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EXPLANATION OF THE NON-HYPERBOLIC KINETICS OF THE GLUTATHIONE *S*-TRANSFERASES BY THE SIMPLEST STEADY-STATE RANDOM SEQUENTIAL Bi Bi MECHANISM

KATHRYN M. IVANETICH,*† RICHARD D. GOOLD‡ and CYNTHIA N. T. SIKAKANA‡

†Biomolecular Resource Center, University of California Medical School, San Francisco, CA 94143-0541, U.S.A.; and ‡Department of Medical Biochemistry, University of Cape Town Medical School, Observatory, 7925, R.S.A.

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Abstract—We have demonstrated that the simplest steady-state random sequential Bi Bi mechanism is sufficient to explain the previously reported non-hyperbolic kinetics of glutathione *S*-transferase 3-3 [Pabst MJ *et al.*, *J Biol Chem* **249**: 7140–7150, 1974; Jakobson I *et al.*, *Biochem J* **177**: 861–868, 1979]. The metabolism of 1-chloro-2,4-dinitrobenzene by rat liver glutathione *S*-transferase isoenzymes 2-2 and 3-3 and of 1,2-dichloro-4-nitrobenzene by isoenzyme 3-4 was shown to exhibit non-hyperbolic kinetics, which are best fit by the simplest steady-state random sequential Bi Bi mechanism. Neither more complex steady-state mechanisms nor the superimposition of product inhibition or enzyme memory on the simplest steady-state mechanism was necessary to generate non-hyperbolic kinetics for the glutathione *S*-transferases.

The GSH *S*-transferases§ (EC 2.5.1.18) are a group of dimeric enzymes which catalyze the conjugation of reduced glutathione (GSH) with a variety of electrophilic molecules. This family of enzymes, therefore, is thought to play a major role in drug biotransformation and the detoxication of xenobiotics [1, 2]. The cytosolic GSH *S*-transferase (GST) isoenzymes of rats, humans and mice have been assigned to three classes, Alpha, Mu and Pi, based on N-terminal amino acid sequences, subtrate specificities, immunological cross-reactivity and sensitivities to inhibitors [3].

The most detailed investigations of the steady-state kinetics of the GSTs have been performed for the metabolism of the electrophilic substrate 1,2-dichloro-4-nitrobenzene (DCNB) by rat liver GST 3-3 (class Mu) [4, 5]. Although the kinetic, equilibrium binding and product inhibition data favor a steady-state random sequential mechanism for this reaction [2, 5–8], the kinetic mechanism of GST 3-3 has not been fully resolved, due to the observation of non-Michaelian (non-hyperbolic) substrate–rate saturation curves. Explanations for the apparently anomalous non-hyperbolic kinetics have included subunit co-operativity, steady-state mechanisms of differing degrees of complexity, and the superimposition of either product inhibition or enzyme memory on these mechanisms [1, 2, 9].

This study confirms the biphasic kinetics previously reported for GST 3-3 with DCNB and also reports non-hyperbolic kinetics for this isoenzyme with 1-chloro-2,4-dinitrobenzene (CDNB) and for the heterodimeric rat liver GST 3-4 (class Mu) with DCNB and CDNB. To determine the extent of applicability of non-Michaelian kinetics to other classes of GST, the homodimeric GST 2-2 (representative of class Alpha) was isolated from rat liver and its kinetic mechanism characterized. Non-hyperbolic kinetics were also observed for GST 2-2, although the biphasic nature of the plots was less pronounced than for the isoenzymes of class Mu.

Various explanations for the non-hyperbolic kinetics are discussed, and it is demonstrated that the simplest steady-state random sequential Bi Bi mechanism, in the absence of products, is consistent with, and sufficient to explain, the non-hyperbolic kinetics of GSTs 3-3, 2-2 and 3-4. Neither more complex steady-state mechanisms nor the superimposition of product inhibition or enzyme memory on the basic steady-state random sequential mechanism is necessary.

