The initial-rate kinetics of mouse glutathione S-transferase YfYf
Evidence for an allosteric site for ethacrylic acid

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INTRODUCTION
The cytosolic glutathione S-transferases (GSTs) exist in multiple forms that can be grouped into three species-independent families that have been termed Alpha, Mu and Pi (Mannervik et al., 1985; Alin et al., 1986). Although structural and immunological relationships between the major forms have been well delineated (Hayes & Mantle, 1986a; Rothkopf et al., 1986; Okuda et al., 1987; Lai et al., 1988) and substrate and inhibitor profiles have been described (Mannervik et al., 1985; Hayes & Mantle, 1986b), detailed kinetic studies have until recently been restricted to members of the Mu family. The initial-rate pattern for the rat form GST A [now known to be a homodimer of Yb1 subunits (subunit 3 in the alternative nomenclature of Jakoby et al., 1984)] was recognized as complex by Pabst et al. (1974), who proposed a model that changed from a sequential mechanism at high concentrations of GSH to a ping-pong mechanism at low concentrations of GSH. However, a detailed analysis of all the available data (including the original data) showed that the complex initial-rate pattern was better explained as being a steady-state random mechanism (Jakobson et al., 1977). More recently Danielson & Mannervik (1988) have proposed a similar mechanism for the other major rat Mu homodimer, GST Yb2Yb2 (4-4 in the alternative nomenclature). Insect forms of GST have also been shown to conform to a steady-state random mechanism (Clark et al., 1984, 1986). Schramm et al. (1984) suggested that rat GST YaYa (1-1 in the alternative nomenclature), a member of the Alpha family, obeyed a rapid-equilibrium random mechanism, although they did not rule out a steady-state random mechanism.

There has been little kinetic work reported for the Pi family, although Ivanetich & Goold (1989) have proposed a rapid-equilibrium random mechanism for human placental GST π. In some respects this is surprising, as this form [described variously as the YfYf, YpYP or 7-7 homodimer in the rat (Kitahara et al., 1984; Jansson et al., 1985; Hayes & Mantle, 1986a)] has received considerable interest as a possible early marker for cancer, as it is markedly increased in preneoplastic nodules in rat liver (for a review see Sato, 1989).

The regulation of expression of the mouse Yf orthologue in liver is known to be up-regulated by testosterone (Hatayama et al., 1986; McLellan & Hayes, 1987), so that high concentrations are expressed in the male. Since this is a rich source of the enzyme, we have examined the reaction mechanism with the model substrate 1-chloro-2,4-dinitrobenzene (CDNB). During this study we obtained evidence consistent with the existence of an allosteric site on the enzyme for the diuretic drug ethacrylic acid (which is also a substrate), and details of these experiments are reported in the present paper.

MATERIALS AND METHODS
Materials
S-(2,4-Dinitrophenyl)glutathione (DNPG) was synthesized by the method of Vince et al. (1971). S-Hexylglutathione–Sepharose was prepared by the method of Mannervik & Guthenberg (1981). Glutathione, S-methylglutathione and ethacrylic acid were purchased from Sigma Chemical Co. Hydroxypatite was purchased from Bio-Rad Laboratories. All other reagents were of analytical standard.

Purification of GST YfYf from mouse liver
GST YfYf was purified from the livers of adult male mice (Laca strain) by the method of Hayes et al. (1987), by using S-hexylglutathione–Sepharose chromatography followed by hydroxypatite chromatography.

Analysis of purified protein
SDS/PAGE was carried out by using the method of Laemmli (1970). Isoelectric focusing was performed in flat-bed polyacrylamide gels in an LKB Multiphor apparatus under native and denaturing (8 M-urea) conditions. Immunoblotting was carried out as described previously (Hayes & Mantle, 1986a).

Abbreviations used: GST, glutathione S-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DNPG, S-(2,4-dinitrophenyl)glutathione.
Protein concentration was determined by measuring the absorbance at 280 nm and by the method of Markwell et al. (1978). The N-terminal amino acid sequence for mouse GST YfYf was determined by using an Applied Biosystems 477A sequencer.

