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SHORT COMMUNICATIONS

Inhibition of glutathione *S*-transferases from rat liver by *S*-nitroso-L-glutathione

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The glutathione *S*-transferases are enzymes involved in the detoxication of a wide variety of electrophilic chemicals of both exogenous and endogenous origin [1, 2]. They have received particular attention recently in two contexts: those of the metabolism of carcinogens and cytotoxic drugs and the development of resistance to them in target cells [3–8], and of the metabolism of pesticides and the development of resistance to them in the target organism [9, 10]. In these contexts, the ability to inhibit these enzymes strongly and specifically would have considerable toxicological consequences and we are currently examining a number of different types of glutathione conjugate with a view to identifying potent inhibitors of these enzymes.

S-nitroso compounds are reported to be very effective nitrosating agents [11]. The simple, one step synthesis of *S*-nitroso-L-glutathione has been reported [12]. That this compound might bind specifically to the enzyme and might irreversibly modify susceptible groups in the active centre has led us to investigate it as an inhibitor of the glutathione *S*-transferases of rat liver.

Methods

Sodium nitrite and 1-chloro-2,4-dinitrobenzene were purchased from BDH Laboratories Ltd., Colnbrook, Bucks., U.K. Pharmacia Polybuffer exchanger and Pharmalyte 8–10.5 were purchased from Sigma Chemical Co. (St Louis, MO). All other chemicals were of the highest available commercial grades.

Experimental animals were Sprague–Dawley rats of both sexes of average weight 220 g. They were stunned and killed by cervical dislocation and the livers were removed and used immediately. *S*-Nitrosoglutathione was synthesised according to the method of Hart [12]. It was recrystallized three times from aqueous ethanol. $C_{10}H_{16}N_4O_7S$, requires C, 35.3%; H, 4.85%, N, 16.42%, S, 9.15%; N, 16.42%; S, 9.15%; found C, 35.7%; H, 4.8%, N, 16.6%; S, 9.5%. Glutathione *S*-transferases 1-1 and 1-2 were purified by affinity chromatography on immobilised GSH-sulfobromophthalein* conjugate [13]. Elution of enzymes from the affinity matrix was effected with 5 mM sulfobromophthalein and the partially purified enzymes were separated using chromatofocusing on PBE 118 matrix as described by Mannervik and Jensson [14]. The enzymes thus obtained were homogeneous as ascertained by electrophoresis on polyacrylamide gels in the presence of SDS [15]. Kinetic analyses were carried out at 25° in 0.1 M-sodium phosphate buffer, pH 6.5. GSH was made up as a stock solution of 60 mM. This was kept frozen in 1 ml aliquots until required. When in use, the GSH solution was kept on ice. The *S*-nitrosoglutathione was made up daily as a stock solution of 60 mM. Reaction rates were measured using a Varian-Cary 210 double beam spectrophotometer. Concentrations of GSH in the assay mixtures varied between 0.1 and 2 mM and those of the electrophilic substrate CDNB were varied between 0.1 and 1.0 mM. Rate measurements were made in triplicate. Final concentrations of *S*-nitrosoglutathione ranged from 0 to 0.8 mM. In some experiments the inhibitor was preincubated with the enzyme before assays were undertaken.

Kinetic data were analysed by fitting the raw data to rate equations by means of a least squares non-linear regression routine, KINETICS 301, written for the Apple IIe. Rate data were unweighted [16].

Results

The glutathione *S*-transferase 1-1 and 1-2 were purified in homogeneous form by the two step affinity chromatography-chromatofocusing procedure described in Methods (see Fig. 1). The total recovery of activity in the isoenzyme peaks shown in Fig. 1 was 39%. Isoenzymes 1-1 and 1-2 accounted for 2.4 and 8.1% of the starting activity, respectively.