EXPERIMENTAL PROCEDURES

Materials were obtained as follows: GSH and Sepharose-6B, Sigma Chemicals (St. Louis, MO); CDNB, 2,2′-dinitro-5,5′dithiodibenzoic acid and dimethyl sulfoxide, Merck Chemicals (Darmstadt F.R.G.); DCNB, EGA Chemie (Steinheim, F.R.G.); and dithiothreitol, BDH Chemicals (Poole, U.K.); *S*-(2,4-dinitrophenyl)glutathione was synthesized and purified by the method of Schramm *et al.* [10]. Rat hepatic GST isoenzymes 3-3 and 3-4 were isolated and characterized as described previously [11]. The purification and characterization of GST 2-2 from rat liver was essentially by the method of Boyer *et al.* [12], except that the DE-52 column was omitted. After purification to homogeneity (judged by SDS–PAGE), the enzyme preparations were stored at 77°K without significant loss of activity over the period of study. Activity toward CDNB and DCNB was assayed spectrophotometrically at

* To whom correspondence should be addressed.

§ Abbreviations: GSH, glutathione; GST, GSH *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; and SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

340 nm by a slight modification of the method of Habig *et al.* [13]. Assays were performed in a Multistat III Plus centrifugal analyzer (Instrumentation Laboratory, Lexington, MA) at 30 ± 0.1° using cuvettes of 0.5 cm pathlength. Initial rates were measured at 5-sec intervals for a total period of 60 sec, commencing 3 sec after initial mixing. The reaction was initiated by the addition of enzyme to 0.1 M potassium phosphate buffer (pH 6.5) containing, in order of addition, CDNB (or DCNB), inhibitor (if applicable) and GSH. The electrophilic substrates were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the reaction mixture was constant at 2% (v/v). Stock solutions of GSH were kept under nitrogen in an ice-bath to prevent oxidation. Non-enzymatic reaction rates served as controls, and were subtracted from enzymatic rates. The enzymatic reaction rate was linear with time for up to 60 sec after initiation, and up to an initial rate of 0.15 O.D./min. Data represent triplicate determinations in a single experiment, but are typical of results from two or more separate experiments. Velocities are expressed in nanomoles per minute per milliliter. Non-linear regression analyses of initial velocity data were performed by the iterative method of Duggleby [14]. Model fitting to rate equations describing bireactant kinetic systems was performed using the BMDPAR (derivative-free) and BMDP3R non-linear least squares regression analysis subroutines of the BMDP statistical package (University of California, Los Angeles). Analysis of the error structure of the experimental data showed that variance increased with velocity but not necessarily in proportion to it; the inverse of the predicted variance (from regression analysis) at any observed experimental velocity was used as a weighting factor at that point. The adequacy of fit of a model was determined by the examination of parameter values, residuals, and the residual sum of squares, and discrimination between models was based on the criteria of Mannervik [15].

RESULTS AND DISCUSSION

In spite of past controversy concerning the kinetic mechanism of the GSTs, it appears to be accepted currently that the random sequential model best fits the kinetic data of the GSTs [1, 2]. However, the deviation of GST kinetic data from Michaelis–Menten rate-behavior has given rise to proposals that product inhibition [6] or an enzyme memory mechanism [2] overlays the basic kinetic mechanism.

The non-hyperbolic kinetics of the GSTs were first observed in early studies of the conjugation of GSH and DCNB by rat liver GST 3-3 [4, 5]. Families of concave-down reciprocal plots were also observed in this investigation for the conjugation of CDNB by GST 3-3 (Fig. 1a) and of DCNB by GST 3-4 (Fig. 1b), when initial rates were measured over extended substrate concentration ranges. For GST 2-2, initial rate measurements performed over an eight-by-eight matrix with regard to the concentrations of GSH (0.04 to 1 mM) and CDNB (0.1 to 1 mM) (192 data points) also gave rise to biphasic (concave-down) double-reciprocal plots versus both substrates (Fig. 2).