**Assay conditions for kinetic studies**

Initial rates with CDNB as the electrophilic substrate were measured with a Gilford 240 spectrophotometer by following the appearance of the product, DNPG, at 400 nm (ε = 2.15 mm⁻¹·cm⁻¹). Although the standard assay for CDNB activity is carried out at 340 nm (Habig et al., 1974), it was necessary to change the assay wavelength to 400 nm for two reasons: (i) to allow the measurement of CDNB activity in the presence of ethacrynic acid without measuring the formation of the GSH-ethacrynic acid adduct (λ_max = 270 nm) and (ii) to allow the use of higher concentrations of DNPG in the product-inhibition studies. The reaction was carried out in 0.1 m-sodium phosphate buffer, pH 6.5, at 30 °C. The concentration of ethanol, the solvent for the stock solution of CDNB, was kept constant at 3.5% (v/v). The reaction was initiated by the addition of enzyme. A linear relationship was found for the initial velocity as a function of the concentration of enzyme in the range 0.5–15 µg/ml and the concentration of enzyme used for initial-rate studies was 2 µg/ml. The concentration of GSH was varied in the range 0.05–2 mM and the concentration of CDNB was varied in the range 0.1–2 mM (the concentration of CDNB cannot be increased above 2 mM for reasons of solubility). For product-inhibition studies, DNPG was added in the concentration range 0.02–0.8 mM with the concentration of one substrate varied as above and the other substrate kept at a fixed concentration. For inhibition studies with ethacrynic acid, the concentration of ethacrynic acid was varied in the range 5–40 µM. The formation of the GSH-ethacrynic acid adduct is not detected at 400 nm, so that the changes in absorption monitored only the formation of DNPG.

The binding of GSH to free enzyme was determined by monitoring the quenching of protein fluorescence upon addition of GSH with the use of Perkin-Elmer MPP-44B fluorometer. The excitation and emission wavelengths were 280 nm and 340 nm respectively.

**Statistical analysis of data**

Initial-rate data were fitted to the equation for a rectangular hyperbola. The inhibition constants were obtained from linear fits of appropriate replots. Where appropriate, the data for some plots of apparent inhibition constant (Y) against the fixed substrate were fitted to the hyperbola:

\[
Y = \frac{qK_sS + K_iK_n}{S + qK_n}
\]

where S is substrate concentration, \(K_i\) and \(K_n\) are dissociation constants for inhibitor and substrate respectively and q is an arbitrary constant. All fitting was carried out by using the computer program ENZFITTER (Leatherbarrow, 1987), which uses an iterative Gauss–Newton procedure.

**RESULTS AND DISCUSSION**

**Analysis of purified protein**

The purified protein appeared as a single band on SDS/PAGE with an apparent M₀ of 24500. On isoelectric focusing the enzyme had a pI of 8.3 under native conditions and 7.3 under urea denaturing conditions. Immunoblotting analysis with antisera to rat liver GSTs revealed cross-reactivity with anti-(rat GST YfYf) serum but not with anti-(Ys subunit), anti-(Yc subunit), anti-(Yk subunit), anti-(Yb subunit) or anti-(Yb₂ subunit) serum.

Fig. 1 shows the N-terminal sequence for the first 25 residues. The analysis revealed differences at positions 11, 12 and 13 when compared with the mouse liver GST YfYf (M-II) N-terminal sequence reported by Mannervik et al. (1985). Although this may be evidence of variants of the mouse Yf subunit, it is noteworthy that our N-terminal sequence shows identity with the rat, human and bovine sequences at positions 12 and 13 and with the rat and human sequences at position 11. We have also purified a form of GST of mobility identical with that of GST YfYf on SDS/PAGE, which cross-reacts with antisera to mouse GST YfYf but which appears later in the elution gradient on hydroxyapatite chromatography. The relationship between this fraction and the major form of GST YfYf is unclear, and all kinetic work reported in the present study was conducted with the major form.