The inhibition studies performed on these enzymes revealed that *S*-nitrosoglutathione was a moderate inhibitor of both of the isoenzymes examined. There was no evidence of irreversible inhibition either when the inhibitor was added immediately prior to the experiment or when the enzyme was preincubated with it for periods of up to 20 min. On the contrary the mode of inhibition was classically competitive with respect to glutathione and non-competitive with respect to CDNB in the case of both enzymes. The results are shown in Table 1 and typical double reciprocal plots are shown in Figs 2 and 3.

It has been reported that, under the conditions employed, there is evidence of reaction between the *S*-nitrosoglutathione and GSH in the reaction mixture [12]. Tests for appearance of nitrite were negative and no significant change in the extinction at 327 nm, due to the nitrosoglutathione, was found over a period of 20 min. The inhibition observed seems unlikely, therefore, to be attributable to the formation of oxidized glutathione.

Discussion

From the data presented above, it appears that *S*-nitrosoglutathione inhibits the glutathione *S*-transferases tested solely by competing with glutathione for its binding site at the active centre of the enzymes. There is no evidence of covalent modification of the enzymes and, under the conditions employed, the inhibitor is stable for the duration of the assays. This being so, this compound may well find use in further mechanistic studies on these enzymes as a non-reactive substrate analogue (as opposed to product inhibitors which may also compete with binding of electrophilic substrates to their binding site). Such analogues, notably the oxy- and desthio-analogues of GSH (γ-L-Glu-L-Ser Gly and γ-L-Glu-L-Ala Gly respectively) have been synthesized by others [17]. The oxy-analogue inhibits the rat liver isoenzymes 3-3 and 4-4 competitively with respect to GSH, the inhibition being characterized by K_i values of 13 and 38 μM respectively [17]. The inhibition of the isoenzymes 1-1 and 1-2 by *S*-nitrosoglutathione is thus very similar in both qualitative and quantitative terms. The synthesis of the oxy- and desthio- analogues is relatively complex and a particular advantage of nitrosoglutathione in this context may therefore be the simplicity of its preparation.

The lack of covalent modification by *S*-nitrosoglutathione of the enzymes examined is perhaps not surprising. The sulphydryl group of glutathione seems most likely to lie adjacent to a site which is required to accommodate a range of hydrophobic and electrophilic substrates. This

* Abbreviations used: GSH, reduced glutathione, CDNB, 1-chloro-2,4-dinitrobenzene.

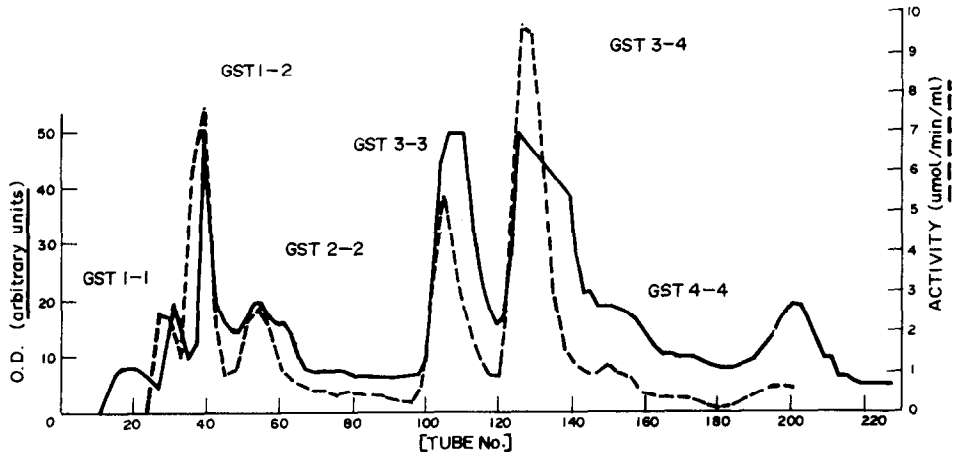

Fig. 1. Purification of glutathione *S*-transferases from rat liver by chromatofocusing. An extract obtained by affinity chromatography was separated into its component isoenzymes on PB 118 [14]. Isoenzymes were identified by their subunit structure as determined on SDS–PAGE [15].