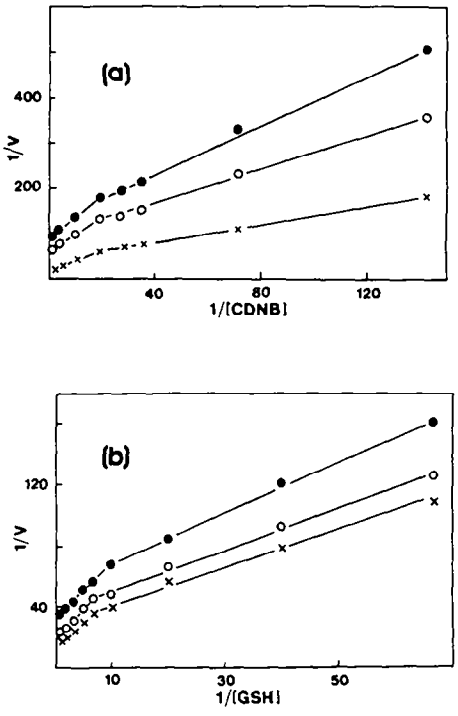
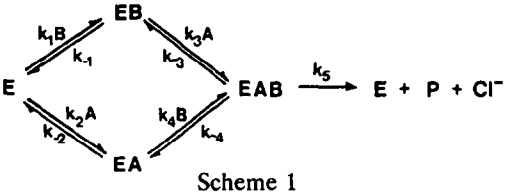


Fig. 1. Kinetics of rat liver GSTs 3-3 and 3-4 in the absence of products. (a) For GST 3-3 (0.1 μM), with CDNB as the varied substrate, GSH concentrations were (●) 0.05, (○) 0.15, and (×) 1 mM. (b) For GST 3-4 (0.2 μM), with GSH as the varied substrate, DCNB concentrations were (●) 0.2, (○) 0.4, and (×) 0.6 mM. Velocities are expressed in nmol/min/mL. Data points are the means of triplicate determinations. Lines drawn are those predicted by fitting the data to Eqn. 1, by non-linear least squares regression analysis, using the BMDP program.

Although a branched ordered sequential/ping-pong mechanism was originally proposed [5] to explain the rate behavior of GST 3-3 for the conjugation of DCNB, a significant body of evidence now favors a steady-state random sequential Bi Bi kinetic mechanism for this reaction [1, 2, 8]. The simplest such mechanism, as applied by Mannervik and Askelöf [8] to GST 3-3, is shown below,



Scheme 1

where E = enzyme, B = DCNB (or CDNB), A = GSH, and P = the GSH·DCNB (or GSH·CDNB) conjugate.

The rate equation corresponding to this mechanism is:

$$v = \frac{V_1AB + V_2A^2B + V_3AB^2}{(K_1 + K_2A + K_3B + AB + K_4A^2 + K_5B^2 + K_6A^2B + K_7AB^2)} \quad (1)$$

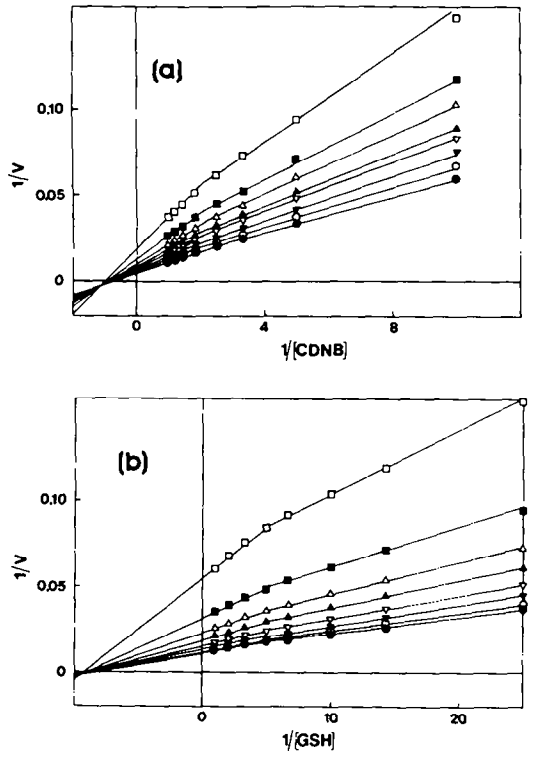


Fig. 2. Kinetics of rat liver GST 2-2 in the absence of products. (a) With CDNB as the varied substrate, GSH concentrations were (□) 0.04, (■) 0.07, (△) 0.1, (▲) 0.15, (▽) 0.2, (▼) 0.3, (○) 0.5, and (●) 1 mM. (b) With GSH as the varied substrate, CDNB concentrations were (□) 0.1, (■) 0.2, (△) 0.3, (▲) 0.4, (▽) 0.55, (▼) 0.7, (○) 0.85, and (●) 1 mM. Velocities are expressed in nmol/min/mL. Enzyme concentration was 0.14 μM. Data points are the means of triplicate determinations. The lines drawn are those predicted by fitting the data to Eqn. 1, by non-linear least squares regression analysis, using the BMDP program.

where V_i (i = 1–3) are constants containing E_T, and V_i and K_j (j = 1–7) are combinations of the rate constants shown in Scheme 1 [8].