**Initial-rate studies with GST YfYf**

Double-reciprocal plots of the initial-rate pattern for murine GST YfYf with GSH and CDNB as substrates were linear and converging, indicating a sequential mechanism. Whether CDNB or GSH was the substrate whose concentration was varied, the same intersection point was observed in the lower left quadrant of a double-reciprocal plot. In order to discriminate between an ordered mechanism and a random mechanism, product-inhibition studies were carried out with DNPG. All product-inhibition patterns were linear. Fig. 2 shows the relationship between inhibition constants (the subscripts s and i refer to slope and intercept constants respectively) and the fixed substrate concentration.

The product (DNPG) was found to be a competitive-type inhibitor with respect to GSH and the \(K_s\) varied hyperbolically with the concentration of CDNB. DNPG was found to be a mixed-type inhibitor with respect to CDNB and both the \(K_i\) and the \(K_n\) increased linearly with the concentration of GSH.

These data are consistent with the rapid-equilibrium random mechanism shown in Scheme 1, where there is formation of an enzyme–GSH–DNPG complex but not of an enzyme–CDNB–DNPG complex. The discriminatory points between this

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**Fig. 1. N-Terminal amino acid sequences for mouse, rat, human and bovine Pi GSTs**

The N-terminal amino acid sequences of mouse, rat, human and bovine Pi GSTs are aligned for comparison. Any differences that occur are outlined by boxes. Key to references: *Mannervik et al. (1985); †Sugioka et al. (1985); ‡Kano et al. (1987), Ålin et al. (1985) and Dao et al. (1984); §Schaffer et al. (1988).
Kinetics of glutathione S-transferase YFY

Fig. 2. Product (DNPG) inhibition of mouse GST YFY

(a) DNPG was found to be a competitive-type inhibitor with respect to GSH. The relationship between the $K_{s}$ with respect to GSH, and the concentration of CDNB is shown. The curve is a fit to eqn. (1). The asymptote is equivalent to $\beta K_{p}$ and the intercept equivalent to $K_{p}$ in eqn. (2). (b) DNPG was found to be a mixed-type inhibitor with respect to CDNB. The relationship between the $K_{s}$ (●) and $K_{ii}$ (■), with respect to CDNB, and the concentration of GSH is shown. The intercepts for the $K_{s}$ and $K_{ii}$ plots are equivalent to $\beta K_{p}$ and $K_{p}$ respectively in eqn. (3). All points plotted are inhibition constants ± S.E.M. ($n = 5$) obtained from linear regression as described in the text.

Scheme 1. Proposed rapid-equilibrium random mechanism for mouse GST YFY with CDNB and GSH as substrates

A refers to GSH, B to CDNB and P to DNPG. The steady-state rate equation (eqn. 4) is given in the text. The release of products is indicated as a single step since the order of release of DNPG and Cl cannot be determined from the available data.

Mechanism and an ordered mechanism are: (i) there is a hyperbolic relationship between the $K_{s}$ [with respect to GSH (A)] and the concentration of CDNB (B), whereas for an ordered mechanism the $K_{s}$ would be insensitive to the concentration of B; (ii) there is a difference in the intercept values for the plots of the $K_{s}$ and $K_{ii}$ [with respect to CDNB (B)] against the concentration of GSH (A), whereas for an ordered mechanism these would be identical. The initial-rate equation for Scheme 1 is given below, in terms of A:

$$v_{o} = \frac{V_{\text{max}}A}{\alpha K_{s} \left( \frac{1 + K_{p} + K_{n} P}{K_{s} P + \beta K_{p}} \right) + A \left( 1 + \frac{\alpha K_{n}}{B} \right)}$$

and in terms of B:

$$v_{o} = \frac{V_{\text{max}}B}{\alpha K_{s} \left( \frac{1 + K_{s} P}{A + \frac{\alpha K_{n}}{A} \frac{P}{\beta K_{p}}} \right) + B \left( 1 + \frac{\alpha K_{s} P}{A + \frac{\alpha K_{n}}{A} \frac{P}{\beta K_{p}}} \right)}$$

