Determination of the Mechanism and Kinetic Constants for Hog Kidney γ -Glutamyltransferase

By JACK W. LONDON,* LESLIE M. SHAW,† DONALD FETTEROLF* and DAVID GARFINKEL*

*Moore School of Electrical Engineering and †William Pepper Laboratory, Department of Pathology, University of Pennsylvania, Philadelphia, PA. 19174, U.S.A.

(Received 24 March 1976)

The initial-velocity kinetics of hog kidney γ -glutamyltransferase were studied. Glutamate γ -(4-nitroanilide) and its 3-carboxy derivative, glutamate γ -(3-carboxy-4-nitroanilide), served as γ -glutamyl donors, and glycylglycine as an acceptor. Reaction products were identified by paper chromatography and amino acid analysis. Inhibited Ping Pong mechanisms and a comprehensive initial-velocity expression were developed which account for the observed simultaneous γ -glutamyl transfer and autotransfer, competitive inhibition by glycylglycine, and non-competitive inhibition by the carboxy donor. The validity of the proposed Ping Pong mechanisms are supported by enzyme-velocity data obtained with constant ratios of acceptor to donor concentrations. Kinetic constants were determined by a non-linear regression analysis. With glutamate γ -(4-nitroanilide) as the donor, Michaelis constants for the donor, acceptor and donor-acting-as-acceptor are 1.87, 24.9, and 2.08 mM respectively. With glutamate γ -(3-carboxy-4-nitroanilide) as the donor, these Michaelis constants are 1.63, 16.6, and 12.3 mM. Glycylglycine competitive inhibition constants with the parent donor and its carboxy derivative are 275 and 205 mM respectively; the non-competitive inhibition constant of the carboxy donor is 34 mM.

The enzyme γ -glutamyltransferase (EC 2.3.2.2) catalyses the transfer of the γ -glutamyl moiety. Although widely distributed, this enzyme is especially active in the kidney (Albert *et al.*, 1961) and in epithelial cells involved in transport processes and the secretion of specialized body fluids (Tate & Meister, 1974). It is of interest because of speculation and investigations concerning its physiological role (Meister, 1973; Elce & Broxmeyer, 1976), and its demonstrated clinical value as an indicator of hepatobiliary disease (Lum & Gambino, 1972).

The overall reaction catalysed by this enzyme is:

where D and A are the donor and acceptor substrates respectively.

The enzyme is not very specific, as a number of peptides, amino acids and synthetic compounds may serve as substrates (Tate & Meister, 1974; Orłowski & Meister, 1963). When the donor and acceptor are different substances, the above reaction is designated as γ -glutamyl transfer; if they are the same substance, it is auto- γ -glutamyl transfer. If the acceptor is water, the reaction is hydrolysis.

The kinetics of this enzyme are often complicated by the simultaneous occurrence of more than one of these modes of catalytic action. Previous kinetic studies of the γ -glutamyl transfer reaction alone

(Tate & Meister, 1974; Elce *et al.*, 1974), or simultaneous hydrolysis and γ -glutamyl transfer (Elce & Broxmeyer, 1976), have suggested a Ping Pong mechanism (Cleland, 1963) for the enzyme, although a sequential mechanism could not be ruled out. Here we consider simultaneous γ -glutamyl transfer and autotransfer. We extend the ratio method of Tsopanakis & Herries (1975) to this multiple-pathway reaction to determine whether the enzyme mechanism is sequential or non-sequential.

Glutamate γ -(4-nitroanilide) and its 3-carboxy derivative, glutamate γ -(3-carboxy-4-nitroanilide), were used as γ -glutamyl donors and glycylglycine as an acceptor. The reaction products were identified by paper chromatography and amino acid analysis. Kinetic measurements were made at 30°C. Inhibited Ping Pong mechanisms are proposed to account for the observed simultaneous γ -glutamyl transfer and autotransfer. A corresponding initial-velocity equation is presented, along with kinetic constants obtained by non-linear regression analysis.

Materials and Methods

Glutamate γ -(4-nitroanilide), glutamate γ -(3-carboxy-4-nitroanilide) and hog kidney γ -glutamyltransferase were all obtained from Boehringer-Mannheim Corp., New York, NY 10017, U.S.A.

Glycylglycine was obtained from Sigma Chemical Co., St. Louis, MO 63178, U.S.A.

