Results and discussion

Fig. 1 summarizes the data and illustrates the dramatic change in extraction ratio (and clearance) of diazepam with a change in protein binding. In the absence of protein, with no binding, the extraction ratio of diazepam is very high, being close to one. Whereas, in the presence of 35 g litre^{-1} albumin (when only 5% of drug is unbound) the extraction ratio is virtually zero. That is, the drug has changed from one of high extraction to one of low extraction simply by changing the degree of protein binding. Such profound changes have not been reported previously to our knowledge.

Two models of hepatic clearance have been proposed Pang & Rowland (1977b). Both models predict that if the degree of binding in the perfusate can be made high enough, then eventually the extraction ratio, of even those drugs which have a high intrinsic clearance, will become low and limited by binding. Our data support this view and stress the need to quote not only the value of the extraction ratio or clearance but also the associated degree of binding of drug within blood.

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Monobromobimane: a substrate for the fluorimetric assay of glutathione transferase

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The glutathione *S*-transferases (EC 2.5.1.18) are a family of isoenzymes that play a vital role in the protection of cells from toxic metabolites of drugs and environmental chemicals (Jakoby 1978; Grover 1982; Ketterer et al 1982). The enzymes conjugate these electrophilic xenobiotic materials to glutathione.

We wish to report that the compound monobromobimane (mBrB) (Kosower & Pazhenchevsky 1980) is a useful new substrate for glutathione transferase(s). It is a non-fluorescent compound that reacts slowly (non-enzymically) with thiols (Kosower et al 1979; Fahey et al 1980), such as glutathione (GSH), to give fluorescent products (Fig. 1). We have discovered that glutathione transferase catalyses the reaction of mBrB with GSH, with the result that glutathione transferase activity can be conveniently assayed with this new substrate. This is achieved by direct measurement of the rate of appearance of the fluorescent conjugate formed within the reaction mixture, i.e. in the presence of an excess of unreacted non-fluorescent mBrB.

Materials and methods

Monobromobimane (mBrB, 3-bromomethyl-2,5,6-trimethyl-lH, 7H-pyrazole[1,2-a]pyrazole-1,7-dione) was synthesized, and purified by chromatography (to remove fluorescent material), as described by Kosower & Pazhenchevsky (1980). Stock solutions were: (a) mBrB (1.0 mM in dry acetonitrile), (b) GSH (3.0 mM in Na_2EDTA, 10 mM, pH 4·5, prepared in de-aerated water and stored at 4 °C), and (c) phosphate buffer (0·05 M, pH 6·5).

Rat liver supernatants, obtained after $CaCl_2$ precipitation of microsomes, served as a source of cytosolic glutathione transferases (Benson et al 1979). When desired, GSH could be removed from this enzyme

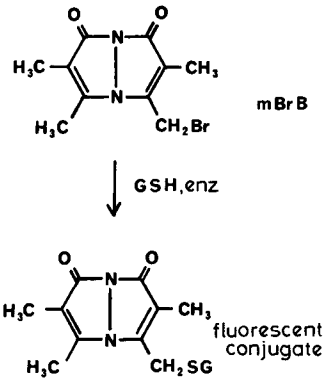

FIG. 1. Structures of mBrB and its fluorescent glutathione conjugate.

* Correspondence.

