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INHIBITION OF RAT LIVER GLUTATHIONE *S*-TRANSFERASES BY GLUTATHIONE CONJUGATES AND CORRESPONDING L-CYSTEINES AND MERCAPTURIC ACIDS

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Abstract—Glutathione *S*-transferases from rat liver were partially purified by ion exchange chromatography. Active peaks, tentatively identified as containing the 1-2, 2-2, 3-3, 3-4, 4-4 and 5-5 isoenzymes were kept for study. The glutathione conjugates, *S*-hexyl-, *S*-benzyl- and *S*-(2,4-dinitrophenyl) L-glutathione were tested as inhibitors of the enzymes. The 1-2, 2-2, 3-3 and 3-4 fractions were inhibited to similar extents by these conjugates. For all enzymes the hexyl conjugate at 0.1 mM concentration was strongly inhibitory, the benzyl conjugate moderately so and the dinitrophenyl compound was only weakly inhibitory. In contrast, the epoxide conjugating activity in the 4-4 and 5-5 peak was barely affected by the substituted glutathiones at 0.1 mM concentrations. Studies on a purified ligandin (isoenzyme 1-2) from rat liver showed that further metabolism of the glutathione conjugates, to the corresponding cysteines or mercapturic acids, resulted in products with inhibitory properties approximately three orders of magnitude less potent than those of the parent *S*-substituted glutathiones.

GSH *S*-transferases, a family of multifunctional enzymes, are known for their wide and overlapping substrate specificities [1]. They catalyze the reaction of reduced glutathione (GSH)† with a wide range of electrophilic compounds in the first step of mercapturic acid formation [2], a pathway whereby many xenobiotics are detoxified and excreted. These enzymes are found in a wide range of organisms. Those most intensively studied are the set of isoenzymes found in rat liver [3] which may be differentiated both by their physical properties and by their differing substrate specificities [4].

It is known that the conjugates produced by this reaction may be quite potent inhibitors of the enzymes producing them [5]. To what extent the various enzymes differ in their sensitivity to different glutathione *S*-conjugates (or the compounds produced by further metabolism of the initial conjugate [6]) is not known and is the subject of the present investigation.

MATERIALS AND METHODS

Chemicals. Sephadex G-100 and QAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden) and TEAE-cellulose from Bio-Rad Laboratories (California). Analytical grade CDNB was purchased from BDH Chemicals Ltd. (Poole, Dorset, U.K.) and DCNB and ENP were from Eastman Kodak Co. (Rochester, NY). Reduced glutathione and NBC were obtained from Sigma Chemical Co. (St. Louis, MO). *S*-substituted L-glutathiones, L-cysteines and *N*-acetyl-L-cysteines were synthesized by variants of the method A of Vince *et al.* [7]. They were each recrystallized twice from aqueous ethanol. They were *S*-(2,4-dinitrophenyl) L-glutathione (m.p. 194° decomp.), *S*-hexyl L-glutathione (m.p. 211°, decomp.), *S*-benzyl L-glutathione (m.p. 190° decomp.), *S*-benzyl L-cysteine, (m.p. 218°, decomp.), *S*-hexyl L-cysteine (m.p. 138° decomp.), hexyl mercapturic acid (m.p. 85°), benzyl mercapturic acid (m.p. 144°).

Animals. Wistar albino laboratory rats (200–400 g, either sex) fed *ad libitum* on pellet diet were used.

Preparation of cytosol. Rats were individually anaesthetized with thiopentone sodium and the livers perfused *in situ* through a portal-vein cannula with approximately 20 ml of ice-cold 0.01 M sodium phosphate buffer pH 7.4 (Buffer A) containing sucrose (0.25 M) until free of blood. The livers were excised and homogenized in two volumes of perfusion buffer. The homogenate was centrifuged at 100,000 *g* for 90 min at 4° using a 60Ti rotor in a Beckman L2-65 centrifuge. The clear supernatant was used immediately.

Separation of cytosolic GSH S-*transferases*. Cytosol from two rat livers was concentrated and dialysed under vacuum against two changes, each of two litres, of buffer A at 4° as described in ref. 8. The dialysed solution (18 ml, approx. 350 mg of protein) was chromatographed on a CM-Sepharose column (2.2 × 15 cm) equilibrated with buffer A. The flow rate was 16 ml/hr and fractions (2.7 ml) were collected.