Enzyme activities were measured by recording the E_{405} , at which wavelength 4-nitroaniline, the cleavage product of the donor, absorbs. A model DU spectrophotometer (Beckman Instrument Co., Fullerton, CA 92634, U.S.A.), fitted with a model 210 cuvette positioner, a model 220 absorbance indicator and optical converter (both from Gilford Instruments, Oberlin, OH 44074, U.S.A.), and a model 100P stripchart recorder (Fisher Scientific Co., Pittsburgh, PA 15219, U.S.A.) were used. All measurements were made at 30°C, in 185 mM-Tris/HCl buffer, pH 8.25.

To determine the form of the initial-velocity equation and its constants, enzyme-activity measurements were made with donor concentrations ranging from 0.20 to 6.0 mM (0.2 to 15 mM with the more-soluble carboxy derivative) and acceptor concentrations from 0 to 120 mM. Eleven sets of data [six with glutamate γ -(4-nitroanilide) as donor, with a total of 180 data points, five with its carboxy derivative totalling 177 points], were obtained, with about 30 measurements per set. All reagents plus enzyme were mixed together and the E_{405} was recorded continuously for 10 min. The final mixture volume was 850 μ l and contained 50 μ l of enzyme preparation.

Descending paper chromatography, as described by Orlowski & Meister (1965), was used to identify the reaction products. For analytical purposes, 0.7 unit of the hog kidney enzyme (1 unit defined as 1 μ mol of substrate transformed/min) was incubated for 5 h at 30°C with 6 mM-glutamate γ -(4-nitroanilide) and 0, 2.46, 6.15, 40.0 and 100.0 mM-glycylglycine in 2.55 ml total volume. After termination of the reactions by the addition of 25 μ l of acetic acid to 0.2 ml of reaction mixture, 10 μ l of each reaction mixture was spotted on Whatman no. 1 paper (30 cm $\times$ 55 cm). The solvent used was pyridine/butanol/water (1:1:1, by vol.). Chromatogram development was allowed to proceed for 7.5 h; the chromatograms were then dried for 15 min at 50°C and sprayed with 0.25 % ninhydrin dissolved in 95 % (v/v) ethanol.

For preparative purposes, 0.7 unit of the enzyme was incubated for 5 h at 30°C in a total volume of 2.55 ml with 6 mM-glutamate γ -(4-nitroanilide) and 2.46 mM-glycylglycine. The reaction was terminated as described above and 150 μ l of reaction mixture was streaked across the Whatman no. 1 paper. Development of the chromatogram was carried out as described above. After drying, 1 cm-wide strips were cut along the length of each side of the chromatogram as well as the middle and sprayed with the ninhydrin reagent. After development of colour, these strips were used as a guide for cutting out the corresponding unstained portions of the preparative chromatogram. Peptide material in the unstained chromatogram was eluted by soaking the cut-out paper strips in 4 ml of water. Amino acid analysis of 6 M-HCl hydrolysates

(24 h under N_2 at 110°C) of the eluted peptide material was with a Durrum D-500 amino acid analyser (Durrum Instrument Corp., Palo Alto, CA 94303, U.S.A.).

A PDP-10 computer (Digital Equipment Corporation, Maynard, MA 01754, U.S.A.) was used for all computer calculations. Kinetic constants were computed by non-linear least-squares-regression analysis. The method of Fletcher & Powell (1963) was used to minimize the deviation between calculated and observed enzyme velocities. Confidence limits for the constants, indicating how well the constants are defined for a given velocity equation with our data, were obtained from the Hessian matrix that this minimization procedure produces (Davidon, 1959). The calculated kinetic constants correspond to the best fit of the velocity equation to all of the data points for that donor compound. The theory and methodology of non-linear regression analysis of enzyme kinetic data has been reviewed elsewhere (Johnson, 1974; Garfinkel *et al.*, 1976).

Results

Activity of γ -glutamyltransferase with glutamate γ -(4-nitroanilide) and glycylglycine as substrates

An investigation was made of the products obtained with glutamate γ -(4-nitroanilide) as donor both without an acceptor and with glycylglycine as an acceptor. Comparison of the descending paper chromatogram shown in Plate 1 with control chromatograms allowed the indicated assignment of the glutamate γ -(4-nitroanilide), glycylglycine and γ -glutamylglycylglycine ninhydrin-positive spots.

