between brackets [ ] the 95% confidence interval. **Between parentheses ( ) % inhibition ± SD at 100 and 2100 μM, for disulfiram and DDTC, respectively.

TABLE 2. The inhibition of rat and human GST isoenzymes by oxidized glutathione (GSSG)

Isoenzyme	GSSG (0.1 mM)	GSSG (1 mM)
Alpha-class		
1-1	29 ± 4.1	54 ± 1.1
2-2	27 ± 0.8	72 ± 2.6
A1-1	16 ± 0.8	58 ± 1.5
A2-2	2 ± 0.1	21 ± 0.6
Mu-class		
3-3	15 ± 1.2	50 ± 3.0
4-4	27 ± 1.1	74 ± 8.7
M1a-1a	15 ± 1.4	54 ± 7.3
Pi-class		
7-7	28 ± 0.5	77 ± 4.0
P1-1	28 ± 3.6	71 ± 3.7
Theta-class		
5-5*	7 ± 0.4	33 ± 1.6

The inhibition (expressed as % inhibition) for the alpha-, mu-, and pi-class GST isoenzymes were determined with CDNB (1 mM) as second substrate and, for the GST theta-class, EPNP (0.5 mM) was used. GSH was held constant at 1 mM. Values were the average ± SD of three incubations. *measured in duplicate.

To determine K_i values for disulfiram, the inhibition of rat GSTs was studied at various substrate concentrations, after which the mechanism of inhibition was examined by graphical methods. Although competitive inhibition was the predominant mechanism of inhibition in most cases (especially versus GSH), there was no isoenzyme that showed a single type of inhibition (data not shown), and, hence, no K_i could be determined. This might be expected because both the DDTC and the GSSG formed, as well as the parent disulfiram, contribute to inhibition. Additionally, especially at low GSH concentrations, inactivation by covalent modification of the enzyme may also contribute to inhibition (see below).

The time-dependent inactivation of the GSTs by disulfiram is shown in Fig. 2. The GST pi-class is, by far, the most sensitive to inactivation by disulfiram. At the first time-point (5 min), the human GST P1-1 was more than 97% inhibited. The human GST A1-1 showed a biphasic inhibition. However, this was due to the sensitivity of human GST A1-1 to reversible inhibition (the inhibition is affected by 1 μM disulfiram, which is present in the enzyme assay). For the other isoenzymes, the rate of inhibition was more or less the same, except for the human GST A2-2. This prompted us to study the role of thiols in the inactivation, because the human GST A2-2 is the only isoenzyme which does not have a cysteine residue [31], and because of the rapid reduction of disulfiram observed (see above). Consistent with the involvement of a thiol group, a partial restoration of catalytic activity occurred under mild reduction with GSH, which could be enhanced by the use of a strong reducing agent (DTT) in most cases (Table 3). However, full restoration of activity was observed only for the human P1-1. This might indicate that, for the other GSTs studied, a different chemical reaction is involved or that the modified thiol of these GSTs are more resistant to reduction by DTT. Finally, inhibition was studied in GST 5-5 and 7-7 in the presence of 2.5 mM GSH. No inhibition could be observed under these conditions (data not shown). GSH protects GSTs from the time-dependent type of inactivation by disulfiram, presumably by reducing them to DDTC, which does not inactivate GSTs in this way (Fig. 2). Only for GST 5-5 was a small, but significant, inhibition by DDTC seen (Fig. 2). Again, for the human A1-1 a decrease is seen initially (at the first time-point), one that can be assigned to reversible inhibition (see above).

DISCUSSION

The present study shows that disulfiram and its reduced form (DDTC) inhibit GST isoenzyme selectively by reversible inhibition, and a time-dependent inactivation was shown for disulfiram.

From the set of experiments with purified GSTs, it is concluded that rapidly formed DDTC is presumably the major factor determining the reversible inhibition of disulfiram *in vitro*. However, disulfiram itself and the GSSG formed upon reduction may also contribute to reversible inhibition. Owing to the activity of glutathione reductase, GSSG is retained at low concentration (20–40 μM) *in vivo* [32] and, thus, GSSG does not significantly contribute to reversible inhibition *in vivo*. DDTC is further metabolized *in vivo* [7–9]. Thus, the *in vivo* reversible inhibition of disulfiram will depend on the rate of metabolism of disulfiram and DDTC, the inhibitory capacity of its metabolites, and general factors such as protein-binding and pharmacokinetic behavior. In general, reversible acting inhibitors of GSTs do not display high selectivity, especially within a GST class [19, 33]. In this case, significant selectivity of DDTC for human A1-1 was observed. Thus, DDTC may be used, for example, to rapidly distinguish between A1-1 or A2-2 or to study the inhibition of human A1-1 in cytosols.

