KINETICS, MECHANISM AND AMINO ACID SPECIFICITY OF SHEEP KIDNEY γ-GLUTAMYL TRANSPEPTIDASE

by

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Prof. Sherwin Wilk

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The City University of New York

Dedicated to my present (Nancy), our past (our parents) and all of our futures (Chavi et al.).

Abstract

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The kinetics of sheep kidney γ-glutamyl transpeptidase was studied using a novel substrate L-α-methyl-γ-glutamyl-L-α-aminobutyrate. When the substrate was incubated with the enzyme in the presence of an amino acid or peptide acceptor, the corresponding L-α-methyl-γ-glutamyl derivatives of the acceptors were formed. In the absence of acceptor only hydrolysis occurred, and no transpeptidation products were detected. The presence of the methyl group on the α-carbon apparently prevents enzymatic transfer of the L-α-methyl-γ-glutamyl residue to the amino group of the substrate itself (autotranspeptidation). When the enzyme was incubated with conventional substrates, such as glutathione or γ-glutamyl-p-nitroanilide and an amino acid acceptor, hydrolysis, autotranspeptidation and transpeptidation to the acceptor occurred concurrently.

Initial velocity measurements in which the concentration of L-α-methyl-γ-glutamyl-L-α-aminobutyrate was varied at several fixed acceptor concentrations,

and either the release of α-aminobutyrate or the formation of the transpeptidation products was determined, yielded results which are consistent with a ping-pong mechanism modified by a hydrolytic shunt. A scheme for such a mechanism is presented. This mechanism predicts the formation of a α-methyl-γ-glutamyl-enzyme intermediate, which can react with an amino acid to form the transpeptidation product; or in the absence of, or in the presence of low concentrations of amino acids, can react with water to form the hydrolytic products. Kinetic derivations for the reaction of the enzyme with the conventional substrate γ-glutamyl-p-nitroanilide predict either linear or non-linear double-reciprocal plots, depending on the contribution of the hydrolytic, autotranspeptidation, or transpeptidation reactions. The results of kinetic experiments confirmed these predictions.

Kinetic expressions for the velocity of formation of either first product or of the individual transpeptidation products were also derived for the general case in which any number of amino acids were incubated with the enzyme and a γ-glutamyl donor. Such expressions were found to be complex, with the K_m and V_{max} values dependent on the concentration of both the acceptors and the amino acids of the incubation medium.

A simplified expression, however, was derived by considering the relative ratio of the rates of formation

of any two transpeptidation products $v_{(1)}/v_{(2)}$. This rate ratio is linearly dependent on the ratio of the concentrations of the precursor amino acids $B_{(1)}/B_{(2)}$ (i.e. $v_{(1)}/v_{(2)} = k_{(1)}/k_{(2)} \cdot B_{(1)}/B_{(2)}$). The ratio, $k_{(1)}/k_{(2)}$, is a constant, and reflects the relative abilities of the amino acids to interact with the γ-glutamyl-enzyme intermediate. This ratio, defined as the reactivity ratio, is independent of the concentration of the enzyme, the γ-glutamyl donor and the amino acids. Reactivity ratios are a measure of the relative efficiencies with which equimolar concentrations of amino acids would serve in the transpeptidation reaction. When amino acids are not present at equimolar concentrations, the relative amount of any two transpeptidation products can be calculated from the reactivity ratio and the relative concentration of the precursor amino acids.

The reactivity ratios of several amino acids towards γ-glutamyl transpeptidase were studied relative to L-alanine in a mixture of plasma amino acids under conditions of pH similar to those existing in vivo. The reactivities, in decreasing order were as follows: glutamine ∿ cystine > methionine > aromatic amino acids > branched chain amino acids ∿ basic amino acids > acidic amino acids. Our results suggest that at physiological concentrations of amino acids found in serum, 30-50 times as much glutamine would be utilized as a substrate in the

transpeptidation reaction as any of the branched chain or aromatic amino acids.

Reactivity ratios were found to be unchanged when any of several L-γ-glutamyl derivatives were used as the γ-glutamyl donor. This finding is consistent with the formation of an identical L-γ-glutamyl-enzyme intermediate from several L-γ-glutamyl donors. Reactivity ratios are changed when a α-methyl-γ-glutamyl derivative, or D-γ-glutamyl derivatives are used as the γ-glutamyl donors, suggesting that the change in structure of the γ-glutamyl-enzyme intermediate affects the interaction of the enzyme with amino acids.

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I. INTRODUCTION

A. General Statement of Theme

This thesis presents studies undertaken to elucidate the function of the enzyme γ-glutamyl transpeptidase[1]. γ-Glutamyl transpeptidase seems to be the enzyme primarily responsible for the in vivo degradation of glutathione. Glutathione (L-γ-glutamyl-L-cysteinylglycine) is a ubiquitous tripeptide whose function (or functions) are just beginning to be explored and appreciated. γ-Glutamyl transpeptidase has, in addition, attained clinical prominence as a serum-enzyme marker, used for the diagnosis of certain pathological states and the following of their clinical course.

This research project was designed to study the kinetics of γ-glutamyl transpeptidase, and to assign a mechanism (or mechanisms) of action to the reactions catalyzed by this enzyme. Using this (these) mechanism(s) as a model, we intend to design experiments to explore the amino acid specificity of this enzyme. We hoped that a clearer understanding of this specificity would clarify this enzyme's role in physiological processes, and possibly, also the physiological function of glutathione.

[1] The Enzyme Commission's name for this enzyme is γ-glutamyl transferase (E.C. 2.3.2.2.). Many current papers and most of the literature published by this laboratory in the past refer to this enzyme as γ-glutamyl transpeptidase. We will, therefore, use γ-glutamyl transpeptidase throughout this thesis to refer to this enzyme.

Several recent reviews (Knox, 1960; Waley, 1966; Meister, 1974a; Meister and Tate, 1976; Orlowski and Karkowsky, 1976) a monograph (Jocelyn, 1972) and several symposia (Colowick et al., 1954; Crook, 1959; Flohe et al., 1974) concerning the metabolism, biochemistry and function of glutathione have been published. A review of only the most salient points covered in these reviews will be presented, in order to place the work contained in this thesis in proper perspective.

B. Glutathione: History, Chemistry, Metabolism, Oxidation-Reduction, Turnover, and Function

1. History

Glutathione, first described by de Rey-Pailhade (1888a,b) was originally named "philothion", because it acted as a biological substrate in the reduction of elemental sulfur to hydrogen sulfide. Several sulfhydryl containing substances (Hefter, 1908; Arnold, 1911a, b) were subsequently discovered to be widely distributed in tissues. In 1921, Hopkins (1921) isolated and partially purified one of these sulfhydryl containing compounds, and identified it as de Rey-Pailhade's philothion. On the basis of degradative studies, Hopkins erroneously assigned it a dipeptide structure containing glutamate and cysteine. He therefore christened it glutathione.

This erroneous dipeptide structure was initially confirmed (Stewart and Tunnicliffe, 1925), but was subsequently challenged on the basis of nitrogen and sulfur analytical data inconsistent with a dipeptide structure (Hunter and Eagles, 1927). Hopkins (1927), at first skeptical of this analytical data, later confirmed it (Hopkins, 1929). It was shown that

glycine, in addition to glutamate and cysteine, is a constituent of glutathione (Hopkins, 1929; Kendall et al., 1929).

On the basis of pK_a measurements L-γ-glutamyl-L-cysteinyl-glycine was suggested as the most probable structure for glutathione (Pirie and Pinhie, 1929). This structural assignment was strengthened by chemical transformation studies of glutathione (Nicolet, 1930; Kendall et al., 1930), and confirmed by its total synthesis (Harington and Mead, 1935).

Much of the subsequent early work on glutathione, both in relation to its synthesis and metabolism, was undertaken in the anticipation that a knowledge of the mechanism involved in the formation and degradation of its peptide bonds would elucidate the general mechanism of protein synthesis. In light of our recently-attained knowledge of the ribosomal nature of protein synthesis, this anticipation was evidently not fulfilled. Nevertheless, studies on the metabolism of glutathione - this thesis included - continue today in the hopes of clarifying the underlying physiological function of this tripeptide.

2. Chemical Properties

Glutathione has two structural features responsible for much of its biochemistry. These two features are its sulfhydryl group (due to cysteine) and the γ-glutamyl bond (the bond linking cysteine to glutamate).

The sulfhydryl group is responsible for most of the catalytic and reactive properties of glutathione. It allows glutathione to participate both in oxidation-reduction reactions and nucleophillic displacement reactions. Because of

its tendency to participate in oxidation-reduction processes, glutathione occurs both in reduced (GSH) and oxidized (disulfide,GSSG) forms. The nucleophillic properties of the sulfhydryl group are such that it readily participates both as an incoming and exiting nucleophile.

The γ-glutamyl bond of glutathione makes it resistant to the actions of most peptidases. In addition, the presence of this bond makes glutathione the most abundant, naturally-occurring substrate for γ-glutamyl transpeptidase.

3. Metabolism

a) Synthesis of Glutathione

The first indication that glutathione was synthesized from its constituent amino acids was the observation (Waelsch and Rittenberg, 1940, 1941) that the administration of [^{15}N] glycine to rats and rabbits caused rapid incorporation of the nitrogen label into glutathione. Incorporation of the nitrogen label into protein was also observed, but it occurred at a much slower rate than into glutathione. It was, therefore, suggested that glutathione might serve as an intermediate in the transfer of free amino acids into proteins.

Synthesis of labeled glutathione from [^{15}N] glycine was subsequently observed by Bloch and his co-workers in isolated liver (Bloch and Anker, 1947), and liver extracts (Johnston and Bloch, 1949). A soluble enzyme system was isolated from pigeon liver extracts which was capable of synthesizing glutathione from L-glutamate, L-cysteine and glycine, in the presence of magnesium ion and an adenine nucleotide (Johnston and Bloch, 1951).

The rate of glutathione formation, when catalyzed by pigeon liver extracts, exhibited lag-times when either L-glutamate and L-cysteinylglycine, or the three constituent amino acids were the substrates. No lag-times, however, were observed when L-γ-glutamyl-L-cysteine and glycine were incubated under the same conditions. These rate patterns for glutathione formation suggested that L-γ-glutamyl-L-cysteine is an intermediate in a multi-step process leading to glutathione formation. This conclusion was supported by the identification of this intermediate when the liver extracts were incubated in an incubation medium that was complete except for glycine. Furthermore, the lag-times for glutathione formation could be abolished if L-glutamate and L-cysteine were preincubated prior to the addition of glycine (Snoke and Bloch, 1952). The synthesis of glutathione was also shown to be a multi-step process from studies on bean seedling extracts (Webster, 1953).

The enzymatic reactions leading to the synthesis of glutathione are (Snoke et al., 1953):

$$\text{L-Glutamate} + \text{L-cysteine} + \text{ATP} \xrightarrow{Mg^{+2} \text{ or } Mn^{+2}} \text{L-}\gamma\text{-glutamyl-L-cysteine} + \text{ADP} + P_i \quad (1)$$

$$\text{L-}\gamma\text{-Glutamylcysteine} + \text{glycine} + \text{ATP} \xrightarrow{Mg^{+2} \text{ or } Mn^{+2}} \text{glutathione} + \text{ADP} + P_i \quad (2)$$

Reaction 1 is catalyzed by γ-glutamylcysteine synthetase, and reaction 2 is catalyzed by glutathione synthetase. A stoichiometric amount of orthophosphate is released from ATP, for each γ-glutamylcysteine (Mandeles and Bloch, 1955) or glutathione (Snoke et al., 1953) molecule formed.

(1) γ-Glutamylcysteine Synthetase

Partially purified preparations of this enzyme have been obtained from several sources, including wheat germ extracts, hog liver, bovine lens, human erythrocytes and sheep brain (see Orlowski and Karkowsky, 1976 for references). An apparently homogeneous preparation of γ-glutamylcysteine synthetase has been isolated from rat kidney (Orlowski and Meister, 1971a).

Rat kidney γ-glutamylcysteine synthetase is a soluble supernatant enzyme. The enzyme has a molecular weight of approximately 92,000 (Orlowski and Meister, 1971b). The enzyme requires either Mg^{+2} or Mn^{+2} for activity. The specificity of this enzyme towards cysteine or an amino acid analog depends on which divalent cation is used (Orlowski and Meister, 1971b). The γ-glutamylcysteine synthetase reaction has a marked specificity for L-glutamate. D-Glutamate, α-methyl-DL-glutamate and RS -β-methyl-DL-glutamate can substitute for L-glutamate, but are much less active. In contrast, a variety of amino acids can substitute for cysteine in this reaction (Rathbun, 1967; Orlowski and Meister, 1971a). The ability of many different amino acids to substitute for cysteine can explain some, but not all of the naturally occurring γ-glutamyl amino acids (Rathbun, 1967).

The mechanism of action of γ-glutamylcysteine synthetase has been investigated. The enzyme, in addition to catalyzing the reversible formation of γ-glutamylcysteine (reaction 1), is also capable of catalyzing the following reactions:

(1) hydrolysis of ATP; (2) phosphorylation by ATP of inorganic phosphate to yield inorganic pyrophosphate, and (3) cyclization of glutamate to form pyrrolidone carboxylate, this cyclization being associated with ATP cleavage. These additional reactions suggest that an enzyme-bound γ-glutamyl phosphate is formed as an intermediate in the synthetase reaction (Orlowski and Meister, 1971b; Meister, 1974a).

Recent kinetic evidence suggests that this reaction is a random BC** mechanism (Yip and Rudolph, 1976). In this random BC mechanism, $Mg(ATP)^{-2}$ binds to the enzyme first, followed by the random binding of glutamate and cysteine. Products (γ-glutamylcysteine, $Mg(ADP)^{-2}$ and P_i) are subsequently released, but the order of this release was not determined. The formation of an enzyme-bound γ-glutamyl phosphate intermediate would thus occur only when glutamate binds before cysteine, representing only one of the two possible pathways available to form the central transitory complex.

(2) Glutathione Synthetase

Snoke (1955) achieved a 5500-fold purification of glutathione synthetase from Brewer's yeast. Shortly thereafter, Webster and Varner (1955) partially purified the enzyme from wheat germ extracts. In addition, partially purified preparations of glutathione synthetase have been obtained from pigeon livers, Escherichia coli, yeast, higher plants, and erythrocytes (see Meister, 1974a and references cited therein). Highly purified preparations of glutathione synthetase have been obtained from yeast (Mooz and Meister, 1967).

** B and C are respectively, the second and third substrates bound to the enzyme.

human red blood cells (Majerus et al., 1971) and bovine red blood cells (Wendel et al., 1972).