The γ -glutamyl glutamate γ -(4-nitroanilide) assignment was based on a spectrophotometric and amino acid analysis. The eluted spot exhibited a maximum at E_{317} , indicating a 4-nitroanilide derivative. On the basis of a molar absorbance of 1.3×10^4 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ at 315 nm for glutamate γ -(4-nitroanilide) (Orlowski & Meister, 1965), the concentration of the eluted 4-nitroanilide derivative was 3.77 μ M. After hydrolysis, an amino acid analysis yielded 6.82 μ M-glutamic acid (93 % of total recovered amino acids). Thus the molar ratio of glutamic acid to eluted 4-nitroanilide derivative is 1.82:1, compatible with γ -glutamyl glutamate γ -(4-nitroanilide) as the structure of the 4-nitroanilide derivative.

Additional evidence for this assignment was obtained by incubating a sample of the eluted 4-nitroanilide derivative with 1.2 units of hog kidney γ -glutamyltransferase and glycylglycine. The molar ratio of 4-nitroaniline enzymically produced to eluted 4-nitroanilide derivative was 1.002:1, confirming the 2:1 ratio of γ -glutamyl groups to 4-nitroanilide parent compound. These results agree with those of a similar study by Orlowski & Meister (1965).

EXPLANATION OF PLATE I

Descending paper chromatography of reaction products

After 5 h incubation at 30°C, terminated reaction mixtures (10 μ l each) were spotted on Whatman no. 1 paper. The chromatogram was developed with pyridine/butanol/water (1:1:1, by vol.) as described in the Materials and Methods section, and the resulting separated peptide spots were developed with 0.25% ninhydrin dissolved in 95% ethanol. The reaction mixtures contained 6 mM-glutamate γ -(4-nitroanilide) dissolved in 185 mM-Tris/HCl, pH 8.25, with 0.7 unit of hog kidney γ -glutamyl-transferase and the following concentrations of glycylglycine (mM): (a) 100; (b) 40; (c) 6.15; (d) 2.46; (e) none. The following spot assignments were made: 1, γ -glutamylglycylglycine; 2, glycylglycine; 3, γ -glutamyl-glutamate γ -(4-nitroanilide); 4, glutamate γ -(4-nitroanilide).

Fig. 1. Effect of glutamate γ -(4-nitroanilide) concentration on γ -glutamyltransferase velocity

Initial velocities ($\mu\text{mol/min}$ per litre) were measured for mixtures of various concentrations of glutamate γ -(4-nitroanilide) donor (0.2–6.0 mM) and glycylglycine acceptor at constant concentrations (mM) of: $\square$, 100; ∇ , 40; Δ , 24.6; $\blacksquare$, 12.3; $\blacktriangledown$, 2.46; $\blacktriangle$, none. Measurements were made at 30°C , in 185 mM-Tris/HCl, pH 8.25. The final mixture volume of 850 μl contained 50 μl of enzyme preparation. The lines were drawn by using the kinetic constant values of Table 1 with eqn. (1).

Fig. 2. Effect of glycylglycine concentration on γ -glutamyltransferase velocity when glutamate γ -(4-nitroanilide) is the donor

Initial velocities ($\mu\text{mol/min}$ per litre) were measured for mixtures of various concentrations of glycylglycine acceptor (0–100 mM) and glutamate γ -(4-nitroanilide) donor at constant concentrations (mM) of: $\square$, 6; ∇ , 1; Δ , 0.5; $\blacksquare$, 0.2. Measurements were made at 30°C , in 185 mM-Tris/HCl, pH 8.25. The final mixture volume of 850 μl contained 50 μl of enzyme preparation. The lines were drawn by using the kinetic constant values of Table 1 with eqn. (1).

This di- γ -glutamyl product results from the auto-transfer of the γ -glutamyl donor substrate. With a high ratio of glycylglycine/glutamate γ -(4-nitroanilide) the primary products are 4-nitroaniline and γ -glutamylglycylglycine, with only a slight indication of glutamate γ -(4-nitroanilide) being present (see Plate 1). The presence of the di- γ -glutamyl product increases as the relative concentration of glycylglycine decreases, indicating an increasing rate of autotransfer. The absence of a glutamic acid product with incubation mixtures containing no glycylglycine, indicates no measurable hydrolysis reaction for glutamate γ -(4-nitroanilide) in the concentration range studied. Thus only γ -glutamyl transfer and autotransfer reactions were observed.