Although sufficient data for detailed mechanistic modelling were not collected in this study for GSTs 3-3 and 3-4, and the contributions of the different subunits of the heterodimer cannot be overlooked, the general rate equation for a steady-state random sequential mechanism (Eqn. 1) was found to be capable of fitting the data for both of these isoenzymes. Although at best two parameters were redundant in the model, it provided a better fit by the criteria of Mannervik [15] for goodness of fit and discrimination between rival models than the branched reaction scheme of Pabst *et al.* [5] or other bireactant models.

The initial velocity data collected for GST 2-2 were considerably more substantial than those collected for GSTs 3-3 and 3-4, and were also best described by the steady-state random model. Where Mannervik and Askelöf [8] reported one redundant parameter when their data for GST 3-3 were fitted to this model, no redundant parameters were found in this study with the data for GST 2-2 (see Table 1). Alternative bireactant models for GST 2-2 were excluded on the basis of lack of convergence, large residual sum of squares, non-random residual distribution, and/or parameter values with high standard deviations.

Table 1. Kinetic constants for the steady-state random sequential mechanism

Constant	Parameter value
V_1	18.8 ± 6.3
V_2	205.8 ± 54.9
V_3	95.5 ± 27.3
K_1	0.001 ± 0.0006
K_2	0.2 ± 0.1
K_3	0.06 ± 0.01
K_4	1.02 ± 0.27
K_5	0.04 ± 0.02
K_6	1.17 ± 0.37
K_7	0.55 ± 0.20
Residual sum of squares	7.13
Mean square error	0.1

The results of fitting the experimental data set for GST 2-2 metabolism of CDNB in the absence of products (presented in Fig. 3) to Eqn. 1 by weighted nonlinear regression analysis, using the BMDP program, are shown. Units of the constants are expressed in their appropriate dimensions by using nmol/min/mL and mM as basic units of velocity and concentration respectively. Values are means ± SD. Data is based on 192 data points.

Reciprocal plots of initial rate data for GST 2-2 generated in the presence of the CDNB·GSH conjugate, *S*-(2,4-dinitrophenyl)glutathione, are shown in Fig. 3. The conjugate showed apparent mixed-type inhibition towards both substrates. No attempt was made to model the product inhibition data, because of the complex nature of rate equations describing steady-state bireactant systems in the presence of the products of the enzymic reaction [16]. However, the data for each varied substrate concentration at fixed co-substrate concentration were found to fit the general rate equation for hyperbolic mixed-type inhibition, as predicted for the steady-state random Bi Bi mechanism [16].

Therefore, the kinetic, equilibrium binding and product inhibition data reported previously for GST 3-3, and those generated in this investigation for GSTs 2-2, 3-3 and 3-4, favor a steady-state random sequential mechanism for all three isoenzymes. Since none of the simple steady-state mechanisms apparently explained the higher-degree dependence of velocity on substrate concentration [7], several alternate models have been proposed over the years to account for the apparently anomalous non-hyperbolic kinetics of GST 3-3 for the conjugation of DCNB and GSH. These suggestions have included subunit co-operativity [17], steady-state mechanisms of differing degrees of complexity [6], and the superimposition of product inhibition [6, 7] or enzyme memory [2] on the latter.