Molecular weight determinations of glutathione synthetase have been reported from yeast (molecular weight 123,000)(Mooz and Meister, 1967), human erythrocytes (molecular weight 150,000)(Majerus et al., 1971) and bovine erythrocytes (molecular weight 122,00) (Wendel et al., 1973). The bovine erythrocyte enzyme was reported to have a subunit molecular weight of 61,000, thus suggesting that glutathione synthetase consists of two subunits, each having a molecular weight of approximately 61,000 (Wendel et al., 1972).

The specificity of yeast glutathione synthetase towards the dipeptide has been studied (Mooz and Meister, 1967). Of the dipeptides studied, L-γ-glutamyl-L-cysteine was the most active substrate. L-γ-Glutamyl-L-α-aminobutyrate and L-γ-glutamyl-L-alanine were somewhat less active. These last two substrates are the probable precursors of the naturally occurring tripeptides ophthalmic acid (γ-L-glutamyl-L-α-aminobutyrylglycine) and norophthalmic acid (γ-L-glutamyl-L-alanylglycine)respectively. The amino acid specificity of glutathione synthetase was also studied. None of the amino acids tested could substitute for glycine. Snoke (1955), however, reported that with pigeon liver glutathione synthetase, aminomethanesulfonic acid, the sulfonic acid analog of glycine, can replace glycine in the synthetase reaction.

The mechanism of action of the yeast glutathione synthetase reaction has also been investigated. On the basis of partial reactions catalyzed by the enzyme, a di-

peptidyl phosphate-ADP-enzyme is postulated as a transient reaction intermediate (Nishimura et al., 1964; Meister, 1974a). For bovine erythrocytes, however, kinetic evidence suggests that glutathione synthetase reacts by a rapid-equilibrium random mechanism. This mechanism suggests that : (1) all substrates are bound to the enzyme in a random order before any products are released; (2) all enzyme-substrate complexes are in thermodynamic equilibrium and (3) the rate-limiting reaction step is the formation of products from the quaternary complex. Based on equilibrium constants, the most abundant of the intermediates of this reaction (e.g. $Mg(ATP)^{-2}$-enzyme, glycine-dipeptide-enzyme) would be the dipeptide-ATP-enzyme complex. It is possible that, with yeast glutathione synthetase, this complex rearranges to the dipeptidyl phosphate-ADP-enzyme complex. The presence of this last intermediate could not, however, be demonstrated with bovine erythrocyte glutathione synthetase (Wendel and Heinle, 1975).

Little is known about the in vivo controlling mechanisms of glutathione synthesis. Although the activity of glutathione synthetase seems to be lower than γ-glutamylcysteine synthetase, the limiting factor for glutathione synthesis may be the availability of γ-glutamylcysteine. Low values of γ-glutamylcysteine (25 μM) were found in hemolysates of human erythrocytes using three different methods to measure the peptide (Wendel et al., 1975b). The synthesis of γ-glutamylcysteine may itself be limited by the availability of cysteine. In addition, physiological levels of glutathione inhibit

γ-glutamylcysteine synthetase. Thus, glutathione exerts feed-back inhibition on its own synthesis. This inhibition may also play a role in the in vivo regulation of glutathione levels (Jackson, 1969; Richman and Meister, 1975).

b) Degradation

The first indication that glutathione could be biologically degraded was the observation (Dakin and Dudley, 1913b) that aqueous extracts of pancreas or pancreatic tissue were capable of inhibiting glyoxalase activity. This glyoxalase-inhibiting activity was destroyed by either heating or digestion with HCl, suggesting an enzymic basis for the inhibition. Preincubation of these pancreatic extracts with tissues that normally contain glyoxalase activity markedly inhibited this activity. The inhibition of glyoxalase activity depended on the length of time of the preincubation. Antiglyoxalase activity, therefore, seemed to be due to direct action of pancreatic extracts on glyoxalase. High antiglyoxalase activity was subsequently found in the kidney of several species including rat, rabbit, pig and horse (Platt and Schroeder, 1934; Woodward et al., 1935). Antiglyoxalase activity was thermolabile, non-dializable and able to be precipitated, thus firmly establishing the enzymic nature of this inhibition (Schroeder et al., 1935).

In 1932 Lohmann (1932) showed that glutathione is a cofactor in the glyoxalase reaction. It was subsequently shown that antiglyoxalase exerts its effect by destroying glutathione. This destruction occurred without the loss of sulfhydryl groups, suggesting that the antiglyoxalase acti-

vity is due to the hydrolysis of glutathione (Girsavicius, 1933).

In the presence of glutathione, antiglyoxalase caused an increase in free carboxylate groups. The products of the action of antiglyoxalase on glutathione were mistakenly identified as γ-glutamylcysteine and glycine, suggesting that glutathione was only partially hydrolyzed (Schroeder et al., 1935). Upon re-examination of the analytical procedures used, however, it was concluded that glutathione was totally hydrolyzed. The total hydrolysis of glutathione was confirmed by the isolation of cysteine (or cystine) in high yields from reaction mixtures containing glutathione and antiglyoxalase (Schroeder and Woodward, 1937).

In addition to glutamate, pyrrolidone carboxylate[2] was identified as a product of the enzymic hydrolysis of glutathione. The relative proportion of glutamate and pyrrolidone carboxylate were dependent on the pH of the incubation medium. Pyrrolidone carboxylate predominated at alkaline pH, and glutamate under acidic conditions. Pyrrolidone carboxylate formation was shown to be a direct result of the scission of the γ-glutamyl bond, and not due to cyclization of free glutamate (Woodward and Reinhart, 1942).

Between 1948 and 1956, the complex nature of antiglyoxalase action on glutathione was substantially clarified. Binkley and Nakamura (1948) used a colorometric method

[2] Synonyms for pyrrolidone carboxylate are: 5-oxoproline, pyroglutamate and 2-oxo-pyrrolidine-5-carboxylate.

which was able to differentiate between cysteine and total cysteine plus cysteinylglycine. The time sequence of cysteinylglycine and cysteine release was consistent with the concept that the hydrolysis of glutathione to cysteine is a two step process with the intermediary formation of cysteinylglycine. The enzyme capable of cleaving glutathione to cysteinylglycine was limited in rat to the kidney. The enzyme capable of hydrolyzing cysteinylglycine was more widespread in rat tissue. It was subsequently shown that cysteinylglycinase activity could be separated from glutathionase activity, the activity responsible for the formation of cysteinylglycine from glutathione (Olson and Binkley, 1950).

Glutamine was shown to markedly enhance glutathionase activity, as measured by cysteinylglycine formation. The suggestion was made that glutamine acted as a soluble cofactor in the following manner (Binkley and Olson, 1951):

$$\text{Glutamine} + \text{enzyme} \longrightarrow \text{enzyme-NH}_2 + \text{glutamate} \qquad (3a)$$

$$\text{Enzyme-NH}_2 + \text{glutathione} \longrightarrow \text{glutamine} + \text{cysteinylglycine} + \text{enzyme} \qquad (3b)$$

Concurrent with the publication of this last paper, showing the activating effects of glutamine on glutathionase activity, several papers by Hanes et al., (1950; 1952) appeared. These papers showed that the decomposition of glutathione in the presence of any one of several amino acids, catalyzed by sheep kidney extracts, yielded new products. These products which were separated by paper chromatography, were identified as γ-glutamyl amino acids. The transfer of the γ-glutamyl

group of glutathione (the donor) to the amino acid acceptor was postulated to occur as follows:

Glutathione + amino acid (or peptide) ⟶

γ-glutamyl amino acid + cysteinylglycine (4)

The enzyme catalyzing this reaction was named γ-glutamyl transpeptidase. The activating effects of glutamine on glutathione degradation could thus also be explained by the ability of glutamine to act as a substrate in the transpeptidation reaction (Hird and **Springell**, 1954).

In 1956, Connell and Hanes described and partially purified an enzyme from liver tissues which was capable of degrading γ-glutamyl peptides to pyrrolidone carboxylate and free amine (reaction 5).

γ-Glutamyl peptide ⟶ pyrrolidone carboxylate + amine (or amino acid) (5)

The enzyme which catalyzes this reaction, and is distinct from γ-glutamyl transpeptidase, was named γ-glutamyl lactamase.[3]

Thus, all the products of glutathione degradation, as described by Woodward and Reinhart (1942), could be accounted for by the actions of γ-glutamyl transpeptidase, γ-glutamyl cyclotransferase and cysteinylglycine dipeptidase.

(1) <u>γ-Glutamyl Transpeptidase</u>

γ-Glutamyl transpeptidase, like glutathione, is widespread in many plants and animal tissues. Early studies, however, suggested that, in mammals, this enzyme was limited to pancreas and kidney. In the early 1960's, several new

[3] Synonym for γ-glutamyl cyclotransferase (E.C. 2.3.2.4.)

synthetic substrates, such as γ-glutamylanilide (Goldbarg et al., 1961), γ-glutamyl-α-aminonitriles (Szewczuk and Orlowski, 1960), γ-glutamylnaphthylamides (Orlowski and Szewczuk, 1961; Glenner and Folk, 1961) and γ-glutamyl-p-nitroanilide (Orlowski and Meister, 1963) were introduced, which both facilitated and enhanced the sensitivity of assaying γ-glutamyl transpeptidase. γ-Glutamyl transpeptidase was subsequently found in most animal tissues studied (Goldbarg et al., 1961). This enzyme was, in addition, found to be a natural constituent both of serum and urine (Szewczuk and Orlowski, 1960). The marked elevation of γ-glutamyl transpeptidase in sera of patients with hepato-billiary disorders, suggested the clinical use of this enzyme as a marker for these disorders (Szewczuk and Orlowski, 1960; Orlowski, 1963; Rosalki, 1975).

γ-Glutamylnaphthylamides have been used as substrates to histochemically locate γ-glutamyl transpeptidase activity. Transpeptidase action on this substrate releases naphthylamine, which can be coupled to a diazonium salt (usually Fast Garnet GBC) to yield a highly colored precipitate. In the kidney, the presence of the enzyme has been demonstrated in the brush borders of the epithelial cells lining the proximal convoluted tubules, and in the lumen of the loop of Henle (Albert et al., 1961; Glenner and Folk, 1961). Transpeptidase activity has also been localized in the brush border region of the villus tip cells (Greenberg et al., 1967), the choroid plexus (Albert et al., 1966) primarily in the apical portion of the epithelial cells (Tate et al., 1973),

isolated brain capillaries (Orlowski et al., 1974), and the basal portions of the epithelial cells of the cilliary body (Ross et al., 1973).

Highly purified preparations of γ-glutamyl transpeptidase were first obtained by Szewczuk and Baranowski (1963), and Orlowski and Meister (1965) from bovine and hog kidney respectively. Homogeneous preparations of this enzyme have been obtained from swine kidney (Leibach and Binkley, 1968), human kidney (Richter, 1969), rat hepatoma (Taniguchi, 1974) ovine kidney (Zelazo and Orlowski, 1976) and rat kidney (Tate and Meister, 1975; 1976; Hughey and Curthoys, 1976).

γ-Glutamyl transpeptidase is a glycoprotein containing up to 36% carbohydrate as hexose, hexosamine and sialic acid (Szewczuk and Baranowski, 1963; Orlowski and Meister, 1965). The enzyme is particle-bound, but can be solublized by either detergent or butanol treatment. Even after solubilization, the enzyme appears to be aggregated with other membraneous material. This aggregation can yield spurious results (Zelazo and Orlowski, 1976), such as high molecular weights and the postulation of multiple isozymic forms for the enzyme. It is possible to disrupt this aggregation by treatment of the enzyme with proteolytic agents, such as ficin (Leibach and Binkley, 1968), trypsin (Richter, 1969; Zelazo and Orlowski, 1976), bromelain (Tate and Meister, 1976) or papain (Hughey and Curthoys, 1976). The high carbohydrate content of γ-glutamyl transpeptidase perhaps protects the enzyme against proteolytic attack. A comparative study of rat kidney γ-glutamyl transpeptidase, prepared

either by solublization with detergent (Triton X-100) or with proteolytic enzyme (papain) suggests, however, that the proteolytic enzyme-treatment does cause excission of a small peptide from the transpeptidase. This peptide is believed to be responsible for the aggregation of the transpeptidase with micelles of detergent, and also possibly for anchoring the transpeptidase in the kidney brush border (Hughey and Curthoys, 1976).

Subsequent to proteolytic treatment, the molecular weights of γ-glutamyl transpeptidase, as obtained from various kidney preparations, were reported to be as follows: porcine (80,000)(Leibach and Binkley, 1968), ovine (92,000)(Zelazo and Orlowski, 1976), rat (68,000) (Tate and Meister, 1976), (66,700-78,000)(Hughey and Curthoys, 1976) and human (80,000-90,000)(Szewczuk, 1966; Richter, 1969; Tate and Meister, 1976). Rat hepatoma and rat kidney γ-glutamyl transpeptidase which were purified without the use of proteolytic enzymes showed molecular weights of 113,000 (Taniguchi, 1974) and 81,000-87,000 (Hughey and Curthoys, 1976), respectively.

Sheep kidney γ-glutamyl transpeptidase could be dissociated in the presence of either sodium dodecylsulfate or 8 M urea into two unequal subunits (molecular weights 65,000 and 27,000). Treatment of the enzyme with dimethylsuberimidate, a peptide cross-linking reagent, prior to dissociation with sodium dodecylsulfate, yielded, a species of molecular weight 92,000, in addition to the two free subunits. This species, composed of one small and one large subunit, represents the structure of the purified enzyme (Zelazo and Orlowski, 1976).

Two unequal subunits have also been obtained from rat (Tate and Meister, 1976; Hughey and Curthoys, 1976) and human (Tate and Meister, 1976) kidney preparations. The functional significance, if any, of the two subunit structure is not known.

γ-Glutamyl transpeptidase catalyzes several reactions. In addition to the hydrolysis of glutathione (reaction 6), and the transpeptidation of amino acids with glutathione (reaction 4), this enzyme also catalyzes the transpeptidation of glutathione with itself (equation 7).

$$\text{Glutathione} \longrightarrow \text{glutamate} + \text{cysteinylglycine} \quad (6)$$

$$\text{Glutathione} + \text{glutathione} \longrightarrow \gamma\text{-glutamylglutathione} + \text{cysteinylglycine} \quad (7)$$

This autotranspeptidation reaction is similar to the transpeptidation reaction, in that glutathione, which has a free amino acid functionality, now serves both as the amino acid acceptor as well as the γ-glutamyl donor.