Selectivity was also found for the inactivation of GST by disulfiram: the pi-class of GST was, by far, the most sensitive to time-dependent inactivation by disulfiram in the absence of GSH. In this case, the specific sensitivity of the pi-class is presumably due to the well-known reactive thiol, identified near or at position 47 in the amino acid sequence of most species studied [34–37]. The enzyme may be inactivated by oxidation with concomitant formation of an intrasubunit disulfide [38] or by a thiol/disulfide exchange reaction with disulfiram and Cys 47 [39, 40]. Disulfiram and related compounds are bound to other proteins both *in vitro* and *in vivo via* formation of mixed disulfides with the free thiol group of various proteins [2, 41, 42]. However, because the inactivation of GSTs was completely abolished in the presence of 2.5 mM GSH *in vitro*, and because the GSH concentration in most eukaryotic cells is generally in the range of 2–10 mM [43], it might be expected that *in vivo* inactivation of GST by disulfiram is only of importance when GSH levels fall dramatically (e.g., by drugs metabolized by GST).

In toxicological research, disulfiram has been used to inhibit cytochrome-P450s [12, 13, 16–18]. It has been shown that disulfiram and DDTC are potent inhibitors of the catalysis of selective substrates of cytochrome P450IIE1 (e.g., of several low-molecular-weight compounds such as benzene, styrene, and most halogen alkanes and alkenes) [44–48]. In animals and humans, a single oral dose of disulfiram produced rapid inhibition of the catalytic activity of cytochrome P450IIE1 using the (semi)-specific substrate N-nitrosodimethylamine and chlorzoxazone [45, 49]. However, rat P450IIB1 was induced markedly between 15 and 72 hr after disulfiram treatment [45]. Furthermore, the inhibitory capacity of DDTC using a nonselective substrate (7-ethoxycoumarin) or a selective substrate (chlorzoxazone) was different: DDTC was not a selective inhibitor of 7-ethoxycoumarin O-deethylation by P450IIE1 (I_{50} value approximately 125 μM), because the P450s IA1, IA2, IIA6, IIB6, IIC8, IIIA3, and IIIA4 were also inhibited [50], and DDTC was a selective inhibitor of chlorzoxanone 6-hydroxylation (I_{50} value approximately 10 μM) [51]. In addition, the I_{50} values for P450IIE1 were, depending on the substrate used, in the same range or significantly higher than the value obtained for human GST A1-1. Thus, in the