The activity of γ -glutamyltransferase as a function of glutamate γ -(4-nitroanilide) concentration was measured with various concentrations of glycylglycine and in its absence. It is apparent from representative data for this study (Fig. 1), that a sizeable auto- γ -glutamyl transfer reaction occurs in the absence of an acceptor. The maximum self-reaction activity is about 25% of the maximum activity measured with both substrates present. Fig. 1 also shows a slight decrease in enzyme activity at the upper limit of glutamate γ -(4-nitroanilide) solubility (approx. 6 mM).

We also measured the activity of γ -glutamyltransferase as a function of glycylglycine concentration with various concentrations of glutamate γ -(4-nitroanilide). Representative data for these experiments are shown in Fig. 2. The donor self-reaction is quite evident by the non-zero activities measured in the absence of glycylglycine (particularly at the higher donor concentrations). Not readily apparent from Fig. 2 is the slight decrease in activity at high glycylglycine concentration with the lower donor concentrations.

A further study was performed in which the enzyme activity was measured for various acceptor and donor concentrations corresponding to certain acceptor/donor concentration ratios. These data are shown as a constant-ratio double-reciprocal plot in Fig. 3.

Activity of γ -glutamyltransferase with glutamate γ -(3-carboxy-4-nitroanilide) and glycylglycine as substrates

As with the glutamate γ -(4-nitroanilide) donor, activity measurements were made for various concentrations of its carboxy derivative with various concentrations of glycylglycine (and in its absence). Typical data are shown in Fig. 4. The auto- γ -glutamyl transfer activity is noticeably less than with the former

Fig. 3. Double-reciprocal plots for constant acceptor/donor concentration ratios

Plots of reciprocal initial velocities versus reciprocal glutamate γ -(4-nitroanilide) concentration for the following constant ratios of glycylglycine/glutamate γ -(4-nitroanilide) concentration: $\blacksquare$, 1:2; $\triangle$, 5:1; ∇ , 10:1; $\square$, 20:1. Lines drawn were obtained by linear regression. Linear plots were also obtained when $1/v$ was plotted against reciprocal glycylglycine concentration, for constant concentration ratios.

Fig. 4. Effect of glutamate γ -(3-carboxy-4-nitroanilide) concentration on γ -glutamyltransferase velocity

Initial velocities ($\mu\text{mol/min}$ per litre) were measured for mixtures of various concentrations of glutamate γ -(3-carboxy-4-nitroanilide) donor (0.2–15 mm) and glycylglycine acceptor at constant concentrations (mm) of: $\square$, 120; ∇ , 40; $\triangle$, 24.6; $\blacksquare$, 12.3; $\blacktriangledown$, 2.46; $\blacktriangle$, none. Measurements were made at 30°C , in 185 mm-Tris/HCl, pH 8.25. The final mixture volume of 850 μl contained 50 μl of enzyme preparation. The lines were drawn by using the kinetic constant values of Table 1 with eqn. (1) modified by the non-competitive-inhibition denominator term (see the text).

Fig. 5. Effect of glycylglycine concentration on γ -glutamyltransferase velocity when glutamate γ -(3-carboxy-4-nitroanilide) is the donor

Initial velocities ($\mu\text{mol/min}$ per litre) were measured for mixtures of various concentrations of glycylglycine acceptor (0–120 mm) and glutamate γ -(3-carboxy-4-nitroanilide) donor at constant concentrations (mm) of: $\square$, 15; ∇ , 10; $\triangle$, 6; $\blacksquare$, 0.5; $\blacktriangledown$, 0.34; $\blacktriangle$, 0.2. Measurements were made at 30°C , in 185 mm-Tris/HCl, pH 8.25. The final mixture volume of 850 μl contained 50 μl of enzyme preparation. The lines were drawn by using the kinetic constant values of Table 1 with eqn. (1) modified by the non-competitive-inhibition denominator term (see the text).

donor, now being at most only about 10% of the maximum activity observed. The carboxy derivative is more soluble than the former donor, and a large decrease in enzyme activity is readily apparent at the higher donor concentrations.

A corresponding study was also made in which the enzyme activity was measured as a function of glycylglycine concentration with various concentrations of glutamate γ -(3-carboxy-4-nitroanilide). Typical data are plotted in Fig. 5. A slight decrease in enzyme activity with high acceptor and low donor concentrations may be discerned.