The first indication for the functioning of this autotranspeptidation reaction was the observation by Hanes et al., (1950) that the incubation of glutathione with extracts of sheep kidney resulted in several ninhydrin positive compounds which on paper chromatography migrated slower than glutathione Slowly migrating species were also observed when other γ-glutamyl compounds were used instead of glutathione. For example, when γ-glutamyltyrosine was incubated with sheep kidney transpeptidase several ninhydrin positive compounds were observed, which migrated on paper chromatography slower than the starting γ-glutamyl amino acid. One of these compounds was isolated and found to contain glutamate and tyrosine, suggesting that

it was γ-glutamyl-γ-glutamyltyrosine. These results were taken as presumptive evidence that γ-glutamyl amino acids could function as amino acid acceptors in the transpeptidase reaction (Hanes et al., 1952). That γ-glutamyl peptides could act as amino acid acceptors was confirmed by Orlowski and Meister (1965). The ninhydrin positive products formed from L-γ-glutamyl-p-nitroanilide in the presence of hog kidney γ-glutamyl transpeptidase were isolated and analyzed. The products found included autotranspeptidation (γ-glutamyl-γ-glutamyl-p-nitroanilide) and higher order **autotranspeptidation** products (γ-glutamyl-γ-glutamyl-γ-glutamyl-p-nitroanilide, and γ-glutamyl-γ-glutamyl-γ-glutamyl-γ-glutamyl-p-nitroanilide). These results clearly show that γ-glutamyl peptides are capable of functioning as amino acid acceptors in the transpeptidase reaction.

With respect to the reactions catalyzed by γ-glutamyl transpeptidase, it has not yet been determined which of these reactions is of physiological importance, in vivo. It has been suggested that in vivo γ-glutamyl transpeptidase may function as a phosphate-independent glutaminase (Curthoys and Kuhlenschmidt, 1975). It has also been suggested that this enzyme is responsible for the hydrolysis of extracellular glutathione (Schulman et al., 1975; Elce and Broxmeyer, 1976). Both these suggestions imply that the hydrolytic reaction catalyzed by this enzyme is of physiological importance. This view is consistent with the increased activity of the hydrolytic, relative to the transpeptidation action of this enzyme, as the pH approaches the physiological range

(Revel and Ball, 1959).

Evidence for the in vivo functioning of the hydrolytic reaction has been obtained by administering large doses of L-γ-glutamyl-L-α-aminobutyrate to mice and measuring the resultant metabolites in the kidney. High concentrations of glutamate and glutamate metabolites were found. A much smaller accumulation of pyrrolidone carboxylate was also found. These results suggest that significant cleavage of the γ-glutamyl bond of γ-glutamyl-L-α-aminobutyrate has occurred without the intermediate action of γ-glutamyl cyclotransferase. A greater accumulation of pyrrolidone carboxylate would be expected if cleavage of the γ-glutamyl bond would have occurred by the cyclotransferase pathway. The hydrolytic action of γ-glutamyl transpeptidase must, therefore be responsible for a significant portion of the cleavage of L-γ-glutamyl-L-α-aminobutyrate, since the hydrolytic action is the only other known way to cause scission of the γ-glutamyl bond (Orlowski and Wilk, 1976).

Evidence for the functioning of the transpeptidation reaction in vivo has also been obtained. Injection of L-phenylalanine or L-methionine into mice led to the accumulation of small quantities of the respective γ-glutamyl derivatives, primarily in the kidney. These γ-glutamyl derivatives were probably formed by the transpeptidation reaction, since these amino acids are not good substrates for γ-glutamyl-cysteine synthetase, the only other known pathway for the formation of γ-glutamyl amino acids (Orlowski and Wilk, 1975).

Additional evidence for the in vivo activity of the

transpeptidase reaction comes from the observation that pyrrolidone carboxylate accumulates in the kidney after the administration of γ-glutamyl derivatives (e.g. γ-glutamylglycylglycine or glutathione) which are not substrates for γ-glutamyl cyclotransferase. Formation of pyrrolidone carboxylate, therefore, requires the intermediate formation of a γ-glutamyl derivative which is a substrate for the cyclotransferase. Such derivatives can be formed by the action of membrane-bound γ-glutamyl transpeptidase in one of two ways (reaction 8 or 9):

L-γ-Glutamylglycylglycine + amino acid ⟶

γ-glutamyl amino acid + glyclyglycine (8)

L-γ-Glutamylglycylglycine + glutathione ⟶

L-γ-glutamyl-γ-glutamylglycylglycine + cysteinylglycine (9)

The γ-glutamyl products formed in this manner are susceptible to cyclization by γ-glutamyl cyclotransferase with formation of pyrrolidone carboxylate (Orlowski and Wilk, 1976). It should be noted that in equation 9, if the γ-glutamyl compound were glutathione instead of γ-glutamylglycylglycine, the reaction would be the equivalent of the autotranspeptidation reaction. Thus, even the autotranspeptidation reaction, which seems to be an artifact of the dissolution of the cellular membrane , may also be functioning in vivo.

The specificity of γ-glutamyl transpeptidase has also been studied. It is generally agreed that all naturally occurring amino acids, with the exception of proline and hydroxyproline, are capable of acting as amino acid acceptors. Small peptides, such as glycylglycine, are among the most active

acceptors. An analysis of the amino acid specificity of γ-glutamyl transpeptidase forms a major portion of this thesis, and a discussion of this topic will be reserved for the "Results" and "Discussion" sections.

With respect to the γ-glutamyl donor, any of several L-γ-glutamyl peptides were capable of substituting for glutathione in the transpeptidation reaction (Hanes et al., 1952). L-γ-Glutamylamides (e.g. L-γ-glutamyl-p-nitroanilide), L-glutamine, glutamic acid γ-ester, D-γ-glutamylamides (e.g. D-γ-glutamyl-p-nitroanilide), D-glutamine and α-methyl glutamine were also found to be capable of acting as γ-glutamyl donors (Orlowski and Meister, 1965).

The mechanism of action of γ-glutamyl transpeptidase was studied in the course of this thesis. Discussion of this topic will be reserved for the "Results" and "Discussion" sections.

It has been suggested the γ-glutamyl transpeptidase and phosphate-independent glutaminase activities are associated with the same enzyme. The association of these two activities with a single enzyme is consistent with the ability of L-glutamine to function as a γ-glutamyl donor in the transpeptidase reaction. In the absence of added amino acids, significant hydrolysis of glutamine (glutaminase activity) is observed. Both transpeptidase and phosphate-independent glutaminase activities appear to be located in the brush border region of the kidney. Both activities, from rat kidney, were found to co-purify, to exhibit parallel developmental patterns and to exhibit coincident mobilities during electrophoresis.

In addition, the glutaminase activity of both γ-glutamyl transpeptidase and phosphate-independent glutaminase were stimulated by maleate and inhibited by amino acids. These results suggest that, with rat kidney, these two glutaminase activities are associated with the same enzyme (Curthoys and Kuhlenschmidt, 1975; Tate and Meister, 1975). Studies with human kidney, however, showed that phosphate-independent glutaminase and γ-glutamyl transpeptidase activity did not co-purify. In addition, γ-glutamyl transpeptidase did not exhibit glutaminase activity. These results suggest that the two activities in human kidney are associated with different enzymes (Miller et al., 1976).

(2) γ-Glutamyl Cyclotransferase

Subsequent to the initial report by Connell and Hanes (1956) regarding γ-glutamyl cyclotransferase, Cliffe and Waley (1958), while studying ophthalmic acid metabolism, reported that calf lens extracts were capable of degrading γ-glutamyl-α-aminobutyrate into pyrrolidone carboxylate and α-aminobutyrate. The decomposition was apparently limited to the dipeptide, since glutamic acid and ophthalmic acid are unaffected by such incubations. In 1967, Kakimoto et al., reported a 14-fold purification of an enzyme capable of converting γ-glutamylglutamine to pyrrolidone carboxylate and free glutamine. This enzyme was named γ-glutamylglutamine lactamase. It was thought that this enzyme was different from the γ-glutamyl lactamase isolated by Connell and Hanes (1956), since the two enzyme preparations displayed differences in specificity towards various γ-glutamyl peptides. The pos-

sibility exists, however, that such specificity differences are artifacts of the purification and storage procedures, and that these enzymes are the same species. This last possibility seems reasonable in view of the fact that the specificity of γ-glutamyl cyclotransferase towards various γ-glutamyl peptides changed during isolation and storage at low temperature. Changes in the electrophoretic behavior of this enzyme were also observed after low temperature storage (Orlowski and Meister, 1973).

The specificity of γ-glutamyl cyclotransferase towards various γ-glutamyl peptides was also studied. Using freshly prepared homogenates of rat tissue, the most active substrates were γ-glutamylglutamine and γ-glutamylmethionine (Orlowski and Meister, 1973). γ-Glutamyl derivatives of branched chain and aromatic amino acids are poor substrates for this enzyme (Orlowski and Karkowsky, 1976). Using a homologous series of γ-glutamyl peptides [$(\gamma\text{-glutamyl})_n$-α-naphthylamide (n=1,2,3)] the most active substrate was γ-glutamyl-γ-glutamyl-α-naphthylamide (Connell and Szewczuk, 1967). γ-Glutamyl-γ-glutamyl-p-nitroanilide was similarly found to be a good substrate for the cyclotransferase (Orlowski et al., 1969). Glutathione was initially reported to be a good substrate for the cyclotransferase reaction (Connell and Hanes, 1956). Later studies have shown, however, that glutathione is not a substrate and that the formation of pyrrolidone carboxylate from glutathione could be explained by the coupled action of γ-glutamyl transpeptidase and γ-glutamyl cyclotransferase acting on glutathione as follows (Orlowski and Meister, 1971c):

2 Glutathione $\xrightarrow{\gamma\text{-glutamyl transpeptidase}}$ γ-glutamyl-glutathione + cysteinylglycine (10a)

γ-Glutamylglutathione $\xrightarrow{\gamma\text{-glutamyl cyclotransferase}}$ pyrrolidone carboxylate + glutathione (10b)

γ-Glutamyl cyclotransferase is widely distributed in animal tissues. High activity of this enzyme is found in kidney, liver, testes and brain (Connell and Szewczuk, 1967; Orlowski and Meister, 1973). Highly purified preparations of this enzyme have been obtained from human and sheep brain (Orlowski et al., 1969), and also pig (Adamson et al., 1971) and rat (Orlowski and Meister, 1973) liver. The enzyme is associated with the soluble fraction of tissue homogenates. Two forms (Isozymes A and B)of this enzyme, having different isoelectric points, were found in all tissues studied (Adamson et al., 1971; Orlowski and Meister, 1973). The specificity and molecular weights of the isozymic forms were approximately the same. The isozymes from pig liver had a molecular weight of approximately 22,000 (Adamson et al., 1971); those from rat liver had a molecular weight of approximately 27,000 (Orlowski and Meister, 1973).

3) Cysteinylglycine Dipeptidase

Cysteinylglycine dipeptidase activity from pig kidney was initially reported to be due to a protein-free ribonucleic acid (Binkley, 1951). The enzymic activity, however, appears to be due to a protein (Semenza, 1957). The association of cysteinylglycinase activity with ribosomes both in swine and rat kidney (Binkley, 1961), and also in Escherichia coli (McCorquodale, 1963), suggest ribosomal contamination

as the reason for the initially reported ribonucleic acid structure. Other proteinases, including leucine amino peptidase, can also hydrolyze cysteinylglycine (Marks, 1970).

(4) ATP-Dependent Pyrrolidone Carboxylate Hydrolase

Two pathways exist for the formation of L-glutamate from glutathione. The first of these pathways is the direct hydrolysis of glutathione by γ-glutamyl transpeptidase (equation 6). A second pathway is the hydrolysis of pyrrolidone carboxylate to L-glutamate, according to equation 11;

$$\text{L-Pyrrolidone carboxylate} + \text{ATP} + 2H_2O \xrightarrow[Mg^{+2}(\text{or } Mn^{+2})]{\text{5-oxoprolinase}} \text{L-glutamate} + \text{ADP} + P_i \qquad (11)$$

L-Pyrrolidone carboxylate is formed from glutathione by the coupled actions of γ-glutamyl transpeptidase and γ-glutamyl cyclotransferase. γ-Glutamyl transpeptidase causes the degradation of glutathione in the presence of an amino acid, with formation of a γ-glutamyl amino acid (equation 4). The resulting γ-glutamyl amino acid then acts as a substrate for γ-glutamyl cyclotransferase to form pyrrolidone carboxylate and a free amino acid (equation 5). The enzyme catalyzing the hydrolysis of pyrrolidone carboxylate is called 5-oxoprolinase.

The first indication that pyrrolidone carboxylate was metabolized, was the observation that intraperitoneal injection of L-[^{14}C] pyrrolidone carboxylate led to rapid $^{14}CO_2$ formation. Eighty-seven percent of the recovered radioactivity, (64% of the total dose) was found in the expired CO_2 (Ramakrishna et al., 1970). Kidney slices were also capable

of converting pyrrolidone carboxylate to CO_2 (Orlowski and Meister, 1970). Rabbit liver preparations converted L- [^{14}C]-pyrrolidone carboxylate to L-[^{14}C] glutamate. The hydrolysis of pyrrolidone carboxylate to glutamate appeared to be enzymatic since it was dependent on ATP and divalent cations (Rush and Starr, 1970) These results taken together suggest that pyrrolidone carboxylate is hydrolyzed to glutamate which is then metabolized to CO_2.

Subsequently, 5-oxoprolinase was partially purified from rat kidney (Van Der Werf et al., 1971). A homogeneous preparation of 5-oxoprolinase was reported to have a molecular weight of 460,000. 5-Oxoprolinase is composed of four subunits, each with a molecular weight of 115,000 (Wendel and Flugge, 1975; Wendel et al., 1975a). Highest 5-oxoprolinase activity in rat was found in kidney, spleen and liver (Van Der Werf et al., 1971). 5-Oxoprolinase activity has also been found in the cilliary body (Ross et al., 1973) and choroid plexus (Okonkwo et al., 1974; Tate et al., 1973). The level of activity of this enzyme in the choroid plexus is higher than in the brain.

4. Oxidation-Reduction Reactions of Glutathione

In addition to the reactions which affect the net concentration of glutathione, there are also enzymic reactions of physiological interest, which utilize the oxidative and reductive properties of the sulfhydryl group of glutathione. These reactions include oxidation, reduction, and transhydrogenation catalyzed respectively by glutathione peroxidase, glutathione reductase and the various glutathione transhydrogenases.

Glutathione peroxidase was first isolated by Mills (1957) as a glutathione-utilizing enzyme which protects hemoglobin from oxidation. The reaction catalyzed by glutathione peroxidase is:

$$2\ \text{Glutathione} + \text{ROOH} \xrightarrow[\text{peroxidase}]{\text{glutathione}} \text{GSSG} + \text{ROH} \qquad (12)$$

In this reaction, GSH reduces hydrogen peroxide (R=H) to water, or a hydroperoxide (R=e.g. t-butyl or linoleic) to an alcohol, and is itself oxidized in the process. Other sulfhydryl compounds can replace glutathione as the reducing agent. Glutathione, however, appears to be the most effective in affecting reduction (Mills, 1959).