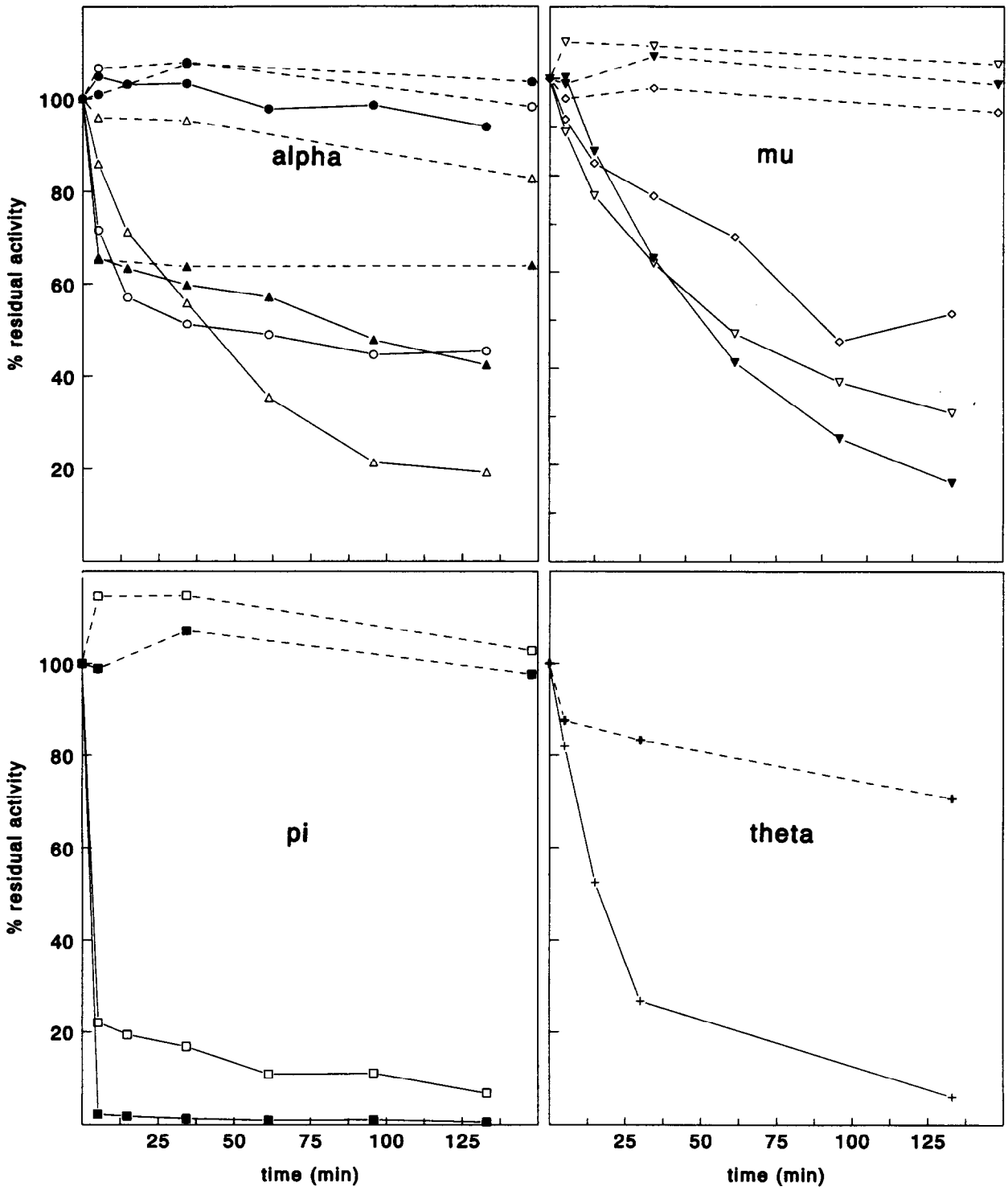

FIG. 2. Time-dependent inactivation of rat and human GST isoenzymes by disulfiram and DDTC. One μM isoenzyme was incubated with 50 μM disulfiram or DDTC at 25°C in 0.1 M potassium phosphate buffer pH 7.4. At appropriate time intervals, samples were drawn from the incubations and assayed immediately for GST activity. The incubations were performed in triplicate (duplicate measurements for GST 5-5). Solid lines and dotted lines were used, respectively, for disulfiram and DDTC. Symbols = alpha-class: △, 1-1; ○, 2-2; ▲, A1-1; ●, A2-2; mu-class: ▽, 3-3; ◇, 4-4; ▼, M1a-1a; pi-class: □, 7-7; ■, P1-1; and theta-class: +, 5-5.

study of biotransformation, it is recommended to use a different P450IIE1 inhibitor than disulfiram because of the nonspecificity and concomitant inhibition of GSTs.

Disulfiram has been shown to inhibit chemically-induced carcinogenesis [52]. Induction of GST by anticarcinogenic compounds is believed to be part of the mechanism of anticarcinogenesis [22, 53]. Administration of disulfiram in the diet for several days induced GST activity approxi-

TABLE 3. Restoration of the catalytic activity of disulfiram-inactivated GST by incubation with glutathione and dithiotreitol (expressed as % remaining activity)

		+ Gluthathione		+ Dithiotreitol	
Isoenzyme	**start***	**35 min**	**65 min**	**10 min**	**110 min**
Alpha-class					
1-1	19 ± 0.9	24 ± 0.8	24 ± 1.2	58 ± 4.6	68 ± 2.3
2-2	45 ± 0.8	60 ± 2.7	64 ± 0.7	83 ± 6.5	81 ± 3.2
A1-1	42 ± 1.1	32 ± 1.0	49 ± 1.9	44 ± 1.9	52 ± 3.1
Mu-class					
3-3	31 ± 4.5	48 ± 4.4	47 ± 0.3	57 ± 2.0	70 ± 2.7
4-4	51 ± 4.5	62 ± 1.2	62 ± 5.9	65 ± 5.7	79 ± 1.9
M1a-1a	16 ± 0.2	26 ± 2.3	32 ± 0.6	40 ± 0.1	49 ± 1.7
Pi-class					
7-7	7 ± 1.4	22 ± 4.8	25 ± 5.0	52 ± 7.9	49 ± 6.0
P1-1	0.6 ± 0.2	22 ± 0.5	30 ± 5.0	85 ± 2.6	103 ± 2.2
Theta-class					
5-5**	6 ± 0.8	9 ± 1.3	9 ± 6.4	28 ± 0.9	32 ± 1.3

* First, the remaining activity of the enzymes (1 μM) that had been incubated for 133 min with 50 μM disulfiram was measured. Next, the enzymes were incubated with 20 mM GSH, after which the activity of the enzymes was determined. Finally, DTT (final concentration 50 mM) was added to the same tubes, and the activity was measured. The experiments were performed in triplicate. Values were average ± SD.
** Measured in duplicate.

mately 2-fold [21–23]. Thus, a distinction presumably has to be made between short- and long-term effects in processes induced by disulfiram in a cell: initial inhibition is followed by induction, a phenomenon observed for other compounds affecting biotransformation enzymes, including P450IIE1 [53]. In this field, it might not only be the GST-disulfiram interaction that triggers induction, but also the GSSG/GSH levels affected by disulfiram, which is involved in the regulation of GST and other biotransformation enzymes [54].

In summary, disulfiram and DDTC have been shown to have GST inhibitory capacity. *In vitro* reversible inhibition by disulfiram was seen for several GST isoenzymes and is, in principle, due to rapidly formed DDTC. Rapid inactivation of GST pi by disulfiram and involving a thiol occurs *in vitro*. *In vivo*, disulfiram is rapidly reduced, leaving reversible inhibition as the main mechanism of inhibition.

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