Discussion

Previous enzyme kinetic studies (Tate & Meister, 1974; Elce *et al.*, 1974) primarily used the probable physiological donor substrate glutathione with methionine as an acceptor. The γ -glutamyl transfer reaction alone was studied by having the glutathione concentration greater than or equal to that of methionine. For a limited number of initial-velocity measurements, Tate & Meister (1974) obtained parallel double-reciprocal plots, highly suggestive of Ping Pong kinetics. A sequential mechanism is still

Fig. 6. Double-reciprocal plots for constant acceptor concentrations

Plots of reciprocal initial-velocities versus reciprocal glutamate γ -(4-nitroanilide) concentration for the following glycylglycine concentrations (mm): ▼, 2.46; ■, 6.15; △, 12.3; ▽, 24.6; □, 40. Lines were drawn by using the kinetic constant values of Table 1 with eqn. (3).

Fig. 7. Double-reciprocal plots for constant donor concentrations

Plots of reciprocal initial-velocities versus reciprocal glycylglycine concentration for the following glutamate γ -(4-nitroanilide) concentrations (mm): ▼, 0.2; ■, 0.5; △, 1; ▽, 4; □, 6. Lines were drawn by using the kinetic constant values of Table 1 with eqn. (3).

possible, since the parallel lines may be slowly converging (Tsopanakis & Harries, 1975). Elce *et al.* (1974), with a larger data set, obtained equally good fits to their data for both sequential and non-sequential rate equations by a non-linear-regression analysis. They judged the Ping Pong rate equation better, since the denominator constant term of the sequential mechanism had confidence limits that included zero.

A study by Elce & Broxmeyer (1976) with glutathione and methionine as substrates and under conditions favouring simultaneous γ -glutamyl transfer and hydrolysis also resulted in parallel double-reciprocal plots. This is inconsistent with the observed simultaneous modes of catalytic action, and they attributed the parallelism to insufficient data precision and greatly differing acceptor and hydrolytic donor Michaelis constants. An isotope-exchange experiment they performed with γ -glutamylmethionine and radioactive methionine resulted in converging double-reciprocal plots, which unfortunately would be expected with both sequential and non-sequential branched mechanisms.

In the present study, any proposed mechanism must account for simultaneous γ -glutamyl transfer and autotransfer. The decrease in enzyme activity at high substrate concentrations should also be expressed. For our data, initial-velocity double-reciprocal plots do not result in straight lines (see Figs. 6 and 7). However, if the reciprocal velocity is plotted as a

function of the reciprocal of one substrate's concentration, for a constant ratio of substrate concentrations (i.e. ratio = $R = [\text{glycylglycine}]/[\text{glutamate } \gamma\text{-(4-nitroanilide)}]$ = constant), then straight lines do result (see Fig. 3). As will be developed below, these observations are consistent only with the simultaneous γ -glutamyl transfer and autotransfer proceeding by Ping Pong mechanisms.

The following reaction steps would occur in the transfer of the γ -glutamyl moiety from the glutamate γ -(4-nitroanilide) donor to the glycylglycine acceptor by Ping Pong mechanisms:

where E is the free γ -glutamyltransferase enzyme; GluNA is the donor glutamate γ -(4-nitroanilide) (or its carboxy derivative); Gly-Gly is the acceptor glycylglycine; NA is the product 4-nitroaniline; Glu-Gly-Gly is the product γ -glutamylglycylglycine; other names refer to enzyme complexes with the substrates (e.g. E·Glu is the γ -glutamyl-enzyme intermediate).

The autotransfer could then proceed by the following steps:

The γ -glutamyl enzyme intermediate combines with a second donor molecule; this complex breaks down to yield the product γ -glutamyl glutamate γ -(4-nitroanilide) (Glu-GluNA).

Further, the activity decrease observed at high glycylglycine concentrations can be attributed to competitive inhibition by the acceptor with the free enzyme:

This mechanism can also account for the activity decrease observed at the high donor concentration by assuming a larger proportion of the glutamyl enzyme intermediate proceeds through the comparatively slower steps 5 and 6.

This mechanism, summarized in Scheme 1, results in the following initial-velocity equation:

In the above equation, [D] and [A] are the donor and acceptor concentrations. All other symbols are defined in Table 1. This initial-velocity equation was derived with the aid of a computer implementation (Rhoads & Pring, 1968) of the method of King & Altman (1956).