Glutathione peroxidase is believed to play a major role in the protection of living cells against oxidative damage. This protective role stems either from the ability of the enzyme to degrade low levels of hydrogen peroxide normally generated in living cells (Cohen and Hochstein, 1963), or its ability to degrade lipid peroxides formed through the oxidation of cellular fatty acids (Neubert et al., 1962; Christophersen, 1966).

Glutathione reductase is the enzyme responsible for maintaining cellular glutathione in the reduced state. In living tissues glutathione occurs primarily in this reduced form. Glutathione reductase catalyzes the following reaction:

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow[\text{Reductase}]{\text{Glutathione}} 2\text{GSH} + \text{NADP}^+ \qquad (13)$$

Glutathione reductase prevents cellular damage by oxidative stress by maintaining cellular sulfhydryl groups in the reduced state. This antioxidant activity of GSH reductase results from two distinct actions of this enzyme. First,

glutathione reductase can directly reduce certain disulfides formed during oxidative stress, such as the mixed disulfides hemoglobin -glutathione (Srivastava and Beutler, 1970) and lens crystallins-glutathione (Srivastava and Beutler, 1973). Second, glutathione reductase can also reduce GSSG to GSH, thereby maintaining a sufficient supply of reduced substrate for the glutathione peroxidase reaction (reaction 12). Since glutathione peroxidase functions to eliminate cellular hydrogen peroxide (or hydroperoxides), the coupling of glutathione peroxidase and glutathione reductase results in the net reduction of the peroxide and the net oxidation of NADPH, with no net change in the oxidation state of glutathione.

The naturally occurring disulfide compounds (e.g. cystine, lipoic acid) can be reduced by two nucleotide-linked pathways (Tietze, 1970). The first of these pathways, represented by reaction 14 is the direct reduction of the disulfide by a reduced nucleotide.

$$R^1SSR^2 + H^+ + NAD(P)H \longrightarrow R^1SH + R^2SH + NAD(P) \quad (14)$$

This reaction is analogous to the reaction catalyzed by glutathione reductase. An alternate path for disulfide reduction is represented by reactions 15 to 17.

$$R^1SSR^2 + GSH \longrightarrow R^1SSG + R^2SH \quad (15)$$

$$R^1SSG + GSH \longrightarrow GSSG + R^1SH \quad (16)$$

$$GSSG + H^+ + NADPH \longrightarrow 2GSH + NADP \quad (17)$$

$$R^1SSR^2 + H^+ + NADPH \longrightarrow R^1SH + R^2SH + NADP \quad \text{:sum} \quad (18)$$

In this case, each half of the disulfide bond is sequentially replaced by reduced glutathione (reactions 15-17), resulting in oxidized glutathione and two free thiol groups.

The oxidized glutathione is subsequently reduced by glutathione reductase. The sum of these last reactions (reaction 18) is equivalent to reaction 14.

The enzymes which catalyze the sequential replacement of disulfide bonds by glutathione are called transhydrogenases.[4] The transhydrogenase activity described by reactions 15 and 16 was first demonstrated by Racker (1953). Such activity is widespread in nature(see review by Orlowski and Karkowsky, 1976).

It is possible to divide transhydrogenase activity on the basis of their disulfide specificity, though such a classification is by no means rigorous. First, there are transhydrogenases which react preferentially with small disulfides such as coenzyme A-glutathione disulfide (Chang and Wilken, 1966). Second, there are transhydrogenases such as glutathione-insulin, transhydrogenase which preferentially utilize proteins or peptides as the disulfide substrate (Katzen and Stetten, 1962).

The physiological function of these transhydrogenases can probably be related to their specificity. The enzymes with specificity towards small disulfides probably maintain these disulfides in their reduced form (Chang and Wilken, 1966). There is probably more than one enzyme capable of reducing small disulfides, as judged by differences in specificity and tissue distributions. The physiological function of transhydrogenases which preferentially utilize proteins or peptides, is probably to either activate or terminate pro-

[4]The Enzyme Commission's name for these enzymes is thiol transferases.

tein or peptide activity (DeLorenzo et al., 1966; Tomizawa, 1962; Katzen and Stetten, 1962; Varandani and Nafz, 1974).

5. Turnover

The first indication for the rapid turnover of glutathione was the observation that [^{15}N]glycine is rapidly incorporated into glutathione of rat and rabbit livers (Waelsch and Rittenberg, 1940, 1941). The calculated half-time of glutathione turnover in rat liver was less than 18 hours. Similar experiments using either DL-[^{15}N]glutamate or [^{15}N]ammonia indicated that the half-time of glutathione turnover in rat and rabbit livers was between two and four hours (Waelsch and Rittenberg, 1942). Since then, the turnover of glutathione in several tissues, including erythrocytes, brain, muscle and kidney, have been reported (Sekura and Meister, 1974).

The first order rate constants for glutathione synthesis vary markedly depending on the tissue studied. Such constants are quite low for human erythrocytes (0.12×10^3 min^{-1}), rabbit muscle (0.11×10^3 min^{-1}) and rat brain (0.17×10^3 min^{-1}), but are much higher in mouse, rat and rabbit liver ($2.9\text{-}5.8 \times 10^3$ min^{-1}) and mouse kidney (24×10^3 min^{-1}). The first order rate constants, however, assume a homogeneous pool for both precursor and products (Sekura and Meister, 1974). That such an assumption is not true for glutathione precursors has been clearly shown for brain, where glutamate metabolism is believed to occur in at least two compartments, or pools (Berl

and Clarke, 1969). Similar heterogeniety of pools seems also to be the case for products. Thus, in kidney, the cortex contains 1.8 fold higher concentrations of soluble-SH groups reactive with DTNB (5,5'-dithiobis(2-nitrobenzoate)) than does the kidney medulla. Since the soluble reactive SH groups are to a large extent glutathione (Boyne and Ellman, 1972), this would suggest that glutathione is not homogeneously distributed between medulla and cortex of the kidney. Similarly, higher concentrations of glutathione are located in the crypt cells, as opposed to the villus tips, of the jejunal mucossa (Cornell and Meister, 1976), indicating that a non-homogeneous distribution of glutathione also occurs in small intestine.

The half-life of glutathione in various tissues is also dependent on the tissue studied. Long half-lives for glutathione were found in rabbit muscle (103 hours) (Henriques et al., 1955), human erythrocytes (96 hours) (Dimant et al., 1955) and rat brain (71 hours)(Douglas and Mortensen, 1956). Short half-lives for glutathione were found in mouse kidney (0.5 hours) (Sekura and Meister, 1974), and in mouse, rabbit and rat livers (2-4 hours) (Sekura and Meister, 1974; Waelsch and Rittenberg, 1942).

It should be noted that turnover does not include glutathione utilization by such processes as oxidation, reduction and transhydrogenation. These processes, in general, yield no net synthesis or degradation of glutathione, and would not be expected to have a significant

effect on net glutathione turnover. It should also be noted that within an organ, a small pool of glutathione with a rapid turnover time could, in performing an important function, account for most of the observed glutathione turnover.

6. Possible Functions of Glutathione

Several functions have been associated with glutathione. These functions include: detoxification of exogenous and endogenous substances, action as a co-enzyme, and maintenance of cellular thiol groups. Glutathione is also the primary physiological substrate of the γ-glutamyl cycle.

a) Detoxification

Glutathione is able to conjugate with certain electrophillic substances, and thereby prepare these substances for eventual excretion (for recent reviews see Boyland and Chasseaud, 1969; Wood, 1970; Boyland, 1971; Chasseaud, 1973; and in Flohe et al., 1974). The reaction of the nucleophillic thiol group of glutathione with the electrophillic substance protects other cellular nucleophillic sites. Conjugation with glutathione limits the lifetime and toxicity of these substances in the body.

Conjugation with glutathione generally occurs enzymically, and is catalyzed by a series of enzymes called glutathione S-transferases (Combes and Stakelum, 1961; Booth et al., 1961). The conjugates formed with glutathione are either excreted unchanged into the bile, or are further

metabolized to mercapturic acids. Mercapturic acids are N-acetyl-S-substituted derivatives of cysteine. They are generally water soluble, and can be excreted into the urine.

The formation of mercapturic acid from the initial glutathione conjugate involves enzymic removal of the γ-glutamyl and glycine moieties of the conjugate, followed by enzymic acetylation. The γ-glutamyl moiety of the conjugate is removed by the action of γ-glutamyl transpeptidase. It is of interest that several S-substituted derivatives of glutathione are more active substrates for γ-glutamyl transpeptidase than glutathione (Tate and Meister, 1974 a).

Several glutathione S-transferase activities have been described. These activities are characterized by the nature of the substrate conjugated to glutathione. Such activities include glutathione S-aryltransferase (Booth et al., 1960; Combes and Stakelum, 1961; Al-Kasseb et al., 1963), glutathione S-alkyltransferase (Johnson, 1966), glutathione S-aralkyltransferase (Suga et al., 1967), glutathione S-epoxide transferase (Boyland and Williams, 1965; Booth et al., 1961), glutathione S-alkenyltransferase (Boyland and Chasseaud, 1967, 1968) and glutathione S-estrogen transferase (Jellinck et al., 1967; Kuss, 1967).

Five enzymes containing glutathione S-transferase activity have been identified in rat liver. These enzymes have been designated glutathione S-transferase A to E. Four of these enzymes, glutathione S-transferase A (Pabst

et al., 1974) B, C (Habig et al., 1974) and E (Fjellstedt et al., 1973) have been purified to homogeniety. Each of these purified enzyme preparations has, to a greater or lesser extent, activity with aryl, alkyl, aralkyl, epoxide and alkenyl substrates.

b) Coenzyme Function

Glutathione can function as a coenzyme in the catalysis of several reactions. These reactions include: hydration, dehydration, isomerization and dehydrochlorination (Jocelyn, 1972).

Glutathione serves as a coenzyme in the hydration and rearrangement of methylglyoxal (pyruvic aldehyde) to lactic acid catalyzed by glyoxalase. Glyoxalase (Neuberg, 1913; Dakin and Dudley, 1913a) is composed of two enzymes: glyoxalase I and glyoxalase II (Crook and Law, 1950; Racker, 1951). Glyoxalase I catalyzes the conversion of methylglyoxal in the presence of glutathione to D-lactoylglutathione. Glyoxalase II catalyzes the hydrolysis of D-lactoylglutathione to D-lactic acid and glutathione. Glyoxalase II isolated from human liver seems to be specific for thiol esters of glutathione, but relatively non-specific for the acyl group (Uotila, 1973). Glyoxalase activity is widely distributed in plants and animals (Dakin and Dudley, 1913b).

The physiological function of the glyoxalase system is not known. Numerous attempts have been made to associate this system with glycolysis. Methylglyoxal, however,

is not an intermediate in the glycolytic pathway. It has also been suggested that the function of glyoxalase is to utilize other naturally occurring keto aldehydes as substrates (Jerzykowski et al., 1973). Glyoxalase may be a promoter (promine) of cell growth, reacting with keto aldehydes that show growth-retarding abilities (retine) (Szent-Gyorgyi et al., 1967).

Glutathione also serves as a coenzyme in the dehydrogenation of formaldehyde, as catalyzed by formaldehyde dehydrogenase. This enzyme reversibly catalyzes the dehydrogenation of formaldehyde, in the presence of NAD, to S-formylglutathione. S-formylglutathione is subsequently hydrolyzed by a second enzyme, S-formylglutathione hydrolase, to formic acid and glutathione (Uotila and Koivusalo, 1974a). Formaldehyde dehydrogenase has a specific requirement for glutathione as a cofactor, which cannot be satisfied by other sulfhydryl compounds (Strittmatter and Ball, 1955). Formaldehyde dehydrogenase has been found in every tissue examined (Uotila and Koivusalo, 1974b).

Glutathione is a necessary cofactor in the dehydrochlorination of dichlorodiphenyltrichloroethane (DDT) to dichloro-2, 2-bis (p-chlorophenyl)-ethylene (DDE), as catalyzed by DDT dehydrochlorinase (Sternburg et al., 1954). Other thiol compounds, with the exception of cysteinylglycine, are ineffective in DDT dehydrochlorination. Cysteinylglycine can serve as a cofactor, but is only 60% as effective as glutathione (Lipke and Kearns, 1959).

DDT dehydrochlorinase activity in flies has been correlated with their degree of resistance to DDT (Sternburg et al., 1954). Glutathione appears to stabilize a tetrameric form of the enzyme. Smaller aggregates of this enzyme are not as active in affecting dehydrochlorination (DiNamarca et al., 1969, 1971).

Glutathione also serves as coenzyme in the cis-trans isomerization of maleylacetoacetate to fumarylacetoacetate, as catalyzed by maleylacetoacetate isomerase (Edwards and Knox, 1956). It also serves as a coenzyme in the isomerization of maleylpyruvate to fumarylpyruvate, as catalyzed by maleylpyruvyl isomerase (Lack, 1961). Maleylacetoacetate and maleylpyruvate are degradative products of homogentisic acid and gentisic acid, respectively. The isomerizing enzymes require glutathione for activity; other thiol compounds cannot substitute for glutathione (Selzer, 1972).

c) Maintenance of Cellular Thiol Groups

Glutathione also acts to maintain the thiol-disulfide status of cells. The thiol-disulfide status of a given cell is defined as the total pattern of thiols and disulfides within the cell (Kosower et al., 1972). There are many agents that have the potential to introduce changes in the cellular thiol-disulfide status. These agents include peroxides, radiation exposure, hyperbaric oxygen, airborne oxidants such as ozone and nitrogen dioxide, and drugs such as primoquine and nitrofurantoin. Such changes

are transitory as long as sufficient glutathione reductase and NADPH are available. Glutathione peroxidase and the glutathione-dependent transhydrogenases can also play an important role in mitigating the effects of oxidation stress. Glutathione peroxidase can divert the oxidation stress from key sulhydryl groups by reacting with oxidants (e.g. peroxides) before these oxidants react with cellular sulfhydryl groups. The glutathione-dependent transhydrogenases are capable of regenerating these sulfhydryl groups, should protection by the peroxidase prove insufficient.

The importance of the reduced-oxidized glutathione status in cellular function can be determined by alteration of this status. Rapid and stoichiometric oxidation of glutathione can be accomplished by the addition of diamide, or other similar thiol-oxidizing agents (Kosower and Kosower, 1969). Among the various cellular sulhydryl groups, diamide preferentially reacts with glutathione (Kosower et al., 1972).