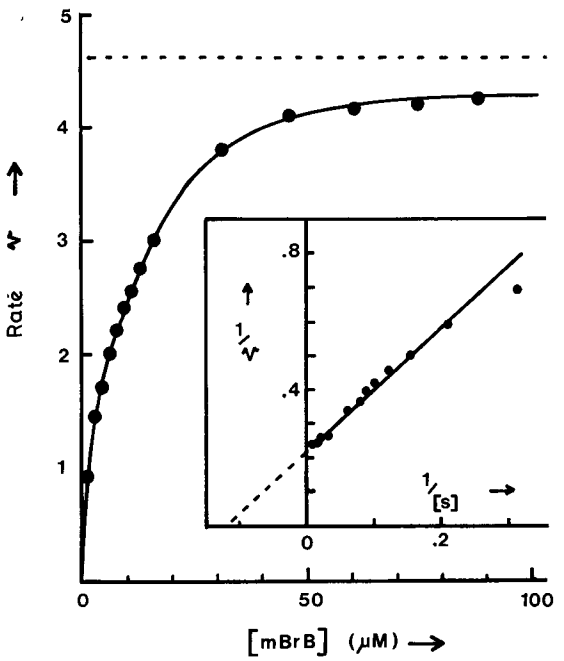

FIG. 2. The effect of increasing the concentration of mBrB on the initial rates (v) of the enzymic formation of fluorescent conjugate (non-enzymic rates have been deducted). The GSH concentration was 100 μM. The rates are given in nmoles of conjugate ml^{-1} min^{-1}; the concentration of cytosolic protein was 2·6 μg ml^{-1}. Experiments were in phosphate buffer (50 mM), pH 6.5 at 24°C. The insert shows the Lineweaver-Burk plot of the data.

preparation by Sephadex G-25 gel filtration. A Perkin-Elmer fluorimeter (Model 1000) (excitation 400 nm, emission 475 nm) was attached to a chart recorder. The instrument scale was adjusted so that a 10 μM solution of bimane conjugate gave a full scale reading of 100 divisions; this was achieved on the least sensitive scale setting.

In a typical incubation, enzyme (5 μl, containing 8 μg supernatant protein) was pipetted into plastic fluorimeter cuvettes containing buffer (3·0 ml) and GSH (100 μM), and the reaction started by addition of mBrB (10 μM final concentration). The increase of fluorescence was followed with time. A rate of increase of 1 division min^{-1} corresponded to 0·1 nmol ml^{-1} min^{-1} of conjugate formation. Cuvettes containing no enzyme showed an initial fluorescence of 1·5 divisions and a non-enzymic rate of 0·5 divisions min^{-1}. All measurements were at 24 °C.

Results

Monobromobimane and GSH were found to react slowly in a non-enzymic manner as observed by a slow linear increase in fluorescence. As expected, the rate of the non-enzymic reaction increased proportionally with increasing concentrations of the two substrates and also with increase of pH (as GSH anion is the true nucleophile with a pK_a of 9·65). Addition of cytosolic glutathione transferase dramatically increased the rate of reaction and could take the reaction rapidly to completion, indicating that monobromobimane is a substrate for GSH-transferase(s). As mBrB is not an endogenous compound but a xenobiotic substance it was by no means certain that it would indeed prove to be an enzyme substrate. The fast rate of reaction observed also makes mBrB a *useful* substrate. Monochlorobimane, which we have also prepared, is a substrate too but its rate of reaction is inconveniently slow. The optimum pH *for the assay* of enzymic activity with mBrB was found to be 6.5. At this pH the non-enzymic reactions were kept to a minimum without appreciably affecting the catalytic activity of the enzyme. Other necessary experiments showed that relatively large amounts of excess mBrB substrate (up to 100 μM) did not quench the fluorescence produced by a typical concentration of conjugated product (2·5 μM).

To characterize the enzymic reaction between mBrB and GSH, kinetic experiments to determine overall Michaelis-Menten parameters were performed. It must be emphasised that the enzyme preparation used was a typical cytosolic preparation of rat liver. As this is known to contain several isoenzymes (Jakoby 1978), the kinetic experiments were carried out to obtain an overall impression of the reaction characteristics and affinities of the substrates. Control experiments, from which enzyme was omitted, characterized the non-enzymic reaction and needed to be included because non-enzymic rates of reaction could not be ignored (especially at higher substrate concentrations). These non-enzymic rates were subtracted from initial total rates to give true enzyme-catalysed rates. At fixed concentration of mBrB (10 μM) and GSH (100 μM), the rate of enzyme reaction was proportional to the amount of enzyme added (in the range 0–5 μg supernatant protein ml^{-1}). This result indicated that this reaction can indeed be used to assay glutathione transferase activity. Fig. 2 shows the increase of the rate of enzymic reaction with increasing mBrB concentration (0–80 μM) at a fixed GSH concentration (100 μM), and Fig. 2 (insert) yields an apparent K_m for mBrB at 8 μM. A good linear Hofstee plot was also obtained. Clearly mBrB is a very high affinity substrate for glutathione transferase. Fig. 3 shows the dependence of the enzymic rate on GSH concentration (0–250 μM) at a mBrB concentration of 50 μM. Hofstee (Fig. 3, insert) and Lineweaver-Burk plots both gave a biphase curve for this data with two apparent K_m values of 24 μM and ca 150 μM for the low and higher ranges of glutathione concentration. This biphasic nature of the kinetics almost certainly has its origin in the heterogeneity of the enzyme preparation.