Although the non-linear concave-down double-reciprocal plots of kinetic data for the GSTs are consistent with negative co-operativity, no experimental evidence has been found for co-operative

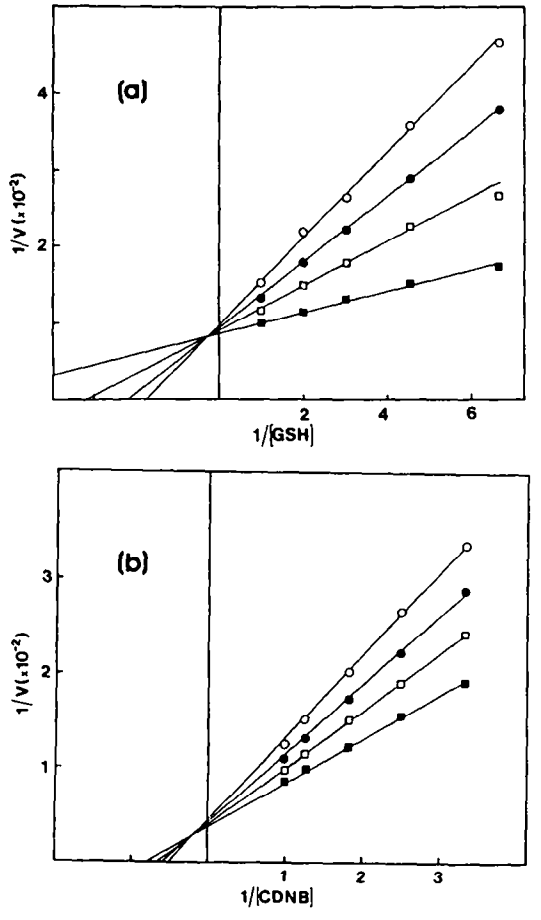


Fig. 3. Inhibition of rat liver GST 2-2 by the product, *S*-(2,4-dinitrophenyl)glutathione. (a) With GSH as the varied substrate at a fixed CDNB concentration of 0.8 mM. (b) With CDNB as the varied substrate at a fixed GSH concentration of 0.5 mM. In both plots, concentrations of the conjugate were: (■) 0, (□) 35, (●) 65, and (○) 100 μM. Velocities are in nmol/min/mL. Enzyme concentration was 0.2 μM. Data points are the means of triplicate determinations. Lines drawn are those predicted by fitting the data to an equation describing hyperbolic mixed-type inhibition, using the BMDP program.

interactions between the subunits of the dimeric GST isoenzymes studied, apparently eliminating this as a possible cause of the non-linearity [2, 17, 18]. It therefore appears that the negative co-operativity of the GSTs is generated kinetically.

A steady-state random mechanism involving kinetically significant enzyme·product complexes was proposed by Jakobson *et al.* [6]. They demonstrated that although the classical rate equation for a steady-state random mechanism (Eqn. 1) fitted the data for GST 3-3 satisfactorily, their more complex model (which included >20 parameters and terms of the 4th degree in concentration of each substrate) fitted the data with a lower residual sum of squares. Although this model was considerably overdetermined (having several redundant parameters and many other parameters with standard deviations of 100%), it was considered to best describe the kinetic mechanism of GST 3-3 [6].

The kinetic complications inherent in an "enzyme memory" mechanism, a complex system of transmission of information through protein–protein interactions [19], have been invoked to explain the apparent deviations of GST 3-3 from Michaelis–Menten rate behavior [2, 9, 20]. We propose here that the non-Michaelian steady-state kinetics observed for GSTs 3-3, 2-2 and 3-4 under initial rate conditions are adequately accounted for in terms of the properties of the rate equation describing the simplest steady-state random sequential mechanism (Eqn. 1). Neither more complex steady-state mechanisms nor the superimposition of product inhibition or enzyme memory on this mechanism is necessary.

For fixed concentrations of DCNB (or CDNB), Eqn. 1 simplifies to:

$$v = \frac{iA^2 + jA}{k + lA^2 + mA} \qquad (2)$$

where: $i = V_2B$, $j = V_1B + V_3B^2$, $k = K_1 + K_3B + K_5B^2$, $l = K_4 + K_6B$, and $m = K_2 + B + K_7B^2$ [16, 21, 22].