The consequences of altering the thiol-disulfide status of glutathione include inhibition of: the thiol-dependent steps in protein synthesis, insect feeding behavior, muscle contraction, ionic transport in rabbit lens, growth of Escherichia coli, development of sea urchin eggs, the feeding response of hydra (Kosower et al., 1972 and references cited therein), and amino acid transport (Hewitt et al., 1974). Diamide treatment also produces changes in the neurophysiological characteristics of frog nerve-

muscle preparations (Werman et al., 1971). The physiological changes induced by diamide are probably mediated by changes in the thiol-disulfide status of enzymes secondary to direct oxidation of glutathione.

d) The γ-Glutamyl Cycle

The enzymes involved in the synthesis and degradation of glutathione have been linked together to form a cycle (figure 1). This cycle called the "γ-glutamyl cycle" was first proposed by Orlowski and Meister in 1970 as a possible enzymic basis for amino acid transport in certain tissues.

The cycle is postulated to function as follows. An amino acid binds to γ-glutamyl transpeptidase at the external membrane surface. This amino acid serves as an acceptor in the transpeptidation reaction with intracellular glutathione. The products of this reaction are cysteinylglycine and a γ-glutamyl amino acid. Cysteinylglycine is hydrolyzed intracellularly by cysteinylglycine dipeptidase to form cysteine and glycine. The γ-glutamyl amino acid is hydrolyzed by one of two pathways to yield free glutamate and free amino acid. One of these pathways would be the direct hydrolysis of the γ-glutamyl amino acid, possibly by the hydrolytic action (reaction 6) of γ-glutamyl transpeptidase. The second pathway would be the coupled reaction of γ-glutamyl cyclotransferase and the ATP-dependent pyrrolidone carboxylate hydrolase on the γ-glutamyl amino acid. Pyrrolidone carboxylate would

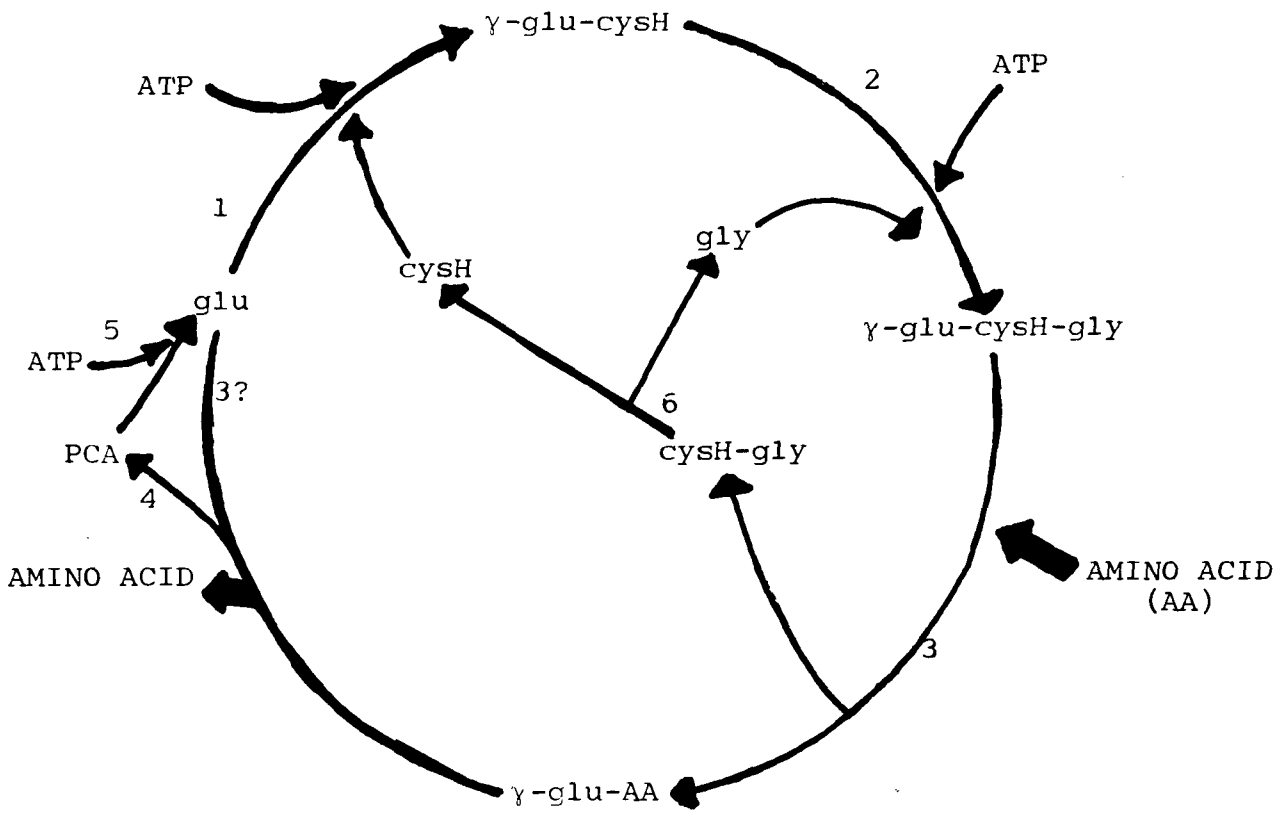


Fig. 1. The γ-glutamyl cycle. 1, γ-glutamylcysteine synthetase; 2, glutathione synthetase; 3, γ-glutamyl transpeptidase; 4, γ-glutamyl cyclotransferase; 5, ATP-dependent pyrrolidone carboxylate hydrolase; 6, peptidase. PCA = pyrrolidone carboxylic acid; AA = amino acid. (Adapted from Orlowski and Meister, 1970).

then be an obligatory intermediate only in the second hydrolytic pathway. The amino acid, as well as glutamate, are released intracellularly.

The glutamate which was formed as a result of the hydrolysis of the γ-glutamyl bond, as well as the cysteine and glycine which are formed by the action of cysteinylglycinase, are utilized as substrates for γ-glutamylcysteine synthetase and glutathione synthetase to regenerate glutathione.

The γ-glutamyl cycle contains all the steps necessary for the functioning of a transport system. These steps are recognition, translocation, release and recovery (Pardee, 1968). A transport system recognizes and binds the molecule to be transported. The bound molecule is then translocated from the extracellular surface to the intracellular surface, where it is subsequently released. The transport system must then undergo a recovery phase, in order that the process can be repeated.

With respect to the γ-glutamyl cycle, the binding of the amino acid to γ-glutamyl transpeptidase is the recognition step. Transpeptidation of the amino acid with glutathione with the formation of a γ-glutamyl amino acid and release of cysteinylglycine, is the translocation step. Hydrolysis of the γ-glutamyl amino acid is the release step and finally, the resynthesis of glutathione is the recovery phase of the transport process. Energy input, a requirement for systems engaged in active transport, occurs dur-

ing the resynthesis of glutathione, and also possibly during the hydrolysis of pyrrolidone carboxylate to glutamate.

The evidence for the functioning of the γ-glutamyl cycle in amino acid transport is, at present, mostly circumstantial. This evidence will be briefly reviewed here (see Orlowski and Meister, 1970; Meister, 1973, 1974b; Orlowski and Karkowsky, 1976 for more extensive reviews).

γ-Glutamyl transpeptidase is a membrane-bound enzyme, requiring detergent treatment for solublization. The enzyme has been histochemically located at sites where high amino acid transport activity occurs. These results are consistent with a recognition site role for this enzyme.

The enzymes of the γ-glutamyl cycle are found in many tissues, including kidney (Orlowski and Meister, 1970; Orlowski and Wilk, 1975), brain , choroid plexus (Tate et al., 1973; Okonkwo et al., 1974), liver (Orlowski and Wilk, 1975), jejunal mucossa (Cornell and Meister, 1976; Ross et al., 1973), cilliary body, lens (with the exception of 5-oxoprolinase) (Ross et al., 1973) and erythrocytes (Palekar et al., 1974). Tissues such as kidney, choroid plexus, cilliary body and small intestine, which are vigorously engaged in amino acid transport, can be differentiated from surrounding tissue by the higher activity of γ-glutamyl transpeptidase contained within them. The findings that the enzymes of the γ-glutamyl cycle and that especially high γ-glutamyl transpeptidase activity are found in tissues engaged in active transport is consistent with

the postulated role of the γ-glutamyl cycle in amino acid transport.

Extremely rapid turnover of glutathione occurs in kidney, but not in tissues such as erythrocytes, muscle or brain. A rapid glutathione turnover rate is consistent with the cycle's postulated rapid degradation and resynthesis of glutathione in the process of amino acid reabsorption in the kidney. Low glutathione turnover rates in erythrocytes, muscle and brain are consistent with the relatively low amino acid transport activity in these tissues.

Several intermediates of the γ-glutamyl cycle, such as γ-glutamyl peptides and pyrrolidone carboxylate, have been found in tissues. The first γ-glutamyl peptides discovered in mammalian tissues were ophthalmic (Waley, 1956). and norophthalmic acid (Waley, 1957) isolated from calf lens. In addition, γ-glutamyl derivatives of valine, leucine, isoleucine, (Buchanan et al., 1962), and ornithine (Lou, 1975) have been found in normal human urine.

Several γ-glutamyl compounds have been found in brain. γ-Glutamylglutamate and γ-glutamylglutamine were the first of several γ-glutamyl peptides isolated from bovine brain (Kakimoto et al., 1964). Six additional γ-glutamyl peptides, γ-glutamylglycine (Kanazawa et al., 1965a), γ-glutamylserine, γ-glutamylalanine and γ-glutamylvaline (Kanazawa et al., 1965b), were isolated from bovine brain. In addition, a substance containing glutamyl, α-aminobutyryl and glycyl residues, suggestive of ophthalmic acid, was also

isolated (Sano, 1970). γ-Glutamyl peptides have also been isolated from monkey brain, including two peptides, γ-glutamylisoleucine and norophthalmic acid, not reported in bovine brain (Reichelt, 1970).

The concentration of these peptides in bovine brain ranges from approximately 30nmoles/gm wet tissue for γ-glutamylglutamine and γ-glutamylglutamate, to about 0.5 nmoles for γ-glutamylvaline (Sano et al., 1966). Relatively high concentrations of γ-glutamylglutamine were found in the intestine, kidney, liver and brain of several species (Kanazawa and Sano, 1967).

Two pathways for the origin of these γ-glutamyl peptides have been suggested. The peptides may be synthesized directly by γ-glutamylcysteine synthetase from the free amino acid and glutamate. These peptides may also be formed by transpeptidation, catalyzed by γ-glutamyl transpeptidase between glutathione and amino acids (Sano, 1970; Orlowski and Wilk, 1975).

Evidence for direct formation of γ-glutamyl amino acids by γ-glutamylcysteine synthetase in vivo comes from the fact that intraperitoneal injection of either DL-C-allylglycine or L-α-aminobutyrate leads to the formation of the corresponding γ-glutamyl compounds, predominantly in the liver. Since these amino acids are good substrates for γ-glutamylcysteine synthetase, and since liver has good γ-glutamylcysteine synthetase activity, but poor γ-glutamyl transpeptidase activity, it seems reasonable, therefore,

that formation of the γ-glutamyl peptides were enzymatically synthesized by the synthetase reaction (Orlowski and Wilk, 1975).

Evidence for the formation of γ-glutamyl peptides by the transpeptidation pathway comes from similar experiments to those described above. Intraperitoneal injection of either phenylalanine or methionine led to only a small accumulation of the corresponding γ-glutamyl amino acids, predominantly in the kidney. Since these amino acids are not good substrates for γ-glutamylcysteine synthetase, but are good substrates for γ-glutamyl transpeptidase, it seems that the accumulation of these γ-glutamyl amino acids is a result of the transpeptidation reaction (Orowski and Wilk, 1975).

Pyrrolidone carboxylate has been found as a natural constituent in the organs of guinea pig, rabbit, rat (Wilk and Orlowski, 1973), and mouse (Van Der Werf et al., 1974; Wilk and Orlowski, 1975). It has also been found in mouse urine (Van Der Werf et al., 1974) and human urine and cerebrospinal fluid (Wilk and Orlowski, 1975). The levels of pyrrolidone carboxylate in mouse tissue were approximately 55 nmole/gm tissue (Orlowski and Wilk, 1975) or 30 nmole/gm tissue (Van Der Werf et al., 1974). Based on enzymic degradative studies of pyrrolidone carboxylate virtually all of this compound in mouse tissues (Van Der Werf et al., 1974), human cerebrospinal fluid, and 40% of that found in human urine (Wilk and Orlowski, 1973) is

of the L-configuration.

2-Imidazolidone-4-carboxylate, a structural analog of pyrrolidone carboxylate is a competitive inhibitor of 5-oxoprolinase (Van Der Werf et al., 1973). Simultaneous administration of pyrrolidone carboxylate and 2-imidazolidone-4-carboxylate results in inhibition of CO_2 formation that normally occurs as a result of pyrrolidone carboxylate metabolism. Administration of the inhibitor to mice causes a 2-3 fold increase both in the concentration of pyrrolidone carboxylate in mouse tissue, and also in its excretion into the urine. Concurrent administration of both the inhibitor and methionine further increased pyrrolidone carboxylate concentrations in tissue by a factor of approximately 1.8. Administration of methionine alone did not result in increased tissue pyrrolidone carboxylate concentrations. Other amino acids such as glutamate, glutamine and valine, when administered in combination with the inhibitor, also significantly raised tissue concentrations of pyrrolidone carboxylate in kidney (Van Der Werf et al., 1974). These results suggest that pyrrolidone carboxylate is a natural cellular constituent which is continuously being formed. These results also suggest that there are interactions between amino acids and pyrrolidone carboxylate formation.

Several inherited disorders of glutathione metabolism have been described during the past several years which show the relationship between amino acid metabolism

and the enzymes of the γ-glutamyl cycle. These diseases include pyroglutamic aciduria, and γ-glutamylcysteine synthetase deficiency.

In 1970 Jellum et al., in Norway, described a new inborn error of metabolism in a 19 year old mentally retarded patient and called it pyroglutamic aciduria. The patient excreted about 30 gm daily of pyrrolidone carboxylate in the urine, and had markedly elevated (>500 times normal) levels of pyrrolidone carboxylate in the plasma. It was first thought that the patient had a deficiency of ATP-dependent pyrrolidone carboxylate hydrolase limited to the kidney, and was therefore unable to convert pyrrolidone carboxylate into glutamate in the kidney (Eldjarn et al., 1972). Subsequent studies, however, have shown that the excessive excretion of pyrrolidone carboxylate is not due to a block in its metabolism. Instead, the defect appeared to be due to its increased formation (Eldjarn et al., 1973).