Table 1. Inhibition of glutathione *S*-transferases 1-1 and 1-2 from rat liver by *S*-nitrosoglutathione: kinetic parameters

Enzyme	Varied substrate*	V_m	K_m	K_i
1.1	GSH	0.893 ± 0.08	0.143 ± 0.047	0.0165 ± 0.005
1.1	CDNB	2.302 ± 0.47	1.35 ± 0.34	0.13 ± 0.01
1.2	GSH	5.19 ± 0.53	0.126 ± 0.04	0.013 ± 0.006
1.2	CDNB	8.6 ± 0.81	0.73 ± 0.10	0.127 ± 0.008

* When GSH was the varied substrate, the concentration of CDNB was held at 1 mM. When CDNB was the varied substrate, the concentration of GSH was maintained at 1 mM.

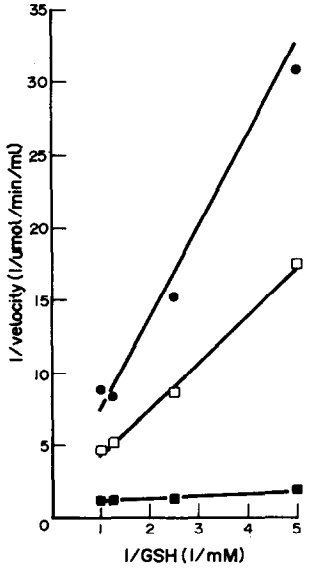

Fig. 2. Inhibition with respect to GSH of glutathione *S*-transferase 1-1 from rat liver, by *S*-nitroso-L-glutathione. Substrate concentrations were varied as described in Methods. Concentration of *S*-nitroso-L-glutathione were: ■, 0; □, 0.4 mM; ●, 0.8 mM.

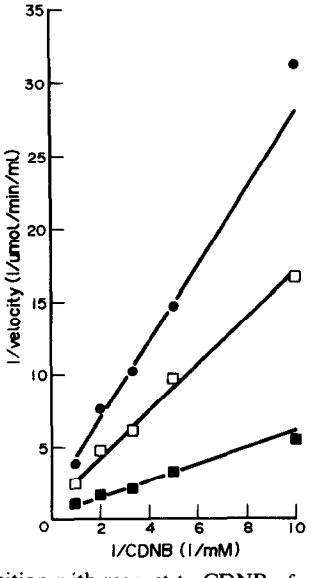

Fig. 3. Inhibition with respect to CDNB of glutathione *S*-transferase 1-1 from rat liver, by *S*-nitroso-L-glutathione. Symbols as in Fig. 2.

hydrophobic cleft is unlikely therefore to include reactive nucleophiles unless the catalytic mechanism proceeds via a covalently modified enzyme intermediate. There is kinetic [18] and stereochemical [19] evidence that this is not the case. The findings of this work are therefore compatible with the sequential mechanisms that have been proposed on kinetic grounds [18].

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Decreased hepatic glutathione *S*-transferase A, AA and L concentration produced by prolonged thyroid hormone administration

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The glutathione *S*-transferases (GST) are a complex group of enzymes that possess several biological functions but the primary function of GST is considered to be one of detoxification [1]. The cytosolic GST forms are comprised of two subunits of approximately similar size and in rat liver the major subunit forms are Ya (M_r 25,500), Yc (M_r 27,500), Yb_1 and Yb_2 (both M_r 26,300). These subunits may combine to produce various GST isoenzymes as summarized in Table 1 but the homodimers of the Ya, Yb_1 and Yc subunits account for approximately 60% of the total hepatic GST [1,2]. Some degree of differentiation of the GST subunits in crude tissue extracts may be obtained by using different substrates [1] but a more reliable approach to quantitating the GST isoenzymes is to measure specifically the concentration of each of the GST using radioimmunoassay.

In the rat, surgical thyroidectomy results in a 30% increase in the hepatic concentration of GST L (YaYa, previously called "ligandin") and normal GST concentrations are restored following daily intraperitoneal