Inspection of eqn. (1) shows that in the absence of glycylglycine acceptor (i.e. [A] = 0), a pseudo-single substrate Michaelis-Menten expression results:

$$v = \frac{V_{mt} \alpha [D]}{(K_M^{DA} + \alpha K_M^D) + [D]} \quad (2)$$

Extending the 'ratio' method of Tsopanakis & Herries (1975) to this branched non-sequential mechanism, we define the ratio of acceptor/donor concentrations as $R = [A]/[D]$. We make this substitution after dividing the numerator and denominator of eqn. (1) by $[D][A]$, and finally take the reciprocal of the result, obtaining:

$$\frac{1}{v} = \frac{c_3}{\left(c_1 + \frac{c_2}{R}\right)} \frac{1}{[A]} + \frac{c_4}{\left(c_1 + \frac{c_2}{R}\right)} \frac{1}{[D]} + \frac{\left(\frac{c_5}{R} + c_6 R + c_7\right)}{\left(c_1 + \frac{c_2}{R}\right)} \quad (3)$$

$$v = \frac{d[\text{NA}]}{dt} = \frac{V_{mt} \left([D][A] + \alpha \frac{K_M^A}{K_M^{DA}} [D]^2 \right)}{\left(K_M^A + \alpha K_M^D \frac{K_M^A}{K_M^{DA}} \right) [D] + K_M^D [A] + \left(1 + \alpha \frac{K_M^D}{K_M^A} \frac{K_M^A}{K_M^{DA}} \right) [D][A] + \frac{K_M^A}{K_M^{DA}} [D]^2 + \frac{K_M^D}{K_M^A} [A]^2} \quad (1)$$

Scheme 1. Reaction scheme for simultaneous γ -glutamyl transfer and autotransfer by γ -glutamyltransferase

Schematic illustration of the forward pathway for simultaneous γ -glutamyl transfer and autotransfer. The enzyme γ -glutamyltransferase is denoted as E, glutamate γ -(4-nitroanilide) (or its carboxy derivative) as GluNA, glycylglycine as Gly-Gly, and 4-nitroaniline as NA. Reverse reactions and competitive inhibition by glycylglycine are not shown. This type of graphical representation of reaction schemes was developed by Cleland (1963).

Table 1. *Kinetic constants for hog kidney γ -glutamyltransferase*

Definition of symbols used for kinetic constants and values (with 95% confidence limits) were obtained by non-linear regression.

Kinetic constant (mm)	Symbol	Definition	Donor ... Glutamate γ -(4-nitroanilide)	Glutamate γ -(3-carboxy-4-nitroanilide)
Acceptor (glycylglycine) Michaelis constant	K_M^A	$\frac{k_{+2}(k_{-3} + k_{+4})}{k_{+3}(k_{+2} + k_{+4})}$	24.9 $\pm$ 2.7	16.6 $\pm$ 2.3
Donor [glutamate γ -(4-nitroanilide or glutamate γ -(3-carboxy-4-nitroanilide)] Michaelis constant	K_M^D	$\frac{k_{+4}(k_{-1} + k_{+2})}{k_{+1}(k_{+2} + k_{+4})}$	1.87 $\pm$ 0.13	1.63 $\pm$ 0.12
Donor-acting-as-acceptor (autotransfer) Michaelis constant	K_M^{DA}	$\frac{k_{+2}(k_{-5} + k_{+6})}{k_{+5}(k_{+2} + k_{+6})}$	2.08 $\pm$ 0.18	12.3 $\pm$ 3.4
Acceptor competitive inhibition constant	K_I^A	$\frac{k_{-7}}{k_{+7}}$	275 $\pm$ 76	205 $\pm$ 46
Donor non-competitive inhibition constant	K_I^D	$\frac{k_{-8}}{k_{+8}} = \frac{k_{-9}}{k_{+9}} = \frac{k_{-10}}{k_{+10}} = \frac{k_{-11}}{k_{+11}} = \frac{k_{-12}}{k_{+12}} = \frac{k_{-13}}{k_{+13}}$	—	34.3 $\pm$ 7.8
γ -Glutamyl transfer turn-over number	T_t	$\frac{k_{+2}k_{+4}}{k_{+2} + k_{+4}}$	—	—
Auto- γ -glutamyl transfer turnover number	T_a	$\frac{k_{+2}k_{+6}}{k_{+2} + k_{+6}}$	—	—
γ -Glutamyl transfer maximum velocity	V_{mt}	$T_t [e_{total}]$	—	—
Ratio of turnover numbers	α	T_a/T_t	0.167 $\pm$ 0.012	0.166 $\pm$ 0.031

in which, $c_1 = V_{mt}$; $c_2 = V_{mt}\alpha(K_M^A/K_M^{DA})$; $c_3 = K_M^A + \alpha K_M^D(K_M^A/K_M^{DA})$; $c_4 = K_M^D$; $c_5 = K_M^A/K_M^{DA}$; $c_6 = K_M^D/K_I^A$; $c_7 = 1 + \alpha(K_M^D/K_I^A)(K_M^A/K_M^{DA})$.