Assays for GSH S-*transferase activity*. The GSH

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† Abbreviations used: GSH, reduced glutathione; ENP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; NBC, *p*-nitrobenzyl chloride; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 3,4-dichloro-1-nitrobenzene; HSG, *S*-hexyl L-glutathione; BSG, *S*-benzyl L-glutathione; DSG, *S*-(2,4-dinitrophenyl-L-glutathione.

S-transferase activity of eluted fractions was monitored at 30° with CDNB, DCNB, NBC and ENP as substrates. The conditions of the assays were as described by Habig *et al.* [9]. Fractions containing the greatest activity with each substrate were retained for studies on their susceptibility to inhibition by substituted glutathiones. HSG, BSG and DSG were used as inhibitors at a concentration of 0.1 mM for each.

Protein estimation. The protein concentration in eluted fractions was monitored by measuring the extinction at 280 nm of the fraction collected.

Preparation of ligandin. Ligandin was purified by the methods described by Hayes *et al.* [8]. The 100,000 *g* supernatant obtained from a 33% rat liver homogenate (w/v) in buffer A containing 0.25 M sucrose was dialysed against 0.01 M Tris/HCl buffer pH 8.8 (Buffer B). The dialysed solution was chromatographed on a column of TEAE-cellulose (2.5 × 100 cm) equilibrated with buffer B. The first protein peak eluted was pooled, concentrated, dialysed against buffer A and incubated (60 min, 4°) with 20 mg of BSP. The mixture was eluted (24 ml/hr) with buffer A through a column (2.5 × 100 cm) of Sephadex G-100. Fractions containing the maximum amount of protein-bound BSP with CDNB activity were pooled, concentrated, dialysed against buffer B and chromatographed on a column of QAE-Sephadex A-50 (30 ml/hr) in buffer B. The single protein peak eluted was pooled and used in the inhibition studies.

Inhibition studies. Hexyl GSH, hexyl cysteine, benzyl GSH, benzyl cysteine and the corresponding mercapturic acids were tested for their ability to inhibit the various glutathione *S*-transferase preparations. The GSH concentration was varied between 1 and 5 mM while the CDNB concentration was held constant at 1 mM. Alternatively, the CDNB concentration was varied from 0.2 to 1 mM while GSH was kept at 1 mM. Benzyl GSH concentration was varied from 0.1 to 1.0 mM. The concentration of hexyl GSH was varied from 0.02 to 0.2 mM. Benzyl- and hexyl-L-cysteines and the corresponding mercapturic acids were varied in the range 0.1–10 mM although the limited solubility of the cysteines restricted the concentrations that could be attained. The assays were carried out in a thermostatted cell compartment at 30° in a Varian-Cary 210 double-beam spectrophotometer. The enzyme activities were measured in 0.1 M-sodium phosphate buffer pH 6.4. Enzyme activities were measured at least in duplicate. The results used are the means of data pairs agreeing to within 6%.

Kinetic data were analysed by means of a non-linear, least squares regression on the raw data pairs [11].

RESULTS

Product inhibition of partially purified GSH S-transferases activities

Five peaks of glutathione *S*-transferase activity were eluted from CM-Sepharose (Fig. 1). Fractions comprising each peak were pooled and the activities with CDNB, DCNB, NBC and ENP were measured. Based on the order of elution of the activity peaks