where $A$, $B$ and $P$ represent concentrations of GSH, CDNB and DNPG respectively. This shows that the difference found in the value for $K_{s}$ and $K_{ii}$, when extrapolated to zero GSH concentration, is explained by the factor $\beta$ in the equation. The intercepts from the plots of $K_{s}$ and $K_{ii}$ (with respect to CDNB) against the concentration of GSH (Fig. 2b) are equal to $K_{s}$ and $\beta K_{p}$ respectively (see Scheme 1). This same factor operates in defining the hyperbola observed in the plot of $K_{s}$ (with respect to GSH) against the concentration of CDNB (Fig. 2a). The hyperbola has an intercept equal to $K_{s}$ and approaches the asymptote $\beta K_{p}$. Table 1 summarizes the values obtained from computer fits. It is significant that Ivanetich & Goold (1989) have described an identical mechanism for the human form of GST YFY (GST π).

The observed quenching of the protein fluorescence upon addition of GSH to the enzyme indicated that GSH forms a complex with free enzyme. A plot of the change in fluorescence against the concentration of GSH was hyperbolic and gave $K_{s} = 0.19 \pm 0.02$ mM. Further evidence in support of a random mechanism could in theory come from binding experiments with CDNB and free enzyme. Binding experiments with CDNB are not straightforward, as this substrate is a fairly reactive electrophile, so that reversible binding has to be distinguished from covalent incorporation. Some evidence that CDNB exhibits saturable binding has been presented by Corrigall et al. (1989). On the assumption that the mechanism is rapid-equilibrium random, the initial-rate data were interpreted according to the equation:

$$v_{o} = \frac{V_{\text{max}}AB}{\alpha K_{s} K_{n} + \alpha K_{n} A + \alpha K_{s} B + AB}$$

where $A$ and $B$ represent concentrations of GSH and CDNB respectively. Table 1 summarizes the kinetic constants. These constants compare very closely with those reported by Ivanetich & Goold (1989) for human GST π. Although a full-length mouse sequence has not yet been reported, there is 85.6% identity between the rat and human sequences (Kano et al., 1987), and it would seem therefore that all the major Yf forms of GST will probably exhibit a highly conserved kinetic mechanism.

An important point to notice is the value for $\alpha = 2.1$ (Table 1). The fact that the initial-rate patterns for GST and CDNB intersect below the [substrate] axis also implies that $\alpha > 1$ and therefore that the binding of either substrate to free enzyme
Table 2. Summary of the inhibition constants for product inhibition of mouse GST YfYf with DNPG

<table>
<thead>
<tr>
<th>Value</th>
<th>From inhibition with respect to GST</th>
<th>From inhibition with respect to CDNB</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_P )</td>
<td>3.2 ± 0.4 ( \mu )M</td>
<td>3.2 ± 2.7 ( \mu )M</td>
</tr>
<tr>
<td>( \beta )</td>
<td>7.2 ± 4.2</td>
<td>3.4 ± 2</td>
</tr>
<tr>
<td>( K_B )</td>
<td>0.58 ± 0.23 ( \mu )M</td>
<td>–</td>
</tr>
<tr>
<td>( \beta K_B )</td>
<td>–</td>
<td>11.0 ± 3.6 ( \mu )M</td>
</tr>
</tbody>
</table>

hinders binding of the other substrate. Similarly the fact that \( \beta > 1 \) (Table 2) indicates that it is more difficult for DNPG to bind to the enzyme–CDNB complex than to the free enzyme. The data suggest some conformational change or steric hindrance. Principato et al. (1988) have shown that binding of S-methylglutathione to rat GST Yb-Yb (4-4) enables the enzyme to utilize 2-mercaptoethanol as the thiol substrate, indicating conformational changes at the active site. We have observed the same phenomenon for mouse GST YfYf (M. F. Phillips & T. J. Mantle, unpublished work), indicating that S-alkylated glutathione binding may induce conformational changes.