If R is held constant, then straight lines will result for both $y = 1/v$, $x = 1/[A]$, $1/[D] = Rx$; and $y = 1/v$, $x = 1/[D]$, $1/[A] = x/R$. Unlike the lines for a simple non-sequential mechanism, these do not converge on the y axis at $1/V_{max}$. Rather, the intercepts of eqn. (3) are functions of R , so that lines for different values of R do not converge on the y axis.

The rate equation for an ordered sequential mechanism would differ from eqn. (1) by having an additional denominator term in $[A]$ as well as a term composed only of constants. Rearrangement of this rate expression would predict curved rather than straight lines for the constant-ratio double-reciprocal plots. Since we obtain straight lines for our constant-ratio data (see Fig. 3), a non-sequential, rather than a sequential, mechanism is indicated for γ -glutamyl-transferase.

As Tsopanakis & Herries (1975) point out, linear constant-ratio double-reciprocal plots may be misleading if the denominator constant term of the sequential rate equation is small, leading to little curvature in the plots. This is unlikely in this case since

no indication of curvature is found in the plots. As further support for Ping Pong mechanisms for the simultaneous γ -glutamyl transfer and autotransfer, non-linear regression analysis showed the extra constant of the sequential rate expression to have confidence limits which include zero, as Elce *et al.* (1974) had found in their model of the γ -glutamyl transfer reaction alone.

The non-linearity of our double-reciprocal plots in which one substrate concentration is held constant, instead of R , is also predicted by eqn. (3) (the curves of Figs. 6 and 7 were generated by using eqn. 3). If the glutamate γ -(4-nitroanilide) concentration is varied (Fig. 6), the curvature of the lines arises from the different proportion of the γ -glutamyl-enzyme intermediate that proceeds through the slower autotransfer pathway. For high glycylglycine concentrations, the lines curve upward at high donor concentrations (i.e. as more autotransfer occurs). At very low glycylglycine concentrations (approx. 2 mM), autotransfer is substantial even at low donor concentrations, resulting in activity increasing non-linearly over the entire concentration range. If the glycylglycine concentration is varied with a constant donor concentration, the activity increases non-linearly with

increasing acceptor concentration, except at low donor concentrations, where competitive inhibition by glycylglycine is apparent.

It should be noted that the glycylglycine inhibition is exaggerated by the reciprocal plots (inverting v and s introduces distortion). The substrate concentration-velocity curves of Fig. 2 give a more accurate indication of the small degree of inhibition. The disadvantages of reciprocal plots have been reviewed elsewhere (Garfinkel *et al.*, 1976).

A non-linear regression analysis was used to obtain the best fit of initial-velocity eqn. (1) to our data. With glutamate γ -(4-nitroanilide) as the donor, a satisfactory fit was obtained (see Figs. 1 and 2). The standard deviation of the calculated curve to the experimental data was 2.56, giving a coefficient of variation of 3.6%. The kinetic constants and their 95% confidence limits determined by this non-linear analysis are also summarized in Table 1.

However, a non-linear regression analysis fitting eqn. (1) to our data with glutamate γ -(3-carboxy-4-nitroanilide) was not completely satisfactory. This analysis clearly showed that our proposed mechanism could not account for the large decrease in activity observed at the higher concentrations of this more-soluble donor. A reasonable addition to our mechanism to account for this decrease would be to propose that the donor is also a non-competitive inhibitor binding to another site on the enzyme. This second site would have a much lower affinity for the carboxy donor, becoming significant only at high donor concentrations [glutamate γ -(3-carboxy-4-nitroanilide) concentrations > 10mM]. Further inter-conversions of an enzyme form would not occur while this site was occupied. This would generate the following reaction steps (the use of the asterisk with the symbols for the enzyme forms signifies that a second site, other than that involved in steps 1-7, reacts with the donor):

Reaction steps 8-13 have the effect of multiplying the entire denominator of eqn. (1) by $1 + ([D]/K_P^D)$; with K_P^D being the dissociation constant of the carboxy donor bound at the second site.