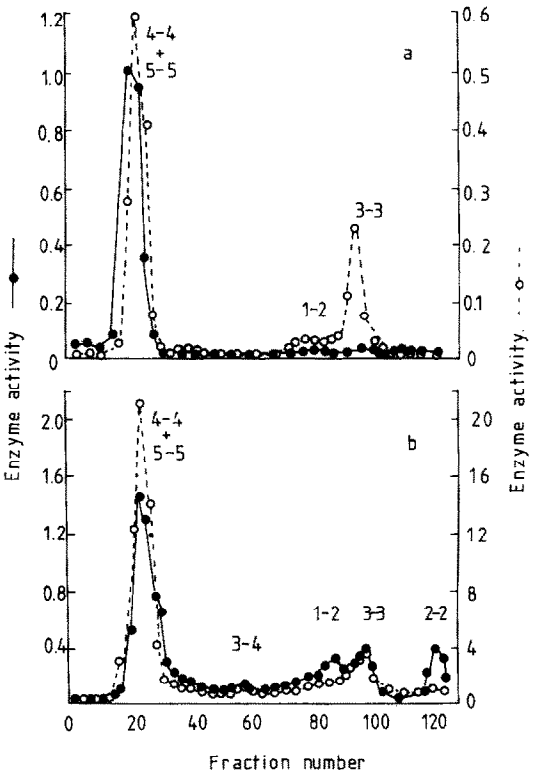

Fig. 1. Separation of isoenzymes of the glutathione *S*-transferases from rat liver by cation exchange chromatography. Chromatography on CM Sepharose at pH 7.4 was carried out as described in Materials and Methods. (a) Enzyme activities were monitored with: ○, 3,4-dichloronitrobenzene; ●, 1,2-epoxy-3-(p-nitrophenoxy)propane (activity units μmol/min/ml). (b) Enzyme activities were monitored with: ○, *p*-nitrobenzyl chloride; ●, 1-chloro-2,4-dinitrobenzene (activity units μmol/min/ml).

and on the activity with the various substrates the various peaks were tentatively classified, in order of elution, as containing isoenzymes 5-5 plus 4-5, 3-4, 1-2, 3-3 and 2-2 [9, 10]. The three conjugates, *S*-hexyl GSH, *S*-benzyl GSH and *S*-(2,4-dinitrophenyl) GSH were then tested for their ability to inhibit the reactions catalysed by these fractions. All were tested with CDNB as substrate at a GSH concentration of 1 mM. For the transferases 1-2, 2-2, 3-3 and 3-4 a remarkably similar response to the conjugates was observed. At a concentration of 0.1 mM, *S*-hexyl glutathione was found to be the most effective inhibitor giving approximately the same degree of inhibition (93 ± 4%) for all of these enzymes. The next most effective was *S*-benzyl glutathione (86 ± 8% inhibition) whereas *S*-(2,4-dinitrophenyl) glutathione was the least effective (28 ± 5% inhibition). The transferase 3-3 was tested in addition with DCNB and NBC as substrates using GSH at 5 mM concentration [9]. The same order of efficacy as inhibitors was seen with the three conjugates although the degree of inhibition was less. At 0.1 mM, hexyl GSH, benzyl GSH and *S*-(2,4-dinitrophenyl) GSH gave 73, 54 and 28% inhibition with DCNB as substrate and 50, 37 and 17% inhibition when NBC was the substrate, respectively.

Table 1. Inhibition by S-substituted glutathiones of the conjugation of CDNB with GSH by glutathione S-transferase 1-2

Inhibitor	K_i (μM) ± S.D. Varied Substance	
	CDNB	GSH
Benzyl GSH	100 ± 7	200 ± 25
Hexyl GSH	47 ± 7	35 ± 5
Benzyl cysteine	n.d.	11000 ± 3000
Hexyl cysteine	n.d.	3200 ± 1400
Benzyl mercapturic acid	11250 ± 2000	8500 ± 1000
Hexyl mercapturic acid	1780 ± 170	1940 ± 180

n.d.—not determined.
Inhibition constants were determined as described in Methods.

In contrast to the above, the epoxide conjugating activity in the 4-4 + 5-5 peak was barely affected by the conjugates at 0.1 mM concentration. Inhibitions of 8, 8 and 14% was observed with the hexyl-, benzyl- and dinitrophenyl glutathiones, respectively.

Studies on purified glutathione transferase 1-2—inhibition by GSH derivatives

The enzyme was purified as described in Materials and Methods. The purified protein examined by SDS-PAGE appeared to consist of equal amounts of Subunits 1 and 2 [10]. It was not further characterized.

The inhibition of this preparation by S-benzyl glutathione and S-hexyl glutathione was studied using CDNB as electrophilic substrate. Under the conditions employed, both conjugates acted in a non-competitive fashion with respect to both substrates (Fig. 2). HSG was the more potent inhibitor of the two. It yielded K_i values of about 0.2–0.5 × those obtained with BSG. The mercapturic acids were found to be poor inhibitors with K_i values in excess of 10^{-3} M. A similar state of affairs was seen with the hexyl- and benzyl-cysteines. In the case of both the cysteines and the mercapturic acids, as with the glutathiones, the S-hexyl substituted compound was a more potent inhibitor than the benzyl compound. Limited solubility in the case of the mercapturic acids and particularly in the case of the cysteines made a precise characterization of the mode of inhibition by these compounds impossible to attain (see Table 1).