The initial-rate pattern for mouse GST YfYf with GSH and ethacrynic acid as substrates gives a value for \( \alpha = 0.83 ± 0.09 \), indicating a marginal enhancement of binding of the second substrate (McCusker et al., 1990). We have also carried out studies with ethacrynic acid as an inhibitor of the GST-YfYf-catalysed conjugation of GSH and CDNB. Ethacrynic acid was found to be a mixed-type inhibitor with respect to GSH. The inhibition with respect to CDNB was unusual in that it gave rise to an inhibition pattern that intersected to the right of the \( 1/V_0 \) axis, in the upper quadrant, on a double-reciprocal plot. Fig. 3 shows, for example, the double-reciprocal pattern for CDNB in the presence of ethacrynic acid at 4 mM-GSH.

As the concentration of ethacrynic acid was increased, the computer fitted to a rectangular hyperbola yielded negative values for both the \( K_m \) and the \( V_{max} \). If the data were plotted as simply \( V_0 \) versus the concentration of CDNB it was clear that at higher concentrations of ethacrynic acid and initial velocity–substrate concentration curve appeared to be linear. Where the fit to a rectangular hyperbola yielded positive values for the \( K_m \) and \( V_{max} \), the \( K_m/V_{max} \) ratio was taken as the reciprocal of the initial slope. Where a negative value was obtained for the \( K_m \), the data (\( v_0 \) versus [CDNB]) were instead fitted to a straight line and the reciprocal of the slope was taken. Replots of these reciprocal slopes against the concentration of ethacrynic acid were found to be linear and a \( K_m \) value was taken from the intercept on the [ethacrynic acid] axis. A plot of \( K_m \) against the concentration of GSH was found to be hyperbolic (see Fig. 4).

The observed increase in the \( K_m/V_{max} \) ratio can be attributed to an increase in the \( K_m \) for CDNB in the presence of ethacrynic acid. However, the double-reciprocal plots also show that \( 1/V_{max} \) decreases as the concentration of ethacrynic acid increases. It would seem therefore that ethacrynic acid is acting simultaneously as a competitive inhibitor and as an uncompetitive activator, giving the observed intersection of lines seen in Fig. 3.

In order to find a possible mechanism that would provide a reasonable model for the observed behaviour, we utilized the computer program EKPLoT (M. F. Phillips, unpublished work), which allows a simulation of the behaviour of any equation with more than one independent variable and presents the user with \( Y \)-versus-\( x_i \) or \( 1/Y \)-versus-\( 1/x_i \) plots for one variable as the second variable (\( x_j \) is altered). Equations were constructed, first

Table 1. Summary of the initial-rate kinetic constants for mouse GST YfYf with GSH and CDNB as substrates

The parameters are evaluated from eqn. (4) where \( A \) and \( B \) refer to concentrations of GSH and CDNB respectively. Values given are means ± s.e.m. (\( n = 5 \)).

<table>
<thead>
<tr>
<th>( K_A )</th>
<th>0.086 ± 0.006 mM</th>
</tr>
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<tbody>
<tr>
<td>( K_B )</td>
<td>0.68 ± 0.27 mM</td>
</tr>
<tr>
<td>( \alpha )</td>
<td>2.1 ± 0.18</td>
</tr>
<tr>
<td>( V_{max} )</td>
<td>0.13 ± 0.01 ( \mu )mol/min per ( \mu )g</td>
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Fig. 3. Anomalous inhibition of mouse GST YfYf by ethacrynic acid

A double-reciprocal plot for inhibition by ethacrynic acid with respect to CDNB shows a linear pattern intersecting to the right of the \( 1/v_0 \) axis. Each line represents a fixed concentration of ethacrynic acid indicated as follows: 0 \( \mu \)M (●), 6.25 \( \mu \)M (○), 12.5 \( \mu \)M (●), 20 \( \mu \)M (●) and 35 \( \mu \)M (▼). The concentration of GSH was fixed at 4 mM throughout. All points plotted represent initial velocities in duplicate.