Subsequently, two sisters, one a new-born, the other three years old, with pyroglutamic aciduria were found in Sweden (Hagenfeldt et al., 1974; Larsson et al., 1974). Increased pyrrolidone carboxylate formation as opposed to a block in its metabolism, was also indicated with these patients (Larsson et al., 1974).

Both Swedish patients, in addition, showed signs of increased hemolysis, and had a markedly reduced level of glutathione in their erythrocytes (Larsson et al., 1974).

Enzyme studies on erythrocytes, placenta, and a culture of skin fibroblasts showed a markedly reduced activity of glutathione synthetase. The activities of γ-glutamylcysteine synthetase,γ-glutamyl cyclotransferase, and pyrrolidone carboxylate hydrolase were all normal (Wellner et al., 1974). The Norwegian patient was also subsequently shown to have a decreased glutathione synthetase activity (Marstein et al., 1976).

The discovery of glutathione synthetase deficiency in patients with pyroglutamic aciduria seemed to provide an adequate explanation for the metabolic disorder. Thus, in the absence of glutathione synthetase, γ-glutamylcysteine is apparently converted into pyrrolidone carboxylate and free cysteine by the action of γ-glutamyl cyclotransferase. The amount of pyrrolidone carboxylate formed exceeds the capacity of pyrrolidone carboxylate hydrolase to convert it into glutamate. The result is an accumulation of pyrrolidone carboxylate and its excretion in the urine. It is likely that more γ-glutamylcysteine is synthesized than normally, due to the low concentration of glutathione in tissues. The low levels of glutathione decrease its feed-back inhibition on γ-glutamylcysteine synthetase.

The amino acid metabolism in the Norwegian patient was studied (Eldjarn et al., 1972; Marstein et al., 1976). The patient had elevated proline levels in the plasma (approximately three times normal). The levels of the other amino acids both in plasma and urine were approxi-

mately normal. An infusion of a mixture of amino acids to this patient caused a massive amino aciduria. This patient showed a marked elevation of amino acids in erythrocytes. In addition, the erythrocytes of this patient were found to contain methionine sulfoxide, an amino acid not found in normal controls. Deproteinized and hydrolyzed preparations of the erythrocytes of this patient and of controls were analyzed for unexplained increases in amino acids. Marked elevations of amino acids, particularly glutamate and glycine, were found. Unexplained amino acid increases were defined as amino acids produced by hydrolysis, in excess of those accounted for by the hydrolysis of the normal cellular constituents such as glutathione and glutamine. This parameter is believed to be a measure of the concentration of small soluble peptides. These results suggest that the patient's erythrocytes contained a high concentration of small peptides. Whether these peptides were γ-glutamyl peptides was not determined.

In addition to glutathione synthetase deficiency, a second enzymic defect related to the enzymes of the γ-glutamyl cycle has been described. Two patients, a sister (35 years old) and a brother (36 years old), have been found who have less than 5% of the normal level of glutathione in their erythrocytes. Biochemical studies have shown a deficiency of γ-glutamylcysteine synthetase (Konrad et al., 1972). A reduction of glutathione, although of a lower degree, was also observed in leukocytes and

skeletal muscles. Studies of the amino acids of the urine showed the presence of amino aciduria. The excretion of both neutral (alanine, valine, asparagine, glutamine, cystine and threonine) and basic (arginine and lysine) amino acids was increased (Richards et al., 1974).

The fact that both inherited disorders of glutathione metabolism (pyroglutamic aciduria and γ-glutamylcysteine synthetase deficiency) are associated with aberrations in amino acid metabolism, is consistent with the involvement of the γ-glutamyl cycle in amino acid transport. The lack of aminoaciduria in the patients suffering from pyroglutamic aciduria was explained by the ability of γ-glutamylcysteine to substitute for glutathione in the transpeptidation reaction with amino acids (Wellner et al., 1974). No γ-glutamylcysteine, however, was found in the erythrocytes of the Norweigen patient. It was suggested that the rapid rate of formation of this compound may be compensated for by an equally rapid rate of its degradation by the cyclotransferase or transpeptidase reactions (Marstein et al., 1976). It is also possible that γ-glutamyl peptides other than γ-glutamylcysteine could substitute for glutathione in the transpeptidation reaction. The γ-glutamyl peptides could be formed either by transpeptidation of the amino acid with γ-glutamylcysteine, or by direct synthesis from glutamate and the amino acid by the now uninhibited γ-glutamylcysteine synthetase.

The high concentration of small soluble peptides found in the erythrocytes of this patient is consistent with the possibility that peptides other than glutathione may function as alternate substrates in the transpeptidase reaction.

Certain limitations must be placed on the hypothesis that the γ-glutamyl cycle is involved in amino acid transport. For example, several distinct transport systems specific for certain groups of amino acids have been described. At the present time, more than one transport system must be postulated to account for all the in vivo and in vitro experimental observations concerning amino acid transport.

The γ-glutamyl cycle, therefore, may be one of several transport systems operating in vivo. To illustrate the point that the cycle cannot account for all amino acid transport is the well documented transport of α-alkyl amino acids, such as cycloleucine and α-aminoisobutyric acid (Christensen et al., 1956). Transport of these amino acids cannot be accounted for by the activity of the γ-glutamyl cycle, since these amino acids are not substrates for γ-glutamyl transpeptidase (Tate and Meister, 1974; Karkowsky et al., 1976; see also "Results" of this thesis).

Furthermore, many cells have little, if any γ-glutamyl transpeptidase, and yet are capable of transporting amino acids. It, therefore, seems unlikely that the cycle functions in amino acid transport in all tissues.

Amino acid transport by the γ-glutamyl cycle would thus be restricted to sites that display high activity of all the enzymes of the cycle (Orlowski, 1963).

The discovery of a patient exhibiting glutathionuria and glutathionemia emphasized that amino acid transport by the γ-glutamyl cycle must be restricted to certain tissues. The patient, a moderately retarded 33 year old white male, had an approximately 6-fold elevation of glutathione in plasma, and a 1500-fold elevation of glutathione in the urine, when compared to normal controls. The patient, in addition, had low levels (approximately 5% normal) of serum γ-glutamyl transpeptidase activity (Goodman et al., 1971). No detectable γ-glutamyl transpeptidase activity was found in lysates of cultured fibroblasts of this patient (Schulman et al., 1975). Amino acid transport in these fibroblasts, however, was normal (Pellefigue et al., 1976).

It was suggested that this patient may have a generalized γ-glutamyl transpeptidase deficiency (Goodman et al., 1971). It seems, however, at present that there is insufficient evidence to substantiate this claim. The deficiency of γ-glutamyl transpeptidase may be limited to tissues such as fibroblasts, liver (the origin of most serum γ-glutamyl transpeptidase) and other tissues not believed to be active in amino acid transport via the γ-glutamyl cycle. Thus normal transport of amino acids would be expected even in the absence of a functioning

cycle. Whether this deficiency of γ-glutamyl transpeptidase in this patient is extended to such organs as kidney, intestinal mucossa etc., which are believed to be active in amino acid transport by the γ-glutamyl cycle, is not known. An absence of γ-glutamyl transpeptidase from these organs would be difficult to reconcile with a role of the enzyme and the γ-glutamyl cycle in amino acid transport. In this case, a difficulty arises from the fact that amino acid concentrations in serum and renal clearance of amino acids in the patient showed no marked abnormalities.

II BACKGROUND OF THIS RESEARCH

In view of the important role γ-glutamyl transpeptidase plays in the turnover of glutathione, and the pivotal role it plays in several of glutathione's postulated functions (i.e. mercapturic acid formation, amino acid transport), we began studies on the kinetics and the mechanism of action of this enzyme. Such studies were hampered by the fact that this enzyme catalyzes three concurrent reactions: hydrolysis (reaction 6), transpeptidation (reaction 4) and autotranspeptidation (reaction 7). In order to lessen the number of on-going reactions occurring in the presence of this enzyme we synthesized the novel substrate, L-α-methyl-γ-glutamyl-L-α-aminobutyrate (Figure 2). This substrate was synthesized enzymically by the action of γ-glutamylcysteine synthetase according to the following reaction:

$$\text{DL-}\alpha\text{-methyl glutamate} + \text{L-}\alpha\text{-aminobutyrate} + \text{ATP} \xrightarrow{Mg^{+2}}$$

$$\text{L-}\alpha\text{-methyl-}\gamma\text{-glutamyl-L-}\alpha\text{-aminobutyrate} + \text{ADP} + P_i \quad (19)$$

The presence of the methyl group on the α-carbon of the γ-glutamyl donor eliminates the autotranspeptidation reaction, so that in the absence of an amino acid, only hydrolysis occurs (reaction 20).

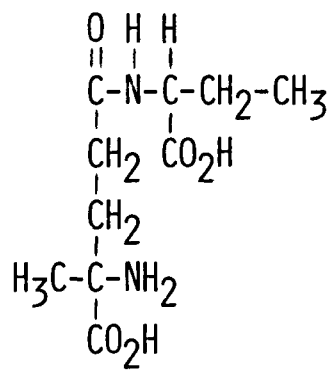


Fig. 2. STRUCTURE OF
L-α-METHYL-γ-GLUTAMYL-L-α-AMINOBUTYRATE

L-α-methyl-γ-glutamyl-L-α-aminobutyrate + H_2O ⟶

L-α-methyl glutamate + L-α-aminobutyrate (20)

In the presence of an amino acid, there is also transfer of the α-methyl-γ-glutamyl group to the amino acid (reaction 21)

L-α-methyl-γ-glutamyl-L-α-aminobutyrate + amino acid ⟶

L-α-methyl-γ-glutamyl amino acid + L-α-aminobutyrate (21)

The elimination of the autotranspeptidation reaction facilitated the study of the kinetics and mechanism of action of γ-glutamyl transpeptidase. These studies form the starting point for this thesis.

III EXPERIMENTAL PROCEDURES

A. Materials

Iodoacetamide, iodoacetic acid, 5,5'-dithiobis(2-nitrobenzoate), p-mercuribenzoate, EDTA, maleic acid, fumaric acid, pyruvic acid, phthalic acid, L-glutamic acid-O-benzyl ester, glutathione, 2-mercaptoethanol, dithiothreitol, L-glutamic acid decarboxylase (Escherichia coli) and all amino acids were obtained from Sigma Chemical Company. [U-^{14}C]-labeled: glycine, L-alanine, L-glutamate, L-cystine and L-methionine were obtained from New England Nuclear Corporation. [U-^{14}C]-labeled: L-leucine, L-glutamine, L-serine, L-valine, L-phenylalanine, L-tyrosine, L-histidine and L-arginine were obtained from Amersham Searle. DL-[1-^{14}C]-α-aminobutyrate was obtained from Schwarz Mann. The γ-glutamyl compounds L-γ-glutamyl-p-nitroanilide, L-γ-glutamyl-α-aminobutyrate, N^2-γ-L-glutamyl-N^6-benzyloxycarbonyl-L-lysine and D-γ-glutamyl-p-nitroanilide have previously been described (Orlowski and Meister, 1965, 1971b, 1973).

1. Synthesis of L-α-Methyl-γ-glutamyl-L-α-aminobutyrate

This compound was synthesized enzymatically from DL-α-methyl glutamate and L-α-aminobutyrate using purified rat kidney γ-glutamylcysteine synthetase (Orlowski and Meister, 1971a). The incubation mixture contained DL-α-methyl glutamate (1.7g;

10 mmol), L-α-aminobutyrate (0.57g; 5.5 mmol), ATP (3.33g; 5.5 mmol), $MgCl_2.6H_2O$ (2.2g; 11 mmol), dithiothreitol (31 mg; 0.2 mmol), 60 ml of 0.2M Tris HCl (pH 6.2). The pH of the solution was adjusted to 8.2 with 5 M sodium hydroxide. Two thousand units of γ-glutamylcysteine synthetase were added and the volume was completed to 100 ml. This mixture was incubated to 37°. The reaction was followed by measuring the formation of inorganic phosphate using the method of Fiske and SubbaRow (1929). When additional phosphate production ceased, the mixture was applied to a Dowex-1 acetate (AG 1-X4; 200-400 mesh) column (35 x 4 cm). The column was washed with 3 liters of H_2O and then eluted in sequence with 2.5 liters of 0.05 M acetic acid, 4 liters of 0.15 M acetic acid, and 4 liters of 0.2 M acetic acid. Fractions of 20 ml were collected and tested for ninhydrin reactive material. L-α-methyl-γ-glutamyl-L-α-aminobutyrate emerged from the column with 0.15 M acetic acid. The fractions containing the product were combined and concentrated by flash evaporation at 40° to a small volume, and then lyophilized. The dried residue was crystallized from ethanol-H_2O. The yield was 0.95g (3.9mmol; 78%). L-α-Methyl-γ-glutamyl-L-α-aminobutyrate appeared as a single ninhydrin positive spot on paper chromatography. The R_f value was 0.56 in solvent A (see Methods) and 0.35 in solvent B; $[\alpha]_D^{20}$ -20.8° (4% in H_2O).

$C_{10}H_{18}N_2O_5 \cdot H_2O$

Calculated: C 45.44, H 7.62, N 10.59

Found: C 45.42, H 7.54, N 10.70

The assignment of the L-configuration to the synthesized α-methyl-γ-glutamyl-L-α-aminobutyrate is based on the specificity of rat kidney γ-glutamylcysteine synthetase for the L-isomer of glutamate (Orlowski and Meister, 1971b). Such an assignment is also supported by measurement of the optical rotation of the remaining unreacted α-methyl glutamate. The α-methyl glutamate was isolated by ion exchange chromatography on a Dowex-1 acetate column and recrystalized twice from a mixture of isopropanol and water. Measurement of the optical rotation of the product yielded an $[\alpha]_D^{20}$ of -10° (4% in 6M HCl), which is close to the value reported by Izumi et al., (1965) for D-α-methyl glutamate. A negative rotation for the D-isomer of α-methyl glutamate was also reported by Kagan et al. (1965).