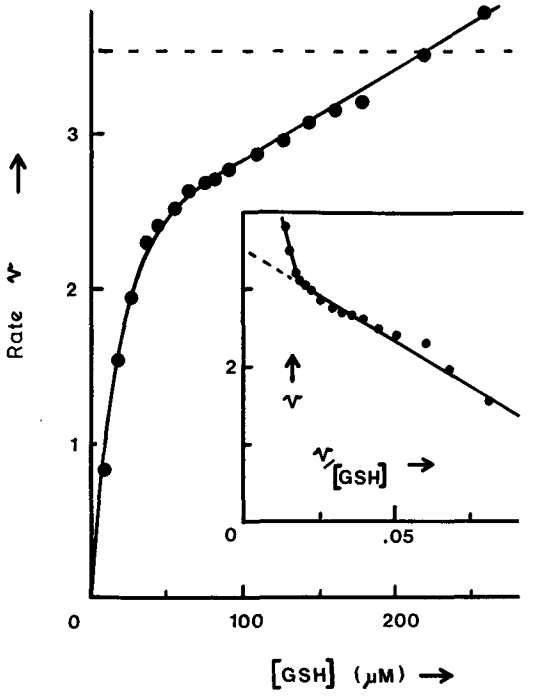

FIG. 3. The effect of increasing the concentration of GSH on the initial rates (v) of the enzymic formation of fluorescent conjugate. The mBrB concentration was 50 μM. Other details as for Fig. 2. (The enzyme preparation lost ca 25% of activity on storage between experiments shown in Fig. 2 and 3). The insert shows the Hofstee plot of the data.

In the absence of added GSH, the addition of *fresh* undiluted liver cytosol (10 μl, containing 2 mg wet weight of liver) to mBrB solution (10 μM final, in 3 ml total volume) rapidly gave a fluorescent product. This arose from the GSH endogenously present in this undiluted enzyme preparation. The small aliquot of cytosol contained a sufficient amount of GSH to give a conveniently measurable concentration of fluorescent conjugate (ca 3 μM) and also contained ample enzyme activity to catalyse the reaction to completion. This endogenous GSH could be removed from the enzyme by Sephadex G-25 gel chromatography. Addition of standard amounts of GSH (0–10 μM, final) to excess mBrB (20 μM) and purified enzyme gave, on completion, proportional amounts of enzymically produced fluorescent conjugate. These observations provide the basis for the development of a highly sensitive and specific fluorimetric assay of endogenous levels of glutathione.

Discussion

Hepatic glutathione transferase preparations are known to contain several glutathione transferase isoenzymes, each isoenzyme having its own spectrum of substrate specificities (Jakoby 1978). Only recently has a very clean separation of rat liver isoenzymes been obtained (Mannervik & Jensson 1982); this has allowed further examination of substrate specificity. Our communication is the first to report a substrate for the assay of glutathione transferase activity by a fluorimetric method. We do not yet know for certain whether monobromobimane is a specific substrate for one particular isoenzyme, or a more general substrate capable of being catalysed by several isoenzymes to its fluorescent conjugate. The biphasic kinetics described here (Fig. 3) and particularly a preliminary separation of isoenzymes that we have carried out both suggest that monobromobimane is a general substrate. It is therefore likely to be valuable reagent for the estimation of glutathione transferase activity of the various mixtures of isoenzymes present in different biological sources. Monobromobimane also has the promise of providing an extremely sensitive enzymic assay of glutathione.

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