Fig. 4. Relationship between the \( K_g \) for inhibition by ethacrynic acid and the concentration of GSH

The \( K_g \) was determined for inhibition by ethacrynic acid with respect to CDNB as described in the text. A hyperbolic relationship was observed between the \( K_g \) and the concentration of GSH. The curve drawn is a fit to eqn. (1). All points plotted are inhibition constants ± s.e.m. (\( n = 5 \)) obtained from linear regression as described in the text.
Scheme 2. Proposed mechanism for the interaction of ethacrynic acid with mouse GST YfYf

A refers to GSH, B to CDNB and I to ethacrynic acid. The rate equation (eqn. 6) is given in the text. In this scheme ethacrynic acid can act as an inhibitor of the enzyme-catalysed conjugation of CDNB and GSH by competing with CDNB at the active site. However, ethacrynic acid can also bind to an allosteric site on the enzyme, thereby altering the $V_{max}$ and possibly the affinity for CDNB and GSH. The altered enzyme (EI) is capable of binding both substrates and catalysing product formation.

in terms of a single substrate and inhibitor and then in terms of two substrates and inhibitor, with terms in the form of [ligand(s)]/dissociation constant(s). The equations were also constructed along the same general form as 'standard' initial-rate equations, i.e. a numerator containing one or more $V_{max}$ terms and a denominator containing a series of equilibrium terms. The behaviour of these equations was simulated and the resulting initial-rate patterns were compared with the experimental data. The basic equation:

$$v_0 = \frac{AB}{N_1 c_1 + N_2 A BI}$$

where $A$, $B$ and $I$ are ligand concentrations, $N_1$ and $N_2$ are arbitrary rate constants and $c_1$ to $c_6$ are arbitrary dissociation constants, was the simplest equation constructed that produced a similar pattern to that observed experimentally. This equation was then examined in terms of a possible enzyme mechanism and the model shown in Scheme 2 was devised. The rapid-equilibrium equation was then derived for the model shown in Scheme 2 and is given below:

$$v_0 = \frac{V_{max,2} A B}{1 + \frac{1}{K_A + K_B + K_c} + \frac{1}{I + I_{AB}} + \frac{1}{A I + I_{AB} + A I_{P}}}$$

where $A$, $B$ and $I$ represent concentrations of GSH, CDNB and ethacrynic acid respectively. This equation was then examined by using the computer program EKPLOT and produced an initial-rate pattern identical with that found experimentally. However, it was found that $V_{max,2}$ must be greater than $V_{max,1}$ and both the $K_i$ and $K_i'$ must be less than the $K_i$.

There are two important terms in the equation. The first is the second numerator term, containing $V_{max,2}$ and $I$, which is responsible for the observed increase in the apparent $V_{max}$ in the presence of ethacrynic acid, provided that $V_{max,2} > V_{max,1}$. In Scheme 2 this is seen as an alternative (faster) flux for DNP formation in the presence of ethacrynic acid. The second crucial feature is the $P$ terms in the denominator, which are responsible for the observed decrease in the apparent $V_{max}/K_m$ ratio in the presence of ethacrynic acid, provided that the enzyme has a greater affinity for I than that for A. In Scheme 2 this is seen as the formation of enzyme-(ethacrynic acid)$_2$ complexes. It is also evident that the binding of ethacrynic acid to free enzyme governed by the equilibrium [ethacrynic acid]/[A] does not preclude the further binding of CDNB and GSH. It is therefore assumed that ethacrynic acid is binding at a site on the enzyme that is distinct from the active site. Since the binding of ethacrynic acid to all other enzyme forms excludes the binding of CDNB but not of GSH, it is assumed here that ethacrynic acid is competing with CDNB at the active site. This is expected, as ethacrynic acid is an alternative substrate to CDNB for YfYf.

The computer program EKPLOT proved to be very useful for analysing a complex equation such as eqn. (6). It was possible to alter the values for parameters and see the effect graphically and in a form directly comparable with the observed experimental results. Once the mechanism shown in Scheme 2 had been identified and eqn. (6) had been derived, values for parameters were constrained within realistic bounds. Table 3 gives the range of values used in the simulations.
of values used and the restrictions on parameter values necessary to produce the experimentally observed pattern.