2. Synthesis of D-γ-Glutamyl-L-phenylalanine

This compound was prepared by a modification (Orlowski and Meister, 1965) of the general procedure of King and Kidd (1949). Phthaloyl-D-glutamic anhydride (2.8g, 0.01 mol) was suspended in 10 ml of glacial acetic acid, and L-phenylalanine (1.74g, 0.01 mol) was added. The mixture was heated to 90° for 15 minutes, which caused the dissolution of the solid material. The resultant solution was then allowed to cool to room temperature, and evaporated under reduced

pressure at 25° to yield an oil. Small quantities of ethanol were added to dissolve the oil, and this solution was evaporated to dryness. The process of dissolving in ethanol and evaporating to dryness was repeated several times to remove the remaining traces of acetic acid. The oil was then dissolved in 20 ml of methanol. Hydrazine hydrate (95%, 0.021 mol) and triethylamine (0.01 mol) were then added, and the solution was allowed to stand at ambient temperature for 48 hours. Methanol was then removed by flash evaporation at room temperature. The residual white solid was suspended in water (40 ml), and the pH of the suspension was adjusted to 3.0 with 1 N HCl. After standing for one hour at room temperature, the mixture was filtered. The pH of the filtrate was adjusted to 5.0 with 1 N NaOH, and the filtrate was applied to a Dowex-1 acetate (Ag 1-X4, 200 to 400 mesh) column (30 x 2.5 cm). The column was washed with 1 liter of water, and eluted, in sequence, with 1.5 liters of 0.05, 0.1, 0.2 and 1 N acetic acid. The third ninhydrin positive compound eluted from the column was D-γ-glutamyl-L-phenylalanine (0.464 g, 1.54 mmol). Acid hydrolysis of this compound (10 mg) in 6 N HCl (1 ml) for 24 hours yielded equimolar amounts of glutamate and phenylalanine as determined by amino acid analysis.

The optical purity of D-γ-glutamyl-L-phenylalanine was determined by treating a similar hydrolysate as the one described above, with L-glutamic acid decarboxylase, and analyzing for α-aminobutyrate on an amino acid analyzer.

Formation of γ-aminobutyrate results from the action of L-glutamic acid decarboxylase on L-glutamic acid. The L-glutamic acid is itself formed by hydrolysis of contaminating quantities of L-γ-glutamyl-L-phenylalanine. The hydrolytic sample was evaporated to dryness and resuspended in acetate buffer (0.3 ml, 0.05 M, pH 5). L-Glutamic acid decarboxylase (Escherichia coli; 0.2 ml, 1 unit) was added, and the samples were incubated at 37° for 3 hours. The suspension was then evaporated to dryness, and analyzed on an amino acid analyzer. The crude D-γ-glutamyl-L-phenylalanine contained approximately 5% of the L-isomer as determined by this method. The D-compound was then further purified by two recrystalizations from methanol-water.

3. Synthesis of L-α-Methyl-γ-glutamyl-L-[1-^{14}C]-α-aminobutyrate

The preparation of this compound is similar to the nonradioactive compound. The incubation mixture contained DL-α-methyl glutamate (7.1 μmol), $MgCl_2 \cdot 6H_2O$ (8.9 μmol), ATP (4.5 μmol), DL-[1-^{14}C]-α-aminobutyrate (5.0 μmol; 50 μCi), Tris-HCl (pH 8.2; 50 μmol) and dithiothreitol (1.5 μmol) in 0.5 ml of H_2O. The pH was adjusted to 8.2 with 5 M sodium hydroxide, 20 units of γ-glutamylcysteine synthetase were added and the mixture was incubated at 37° for 3 h. The mixture was applied to the top of a Dowex-1 acetate (AG 1-X4; 200-400 mesh) column (5 x 0.5 cm). The column was washed with 3 ml of 0.05 N acetic acid and all the unreacted DL-[^{14}C]-α-aminobutyrate was eluted. The product was subsequently eluted with 8 ml of 1.5 N acetic acid. This frac-

tion contained both the unreacted DL-α-methyl glutamate and L-α-methyl-γ-glutamyl-L-[^{14}C]-α-aminobutyrate. No attempt was made to separate these compounds. Aliquots of this last fraction containing approximately 2.5 x 10^6 cpm were lyophilized and stored at -20°.

4. Enzyme

The γ-glutamyl transpeptidase used in these experiments was prepared in this laboratory from sheep kidney cortex (Zelazo and Orlowski, 1976), using a modification of a previously described procedure (Orlowski and Meister, 1965). The enzyme was homogeneous as judged by polyacrylamide gel electrophoresis at several pH values. The specific activity of the enzyme when determined with γ-glutamyl-p-nitroanilide was 510 μmol of product formed per minute per mg enzyme. In the presence of 20 mM glycylglycine the specific activity increased to 1,255 μmol of product formed per minute per mg enzyme.

B. Methods

1. Miscellaneous

All amino acid analyses were carried out on a Technicon TSM amino acid auto-analyzer using a lithium citrate buffer system. Samples were dissolved in 0.2 ml of lithium citrate buffer (pH 2), and 25 μl samples were taken for analysis. Norleucine served as the internal standard.

Radioactivity unless otherwise stated, was determined in a Nuclear of Chicago Iso Cap 300 scintillation counter. An aliquot (usually 1 ml) of the radioactive solution was mixed with 10 ml of Bray's solution (1960) and counted.

The activity of the enzyme with γ-glutamyl-p-nitroanilide was measured by the procedure described by Orlowski and Meister (1965).

Paper chromatography was performed in two solvent systems. Solvent A consisted of 1-butanol/acetic acid/H_2O (60/15/25); Solvent B consisted of 1-butanol/pyridine/H_2O (1/1/1).

Kinetic measurements were carried out at 37°. The nomenclature of Cleland (1963) was used for the description of the kinetic mechanisms. The nomenclature of Folk (1969) was used for the description of the kinetic constants. Any new rate equations described here were derived by assuming steady state conditions. The measurements were carried out under initial velocity conditions in the absence of added products. Unless otherwise specified all kinetic data were expressed as conventional double-reciprocal plots (Lineweaver and Burk, 1934). All initial velocity data were weighed equally. The concentration of amino acids used to calculate the reactivity ratio was the **amino acid's average concentration** during the course of the incubation. This value did not deviate from the initial value of the amino acid by more than 10%.

2. Measurement of L-α-Methyl-γ-glutamyl-L-α-aminobutyrate Hydrolysis

The hydrolytic activity of the enzyme toward α-methyl-γ-glutamyl-L-α-aminobutyrate was determined by measuring the release of L-α-amino[^{14}C]butyrate from the radioactive substrate. The reaction mixture (final volume, 0.2 ml) con-

tained L-α-methyl-γ-glutamyl-L-α-amino[^{14}C]butyrate, 0.1 M Tris-HCl buffer (pH 9.0), and enzyme. Incubations were carried out at 37^{o} and the reactions were terminated by adding 25 µl of 1.5 N acetic acid. The entire contents of the tubes were applied to the top of a Dowex-1 acetate (Ag 1-X4; 200 to 400 mesh) column (2.5 x 0.5 cm). The tubes were rinsed with 0.65 ml and 0.75 ml of 0.05 N acetic acid which were also applied to the column. The eluent containing L-α-amino[^{14}C]-butyrate was collected in a scintillation vial and counted.

3. Measurement of Transpeptidation

The incubation mixtures used to measure the rate of transpeptidation were the same as described for the hydrolytic reaction except that unlabeled α-methyl-L-γ-glutamyl-L-α-aminobutyrate was used as the γ-glutamyl donor and L-[^{14}C]-methionine was used as the acceptor. The reactions were terminated by the addition of 1 ml of 0.05 N acetic acid and cooled in an ice bath. The entire contents of the reaction tube were placed on a Dowex-1 acetate (AG 1-X4; 200-400 mesh) column (2.5 x 0.5 cm). Two 1-ml aliquots of 0.05 N acetic acid were used to rinse the reaction tubes and were applied to the column. An additional 7 ml of 0.05 N acetic acid was used to wash the column free of labeled methionine. Nine milliliters of 1.5 N acetic acid was used to elute L-α-methyl-γ-glutamyl-L-[^{14}C]methionine from the column. A 1.5 ml aliquot from this last eluent was counted.

The data obtained in the kinetic experiments with α-methyl-γ-glutamyl-L-α-aminobutyrate were analyzed for us

by Dr. Carl Johnson (Department of Pharmacology, Mt. Sinai School of Medicine) using a MLAB[5] program run on the NIH PROPHET system. The program used here is similar to the modelaide program described by Chung et al., (1970) in the analysis of guinea pig liver transglutaminases. The values of the kinetic constants reported here are those which represent the best compromise in fitting theoretical curves to the observed data for both the release of L-α-aminobutyrate and the formation of α-methyl-γ-glutamyl-L- [^{14}C]-methionine, when the data from both products were analyzed concurrently. The standard errors reported here were calculated assuming that they could be described by a linear model.

4. Measurement of Reactivity Ratios

Reactivity ratios (for definition of term see "Results") can be determined when two or more amino acid acceptors are incubated with γ-glutamyl transpeptidase and a γ-glutamyl donor. Two experimental methods were used to determine the ratios of transpeptidation product formation.

a) Method 1

This procedure allows the simultaneous determination

[5]The PROPHET System is a specialized computer resource developed by the Chemical/Biological Information-Handling Program of the National Institutes of Health. (A detailed description of the system's features appear in Castleman et al., 1974. See also Raub, 1974). A description of the MLAB program is contained in the manual, MLAB: An On Line Modeling Laboratory, Division of Computer Research and Technology, National Institutes of Health, Bethesda, MD.

of several transpeptidation products using an amino acid analyzer. The incubation mixture (consult individual tables and figures for exact concentrations used) contained glutathione, dithiothreitol, glycylglycine, α-aminobutyrate and methionine in a Tris-HCl buffer. The reaction was initiated by the addition of sheep kidney γ-glutamyl transpeptidase. At the end of the incubation period the reaction was terminated by boiling the incubation mixture for 3 minutes. Additional dithiothreitol was then added to assure that all sulfhydryl groups were in the reduced state. The sulfhydryl groups were then converted into their S-alkyl derivatives by the addition of excess iodoacetamide (Zelazo and Orlowski, 1976), and the solution was applied to an analytical grade Dowex H^+ (Ag 50-X4, 100 to 200 mesh) column(2.5 x 0.5cm). The column was washed with 7 ml of water, and eluted with 5 ml of 3 N NH_4OH. The ammonia eluate was evaporated to dryness at 45°C by flash evaporation. The residue was dissolved in lithium citrate buffer and analyzed on an amino acid analyzer. The various γ-glutamyl compounds of interest; S-acetamido-γ-glutamylglutathione, γ-glutamylglycylglycine, γ-glutamyl-α-aminobutyrate and γ-glutamylmethionine emerge from the column as separate sharp peaks, and can be determined quantitatively.

b) Method 2

This procedure allows the determination of the rate of formation of any one of several transpeptidation products which are produced when a γ-glutamyl donor is incubated with

a mixture of amino acid acceptors. The transpeptidation product is measured by isolating on a Dowex-1 (acetate) column and counting the γ-glutamyl ^{14}C-amino acid formed in an incubation medium containing the precursor ^{14}C-amino acid of known specific activity. A second transpeptidation product can similarly be measured by utilizing a parallel incubation medium of identical composition as the first, with the exception that a second ^{14}C-amino acid acceptor is used while the concentration of the first amino acid, although unlabeled, remains the same. In a similar manner any number of transpeptidation products can be simultaneously measured from an incubation medium by utilizing an equivalent number of parallel incubations, each containing a different labeled amino acid. The relative rates of formation of the transpeptidation products can be used to calculate the reactivity ratios of the precursor amino acids according to equation 34.

The incubation medium (final volume, 0.2 ml) contained alanine (15 mM), methionine (5 mM) and the γ-glutamyl donor in 140 mM Tris-HCl buffer (pH 8.7). Parallel incubation experiments were performed each containing the above incubation medium. Tracer quantities of L-[^{14}C]alanine were added to the first incubation medium and tracer quantities of L-[^{14}C]methionine were added to the second incubation medium . The incubation was initiated by the addition of sheep kidney γ-glutamyl transpeptidase. At the end of

the incubation period the reaction was stopped by the addition of 1 ml of 0.05 N acetic acid. The incubation mixture was applied to the top of a Dowex acetate (AG 1-X4, 200 to 400 mesh) column (2.5 x 0.5 cm). The column was washed with 7.5 ml of 0.05 N acetic acid which eluted all the unreacted ^{14}C-amino acid. The column was then eluted with 5 ml of 1.5 N acetic acid which eluted the γ-glutamyl ^{14}C-amino acid. The γ-glutamyl amino acid was counted as described above.

c) Measurement of Amino Acid Reactivity Ratios in Serum

The procedure that was used to determine the reactivity ratio of serum amino acids was based on the principle outlined under method 2. The reactivity ratios of all amino acids were measured relative to L-alanine arbitrarily set as 1. Pooled normal human serum was used in all experiments. Each incubation mixture contained a tracer amount of a different ^{14}C-amino acid in 0.2 ml of human serum containing 7 mM N^2-γ-L-glutamyl-N^6-benzyloxycarbonyl-L-lysine as the γ-glutamyl donor. The mixtures were pre-incubated under an atmosphere of 95% oxygen-5% carbon dioxide. The pH of the medium was readjusted to 7.3 by the addition of 0.3 N NaOH. Such an adjustment was necessary to neutralize pH changes caused by the addition of the acidic γ-glutamyl donor. The reference incubation mixture was identical to the incubation described above, with the exception that [^{14}C]alanine replaced the labeled amino acid. The reaction was initiated by the addition of sheep kidney γ-glutamyl transpeptidase,

which had been evaporated to dryness under a stream of nitrogen and resuspended in serum. Blanks containing the entire incubation medium with the exception of the enzyme were analyzed concurrently. Such controls were necessary to compensate for any acidic ^{14}C-labeled compounds either initially present as impurities in, or generated by the action of human serum on the labeled amino acids. These controls would, therefore, compensate for any transpeptidation products generated by the relatively low levels of γ-glutamyl transpeptidase (approximately 3% of the activity of the added sheep kidney enzyme normally found in the serum) (Szewczuk and Orlowski, 1960).

At the end of the incubation period all experiments except those measuring the rate of γ-glutamyl-[^{14}C]arginine and γ-glutamyl-[^{14}C]histidine formation (see below) were terminated by the addition of 1 ml of 0.05 N acetic acid. These were then applied to a Dowex acetate (Ag 1-X4, 200 to 400 mesh) column (2.5 x 0.5 cm). The free ^{14}C-amino acid of each incubation experiment was eluted from the column with 7.5 ml of 0.05 N acetic acid. The sole exception was [^{14}C]glutamate which was eluted from its column with 11 ml of 0.5 N acetic acid. The γ-glutamyl ^{14}C-amino acid derivatives except those of cystine, phenylalanine, tyrosine and glutamate were then eluted with 5 ml of 1.5 N acetic acid. γ-Glutamyl-[^{14}C]tyrosine was eluted with 6 ml and γ-glutamyl-[^{14}C]glutamate was eluted with 8 ml of 3.0 N acetic acid solution. The γ-glutamyl ^{14}C-amino acids were counted as

described previously.