An analogous expression can be derived for constant concentrations of GSH. These simplified rate equations (like Eqn. 2) are 2/1 functions, i.e. in reciprocal form (after dividing through by A^2) $1/A^2$ appears in the numerator and $1/A$ appears in the denominator [16]. The presence of squared terms in the rate equations means that the steady-state random mechanism *predicts* non-linear reciprocal plots with either substrate [16, 21–23]. In fact, several distinct types of non-hyperbolic kinetics can be obtained in the steady-state random system. For example, plots of v versus substrate concentration may exhibit zero, one or two inflection points if one route to *EAB* is kinetically more favorable than the other [16, 21, 22].

Only under certain conditions, such as with saturating substrate concentrations or where special relationships exist between the kinetic parameters and the constant concentration of one substrate, will Eqn. 2 simplify so that linear double-reciprocal plots are obtained for the simplest steady-state random Bi Bi mechanism [16, 21, 23]. For example, kinetically generated negative co-operativity of the GSTs (discussed above) should disappear as the concentration of the fixed substrate becomes high enough to essentially force the reaction into an ordered mechanism. It was not possible to observe such a trend in this investigation because of the limited solubility of DCNB and CDNB in aqueous solution. The trend might be observable with GSH concentrations in the range of 100–1000 mM.

The required conditions for the concave-down double-reciprocal plots which are observed experimentally are met when ji/im, ki/mj, $i^2k/j(mi-jl)$ and K_6A^2/m are all less than unity for fixed concentrations of electrophilic substrate (or if the equivalent conditions hold for fixed GSH concentrations) [16, 21]. Using the reported values of V_i and K_i for the metabolism of DCNB by GST 3-3 [8], it can be shown that Eqn. 2 predicts concave-down double-reciprocal plots over the whole range of substrate concentrations used experimentally (data not shown). Conditions for linearity of double-reciprocal

plots would only be met, using these V_i and K_i values, at very much lower concentrations of GSH ($\leq 2\ \mu M$) or DCNB ($<5\ \mu M$) or at saturating concentrations of either substrate.

Using converged values of V_i and K_i for the metabolism of CDNB by GST 2-2 (Table 1) it was also found that concave-down double-reciprocal plots are predicted over the whole range of substrate concentrations used experimentally (data not shown). As was the case for the reported values of V_i and K_i above [8], conditions for linearity of the double-reciprocal plots would only be met at very low concentrations or at saturating concentrations of either substrate.

Generally, in steady-state random bisubstrate systems with a preferred (but not exclusive) kinetic pathway to a ternary complex, there is no need to assume subunit–subunit interactions or ligand-induced conformational changes in order to explain non-hyperbolic velocity curves [16]. This is because the complexities inherent in bisubstrate enzymic reactions can adequately account for apparent allosteric phenomena without requiring protein–protein interactions. The non-hyperbolic kinetics of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthetase of *Rhodomicrobium vannielli* [24] and phosphofructokinase of *Escherichia coli* [21] have been interpreted previously in terms of such a kinetic model.

The proposal that protein–protein interactions are not required to explain the non-hyperbolic kinetics of the GSTs is particularly interesting in view of the implications of a recent demonstration that solvation of the active-site could contribute significantly to the inactivation of the GSTs [25]. Protein–protein interactions have been proposed to explain various phenomena related to the inactivation of various GSTs, and the protection from this inactivation that is afforded the enzyme by preincubation with GSH and several proteins [19]. These phenomena, as well as several other features (such as the pH-dependence) of the inactivation of the GSTs, are equally well (and more simply) accounted for in terms of the loss of catalytic activity that results from differing degrees of solvation of the enzyme active-site. The role of enzyme memory in the kinetic mechanism of the GSTs is, therefore, currently being re-evaluated in this laboratory.

In conclusion, the simplest steady-state random sequential Bi Bi mechanism is consistent with and sufficient to explain the previously reported non-hyperbolic kinetics of GST 3-3 [8] and those observed here for GSTs 3-3, 3-4 and 2-2. This is the simplest mechanism consistent with the kinetic, equilibrium binding and product inhibition data. Therefore, invoking Occam's razor*, neither more complex steady-state mechanisms nor the superimposition of product inhibition or enzyme memory on a steady-state random sequential mechanism is required to explain the non-Michaelian kinetics of the three GST isoenzymes.

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* William of Occam's (1280–1349) dictum—*Entia non sunt multiplicanda practer necessitatem.*

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