After simulation of the inhibition by ethacrynic acid with respect to CDNB, the slope replot was found to be hyperbolic. However, the curvature was not pronounced and, depending on the choice of parameter, values could easily be interpreted experimentally as a linear function (see Fig. 5). If a linear approximation was taken, it was found that the intercept on the [ethacrynic acid] axis ($K_a$) showed a hyperbolic relationship with GSH concentration, as was found experimentally (see Fig. 4). This hyperbola had an intercept equal to $K_a$ and approached the asymptote $aK_a$, i.e. as the concentration of GSH is increased the inhibition shifts from the enzyme-(ethacrynic acid)$_2$ complex to the enzyme-GSH-(ethacrynic acid)$_2$ complex, as one might expect. The simulation also indicated that the intercept replot was markedly hyperbolic. This was also seen experimentally. For some of the data, however, $1/V_{\text{max}} = 0$. This was due to the occurrence of apparently linear $V_e$ versus-[CDNB] plots, presumably due to a shift in the $K_a$ for CDNB well beyond the maximum concentration of CDNB used in the assay.

The simulation also predicted mixed-type inhibition by ethacrynic acid with respect to GSH, under the conditions presented in Table 3. Furthermore, this inhibition was abolished by increasing the concentration of CDNB. This was also observed experimentally. If the concentration of CDNB was increased further in the simulation it was found that there was a shift from inhibition to activation by ethacrynic acid with respect to GSH.

The fact that the enzyme can catalyse the conjugation of ethacrynic acid and GSH to form an alternative product need not be considered. The formation of this product is not being measured with the assay system used and therefore, in a rapid-equilibrium system, ethacrynic acid will behave as though it were a dead-end inhibitor (Segel, 1975). Interestingly, ethacrynic acid was found to be a potent inhibitor of the enzyme conjugation of CDNB and GSH by mouse GST YbYb but not an activator (M. F. Phillips & T. J. Mantle, unpublished work).

There is a similarity between the effects of ethacrynic acid on the enzymatic conjugation of CDNB and GSH by GST YfYf and the effects of ADP on the oxidative deamination of glutamate by glutamate dehydrogenase with NAD$^+$ as cofactor (Frieden, 1959). ADP acts as a competitive inhibitor at the nucleotide-binding site on the enzyme and as an activator, increasing $V_{\text{max}}$, by destabilizing an enzyme-NAD(P)H-glutamate abortive complex (Iwatsubo & Pantaloni, 1967). Furthermore, this effect is pH-dependent (Markau et al., 1972). This results in a similar intersection of lines in the right upper quadrant of double-reciprocal plots to that observed for mouse GST YfYf.

There is good evidence for the presence of a binding site on GST YaYc for bilirubin (Vander Jagt et al., 1982) and lithocholate (Strange et al., 1977) at a site distinct from the catalytic centre. The presence of such a binding site supports the view that some GSTs have an important role in the intracellular transport of hydrophobic ligands. Hayes & Mantle (1986b) observed activation/inhibition effects for both chenodeoxycholate (on GST YcYc, YbYb, and YbYb) and lithocholate (on GST YbYb) that were pH-dependent. Caccuri et al. (1990) have identified two distinct binding sites for haem in placental GST $\pi$, and Xia & Chen (1989) have reported the allosteric inhibition of GST $\pi$ by bilirubin. Clearly there is at least one binding site on GST $\pi$, distinct from the catalytic centre, that may play a role in the regulation of the enzyme. It would seem therefore that allosteric regulation of GSTs might be a more common occurrence than has been thought up to now. Further work is necessary to determine the significance of the observed allosteric activation, but perhaps this activation allows the enzyme to act more efficiently in the presence of more than one electrophilic substrate, as must surely be the case in vivo. Mutual activation by alternative electrophilic substrates would help to alleviate or cancel out to some extent the inhibition resulting from competition at a single site.

We are grateful to Ms. G. Robinson for the N-terminal analysis and to Dr. Bruno Orsi for his valuable discussions. We also thank the Irish Cancer Society for financial support.

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Received 26 October 1990; accepted 12 November 1990