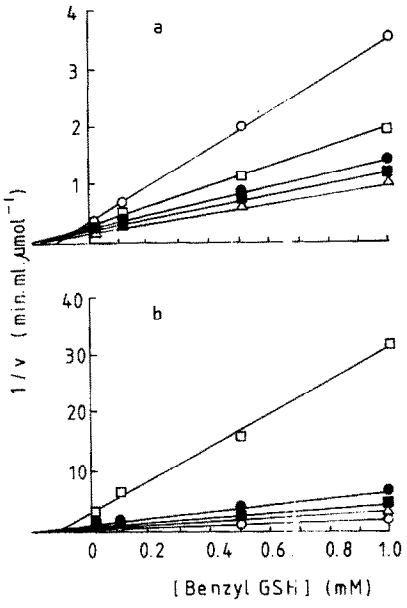

Fig. 2. Inhibition of rat liver glutathione S-transferase 1-2, by S-benzyl L-glutathione. Enzyme assays were carried out at pH 6.4, 30° as described in Materials and Methods. (a) The concentration of CDNB was held constant at 1 mM and the concentration of GSH was: ○, 1 mM; □, 2 mM; ●, 3 mM; ■, 4 mM and △, 5 mM. (b) The concentration of GSH was held at 1 mM and that of CDNB at: ○, 1 mM; □, 0.8 mM; △, 0.6 mM; ●, 0.4 mM and ■, 0.2 mM.

DISCUSSION

It has been appreciated for some years that individual glutathione S-transferases from rat liver were susceptible to marked inhibition by S-substituted glutathiones [5, 12–14]. What has not been clear is how representative of the different enzymes these inhibition studies were and what their relation to the situation *in vivo* was. Was there likely to be significant inhibition *in vivo*? Did the products of one enzyme's action inhibit other isoenzymes equally well? Did different conjugates display preferential affinities for one group of isoenzymes, reflecting substrate specificities, or were they equally efficacious in all instances? It was to approach these questions that the present work was undertaken.

The results indicate that accumulation of conjugates *in vivo* might well lead to pronounced inhibition. At 0.1 mM hexyl-GSH concentration inhibitions of the order of 70% were observed at the physiologically realistic GSH concentration of 5 mM. At 1.0 mM GSH, a concentration that might be seen under conditions of glutathione depletion, the inhibition fell in the range 90–98% for the group of transferases 1-2, 2-2, 3-3 and 3-4. It is clear then, that even modest accumulation of the products of this reaction may cause massive inhibition of an important detoxication system, with potentially severe toxicological results.

It is obvious from the results shown here, which agree with those of other workers [5, 12–14] that different conjugates inhibit to differing extents. The

S-benzyl glutathione is generally a less potent inhibitor than the *S*-hexyl derivative (cf. ref. 12) and the *S*-(2,4-dinitrophenyl) glutathione less so again. Within the group of transferase isoenzymes discussed above, there is little individual variation in responsiveness to these inhibitors. Each is strongly inhibited by HSG, moderately by BSG and weakly by DSG. These enzymes, therefore, appear to constitute a group which would suffer from mutual product inhibition. The product of the reaction catalyzed by one enzyme with a particular substrate might, if allowed to accumulate, inhibit the conjugation of a quite different substrate, catalyzed by a different isoenzyme.

This situation is avoided by two means: elimination of the GSH conjugates from the cell, as for instance, from liver cell to bile, and further processing of the conjugates to substituted cysteines and *N*-acetyl cysteines [6]. With the weak inhibitory properties demonstrated in this work, these types of conjugate show less potential for causing serious toxicological consequences than do the parent conjugates, by about three orders of magnitude. It is clear that the hydrolysis of the initially formed GSH conjugates is important insofar as a potentially incapacitating product inhibition of the transferases is thereby avoided. Our limited data do not suggest that acetylation of the substituted cysteines changes their inhibitor properties further. The function of the acetylation may be to confer a net charge to the molecules at physiological pH to prevent renal absorption and thereby facilitate their excretion. The glutathione transferase 1-2 preparation is assumed, from the results discussed above, to be representative of the group of transferases discussed so far.

In contrast with the above results, it was found the epoxide-conjugating activity in the 4-4/5-5 peak was virtually unaffected by the substituted glutathiones and of those tested, DSG was the most effective. These results are consistent with those of Meyer *et al.* [15], who report that transferase E does not bind to immobilized HSG. This enzyme therefore appears not to be in a functional group with the other transferases. The conjugation of epoxides by this enzyme should not be affected by occurrence of other types of conjugation catalyzed by the other transferases.

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