A non-linear regression analysis was successful in fitting this modified version of eqn. (1) to all of our data with glutamate γ -(3-carboxy-4-nitroanilide) as the donor. The standard deviation of the calculated from the experimental curve was 2.86, with a coefficient of variation of 4.7%. The constants obtained are also summarized in Table 1.

It should be noted that attempts to fit the observed inhibition by allowing second-site interaction with the free enzyme only (step 8), or the free enzyme and the γ -glutamyl enzyme (step 10), were unsuccessful.

From Table 1 it is evident that the ratio of turnover numbers, α , does not depend on whether glutamate γ -(4-nitroanilide) or its carboxy derivative is used as the donor. The Michaelis constant for the donor, in its proper role as a donor, K_M^D , also is not very different for the two donor substrates. Likewise, considering the large confidence limits of the glycylglycine substrate inhibition constants, K_I^A , they do not vary much with the donor substrate used. (The large confidence limits indicate a loosely defined parameter. This lack of definition results from there being only a slight inhibition over the glycylglycine concentration range studied.) However, the other kinetic parameters are a function of which chemical is the donor substrate. The sixfold increase in the Michaelis constant for the donor acting as an acceptor K_M^{DA} , when the carboxy derivative is the substrate, indicates that the

Fig. 8. Contribution of auto- γ -glutamyl transfer to total measured γ -glutamyltransferase velocity

Eqn. (4) was used to calculate the fraction of total enzyme velocity arising from autotransfer as a function of acceptor/donor concentration ratios. —, Glutamate γ -(4-nitroanilide) is the donor; ----, glutamate γ -(3-carboxy-4-nitroanilide) is the donor.

cause of the decrease in autotransfer is a lesser facility of this substrate to function as an acceptor. This is also reflected in the decrease in the glycylglycine Michaelis constant K_M^A , when the carboxy derivative is the donor; the lack of competition from the donor increases the efficiency of the acceptor, making the enzyme saturated with lower concentrations of glycylglycine.

Eqn. (3) may be rearranged to obtain the following relationship for the fraction of the total observed enzyme velocity that is due to auto- γ -glutamyl-transfer:

$$\frac{v_{\text{auto}}}{v_{\text{total}}} = \frac{1}{1 + (K_M^{\text{DA}}/\alpha K_M^A)([A]/[D])} \quad (4)$$

In eqn. (4), v_{auto} is the autotransfer velocity, and v_{total} is the total observed velocity. This fraction is graphically presented in Fig. 8 as a function of the acceptor/donor concentration ratio. The fraction is of course 1 with no glycylglycine acceptor present, falling to 0.09 at a 20:1 ratio for glutamate γ -(4-nitroanilide), and 0.01 for the same ratio and the carboxy derivative as donor.

This study was supported by grants GM16501 and RR15 from the National Institutes of Health. We are grateful to Miss A. Bellow for the amino acid analyses.

References

- Albert, Z., Orlowski, M. & Szewczuk, A. (1961) *Nature (London)* **191**, 767–768
- Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 104–137
- Davidon, W. C. (1959) *A. E. C. Res. Dev. Report*, ANL-5990 (Rev.), U.S. Dept. of Commerce, Washington, DC
- Elce, J. S. & Broxmeyer, B. (1976) *Biochem. J.* **153**, 223–232
- Elce, J. S., Bryson, J. & McGirr, L. G. (1974) *Can. J. Biochem.* **52**, 33–41
- Fletcher, R. & Powell, M. J. D. (1963) *Comput. J.* **6**, 163–168
- Garfinkel, L., Kohn, M. C. & Garfinkel, D. (1976) *CRC Crit. Rev. Bioeng.* in the press
- Johnson, L. E. (1974) *CRC Crit. Rev. Bioeng.* **2**, 1–36
- King, E. L. & Altman, C. (1956) *J. Phys. Chem.* **60**, 1375–1378
- Lum, G. & Gambino, S. R. (1972) *Clin. Chem.* **18**, 358–362
- Meister, A. (1973) *Science* **180**, 33–39
- Orlowski, M. & Meister, A. (1963) *Biochim. Biophys. Acta* **73**, 679–681
- Orlowski, M. & Meister, A. (1965) *J. Biol. Chem.* **240**, 338–347
- Rhoads, D. G. & Pring, M. (1968) *J. Theor. Biol.* **20**, 297–313
- Tate, S. S. & Meister, A. (1974) *J. Biol. Chem.* **249**, 7593–7602
- Tsopanakis, D. & Herries, D. G. (1975) *Eur. J. Biochem.* **53**, 193–196