The experiments measuring γ-glutamyl-[^{14}C]histidine** and γ-glutamyl-[^{14}C]arginine** formation were terminated by the addition of 0.5 ml of boiling water, and then boiled for 2 minutes. Tris-HCl buffer (0.8 ml, pH 9.0) was then added to the incubation tube and the contents of the tube applied to a Dowex acetate (AG 1-X4, 200 to 400 mesh) column (6.0 x 0.5 cm). The ^{14}C-amino acids were eluted from the column with 7.5 ml of water. The γ-glutamyl ^{14}C-amino acid derivatives were eluted with 5 ml of 0.1 N acetic acid and counted as described previously.

The concentration of the various serum amino acids was determined on an amino acid analyzer. Five volumes of a saturated picric acid solution was added to 0.5 ml of serum containing norleucine as an internal standard. Protein was removed by centrifugation. An aliquot of the supernatant solution was applied to the top of a Dowex chloride (AG 2 - X8 , 100 to 200 mesh) column (3.0 x 0.5), and the column was washed with 7 ml of 0.01 N HCl. The eluant was evaporated to dryness at 45°C under vacuum and processed for amino acid analysis.

5. Experiments on Rabbit Choroid Plexus

Dutch-belted rabbits, of either sex, were killed by decapitation. The brains were rapidly removed and placed in ice-cold oxygenated Hepes-buffered medium: 119 mM NaCl, 5 mM KCl, 0.75 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM NaH_2PO_4, 1mM

** These compounds are not retarded by a Dowex-1 resin under acidic conditions.

$NaHCO_3$, 10 mM glucose, 15 mM HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid) with the pH adjusted to 7.37 with NaOH. The choroid plexuses from the lateral and third ventricles were removed from the brain as quickly as possible and used as described below. Each choroid plexus weighed approximately 5 mg.

a. Determination of Cellular Spaces

Total cellular space was determined by the weight loss of the choroid plexus due to drying. Inulin space was used to determine extracellular space. The choroid plexuses were incubated for 2 hours at 37° in an oxygenated HEPES-buffered medium (0.4 ml) containing [^{14}C]carboxy-inulin (approximately 0.2 µCi). A 25 µl aliquot of the incubation medium was removed to determine the concentration of [^{14}C]-carboxy-inulin in the medium.

At the end of the incubation, the choroid plexuses were removed from the incubation medium, rinsed by shaking for 1 or 2 seconds in ice-cold HEPES-buffered medium, and blotted on filter paper. Each plexus was placed on a pre-tared weighing boat, weighed, and then dessicated overnight. The weighing boat with dried choroid plexus was again weighed the following morning. The weight loss, which is equivalent to the total cellular water, was 81±1 percent (n=15) of the total tissue weight. The dry choroid plexus was then placed in a small test tube containing 0.5 ml of 2.5 N NaOH, and digested at 37°for 3 hours with periodic vortexing. A 0.1 ml

aliquot of the choroid plexus digest was placed in a plastic scintillation vial with 10 ml of PCS solution (Amersham/Searle), and counted on a Beckman LS-230 liquid scintillation counter. The aliquot of [^{14}C] carboxy-inulin previously taken from the incubation medium, together with 0.1 ml of 2.5 N NaOH,was placed in a plastic scintillation vial, and similarly counted. The volume of the extracellular space can be determined by dividing the total radioactivity in the tissue by the concentration of [^{14}C] carboxy-inulin in the incubation medium. Extracellular space, as determined in this manner, was 47 $\pm$ 2 percent (n=9) of the total cellular water. Intracellular water was therefore 53% of the total cellular water, or 43.2 percent (.81 x .53) of the weight of the choroid plexus.

b. Determination of Glutathione Concentrations in the Choroid Plexus

Freshly obtained choroid plexuses, or plexuses incubated in HEPES-buffered medium, were rinsed in cold buffer, blotted dry, and weighed. They were homogenized with 1 ml of 5% (w/v) trichloroacetic acid in 0.01 N HCl, and analyzed for total glutathione (GSH + GSSG) by the method of Tietze (1969). The intracellular concentration of glutathione was determined by dividing the total amount of glutathione in the choroid plexus by the intracellular volume.

IV RESULTS

A. Inability of α-Alkyl Amino Acids to Act as Acceptors in the Transpeptidase Reaction

When L-α-methyl-γ-glutamyl-L-α-aminobutyrate was incubated with γ-glutamyl transpeptidase, and the products of the reaction were studied by paper chromatography with either Solvent A or B, only the hydrolytic products, L-α-methyl glutamate and L-α-aminobutyrate were detected. The formation of only the hydrolytic products indicated that the substitution of a methyl group for a hydrogen on the α-carbon, abolished the ability of amino acids to act as acceptors. In contrast, when L-γ-glutamyl-p-nitroanilide was incubated under the same conditions, transpeptidation and higher order transpeptidation products, in addition to the hydrolytic products, were seen on paper chromatography.

The inability of amino acids, in which the α-hydrogen has been substituted by an alkyl group, to act as an acceptor in the transpeptidase reaction was confirmed by incubating L-γ-glutamyl-p-nitroanilide with α-alkyl amino acids such as DL-α-methyl-m-tyrosine, DL-α-methyl glutamate and DL-cycloleucine. In none of these experiments could any formation of transpeptidation products be detected. When the protein amino acids, L-tyrosine, L-glutamate or L-isoleucine, were incubated under identical conditions as the α-alkyl amino acids, transpeptidation products were observed in both, or at least in one of the solvent systems used (Table I).

TABLE I

DETECTION OF TRANSPEPTIDATION PRODUCTS BY PAPER CHROMATOGRAPHY IN AN INCUBATION MIXTURE CONTAINING γ-GLUTAMYL TRANSPEPTIDASE, A DONOR AND VARIOUS AMINO ACID ACCEPTORS

The reaction mixtures (final volume, 0.2 ml) contained the γ-glutamyl donor, the α-alkyl or α-hydrogen amino acid and 80 ng of γ-glutamyl transpeptidase in a Tris-HCl buffer (0.1 M, pH 9.0). Two tubes for each reaction mixture were incubated. One of the tubes was incubated for 10 min, the other for 180 min prior to chromatography. The reactions were terminated by boiling in water for 2 min. Aliquots of the reaction mixture (15 µl) were applied to a Whatman no. 1 filter paper and chromatographed (descending chromatography) overnight (16 to 20 hr) in either Solvent A (1-butanol/acetic acid/water; 60/15/25/) or Solvent B (pyridine/1-butanol/water; 1/1/1). Transpeptidation product formation was determined by the appearance of ninhydrin-positive spots which could not be accounted for by the amino acid or the products of the reaction of the donor alone.

Expt. No.	Donor	Acceptor	Transpeptidation Product (R_f)	Solvent System
1.	L-α-Methyl-γ-glutamyl-L-α-aminobutyrate (10 mM)	L-α-methyl-γ-glutamyl-L-α-aminobutyrate (10 mM)	not detected	A
2.	"	"	"	B
3.	L-γ-Glutamyl-p-nitroanilide (5 mM)	L-γ-glutamyl-p-nitroanilide (5mM)	L-γ-glutamyl-L-γ-glutamyl-p-nitroanilide (0.31)	A

4.	"	"	" (0.38)	B
5.	"	DL-cycloleucine (20 mM)	not detected	A
6.	"	"	"	B
7.	"	L-isoleucine (10 mM)	L-γ-glutamyl-L-isoleucine (0.50)	A
8.	"	"	not detected	B
9.	"	DL-α-methyl glutamate (20 mM)	not detected	A
10.	"	"	"	B
11.	"	L-glutamate (10 mM)	L-γ-glutamyl-L-glutamate (0.14)	A
12.	"	"	" (0.08)	B
13.	"	DL-α-methyl-m-tyrosine (20 mM)	not detected	A
14.	"	"	"	B
15.	"	L-tyrosine (10 mM)	not detected	A
16.	"	"	L-γ-glutamyl-L-tyrosine (0.51)	B

Furthermore, no enhancement of p-nitroaniline release was detected when α-alkyl amino acids were incubated with either L-γ-glutamyl-p-nitroanilide or D-γ-glutamyl-p-nitroanilide (Table II). The inability of α-alkyl amino acids to enhance p-nitroaniline release from either D-, or L-, γ-glutamyl-p-nitroanilide indicates that these amino acids are not suitable acceptors for γ-glutamyl transpeptidase. It should be noted that an enhancement of p-nitroaniline release would have been more readily detectable with the D-substrate, since it has been shown that amino acid acceptors activate the release of p-nitroaniline from D-γ-glutamyl-p-nitroanilide to a much greater extent than from the L-isomer (Orlowski and Meister, 1965).

B. α-Methyl-γ-glutamyl-L-α-aminobutyrate as a γ-Glutamyl Donor

When L-α-amino acids, such as phenylalanine or methionine, were added to an incubation mixture containing L-α-methyl-γ-glutamyl-α-aminobutyrate and the enzyme, formation of the corresponding L-α-methyl-γ-glutamyl amino acid derivatives could readily be observed by paper chromatography. Furthermore, when L-[^{14}C] methionine was used as the amino acid acceptor in a similar incubation mixture, a radioactive compound in addition to [^{14}C] methionine, could be eluted from a Dowex-1 acetate column. This compound was eluted with 1.5 N acetic acid, consistent with its structure being the acidic peptide, L-α-methyl-γ-glutamyl-L-[^{14}C]methionine. The time course for the formation of this peptide was ap-

TABLE II

EFFECT OF α-ALKYL AMINO ACIDS ON THE RELEASE OF p-NITROANILINE FROM EITHER L-, OR D-, γ-GLUTAMYL-p-NITROANILIDE

The reaction mixture (final volume, 2 ml) contained either L-, or D-, γ-glutamyl-p-nitroanilide (5 mM), $MgCl_2$ (10 mM), 34 ng of enzyme, and the α-alkyl amino acid in a Tris-HCl buffer (0.1 M, pH 9.0). Incubations were for 10 min. The reactions were terminated by the addition of 2 ml of 1.5 N acetic acid.

Acceptor	Relative Activity with γ-Glutamyl Donors	
	L-γ-Glutamyl-p-nitroanilide	D-γ-Glutamyl-p-nitroanilide
None	100	100
DL-α-Methyl-m-tyrosine (10mM)	100	99
DL-Cycloleucine (10 mM)	103	102
DL-α-Methyl glutamate (10mM)	96	98
L-α-Methyl-γ-glutamyl-L-α-aminobutyrate (5 mM)	98	98

proximately linear for three hours and the peptide accounted for approximately 25% of the initial amount of the γ-glutamyl donor. After 20 hours, this peptide accounted for the breakdown of 60% of the γ-glutamyl donor initially present. In addition, when an amino acid was incubated with L-α-methyl-γ-glutamyl-L-α-aminobutyrate, the rate of formation of L-α-aminobutyrate was enhanced. These results are consistent with L-α-methyl-γ-glutamyl-L-α-aminobutyrate acting as a γ-glutamyl donor in the γ-glutamyl transpeptidase catalyzed reaction.

L-α-Methyl-γ-glutamyl-L-α-aminobutyrate binds to the same site of the enzyme which interacts with the model substrate L-γ-glutamyl-p-nitroanilide. This conclusion is derived from the observation that this substrate acts as a competitive inhibitor with respect to γ-glutamyl-p-nitroanilide in the presence of 80 mM glycylglycine as the acceptor. Double reciprocal plots (Fig. 3) at several L-α-methyl-γ-glutamyl-L-α-aminobutyrate concentrations show a competitive type of inhibition. The K_I of L-α-methyl-γ-glutamyl-L-α-aminobutyrate calculated from these results is 5.2×10^{-2}M.

C. Studies on the Hydrolytic Reaction of γ-Glutamyl Transpeptidase

The use of L-α-methyl-γ-glutamyl-L-α-aminobutyrate which acts as a γ-glutamyl donor, but not as an acceptor affords the opportunity to study the hydrolytic reaction in the absence of any transpeptidation reaction.

The pH optimum for both the hydrolytic and transpeptidation reactions is shown in Figure 4. The hydrolytic reaction has a broad optimum centered around pH 6.7. The

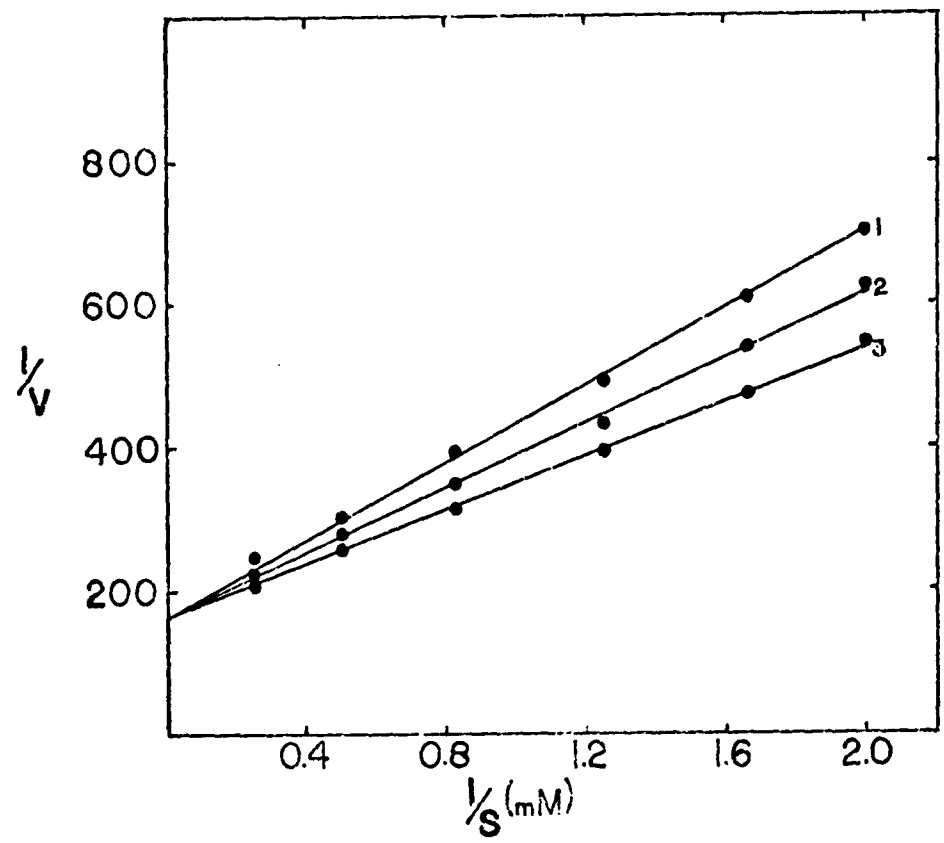
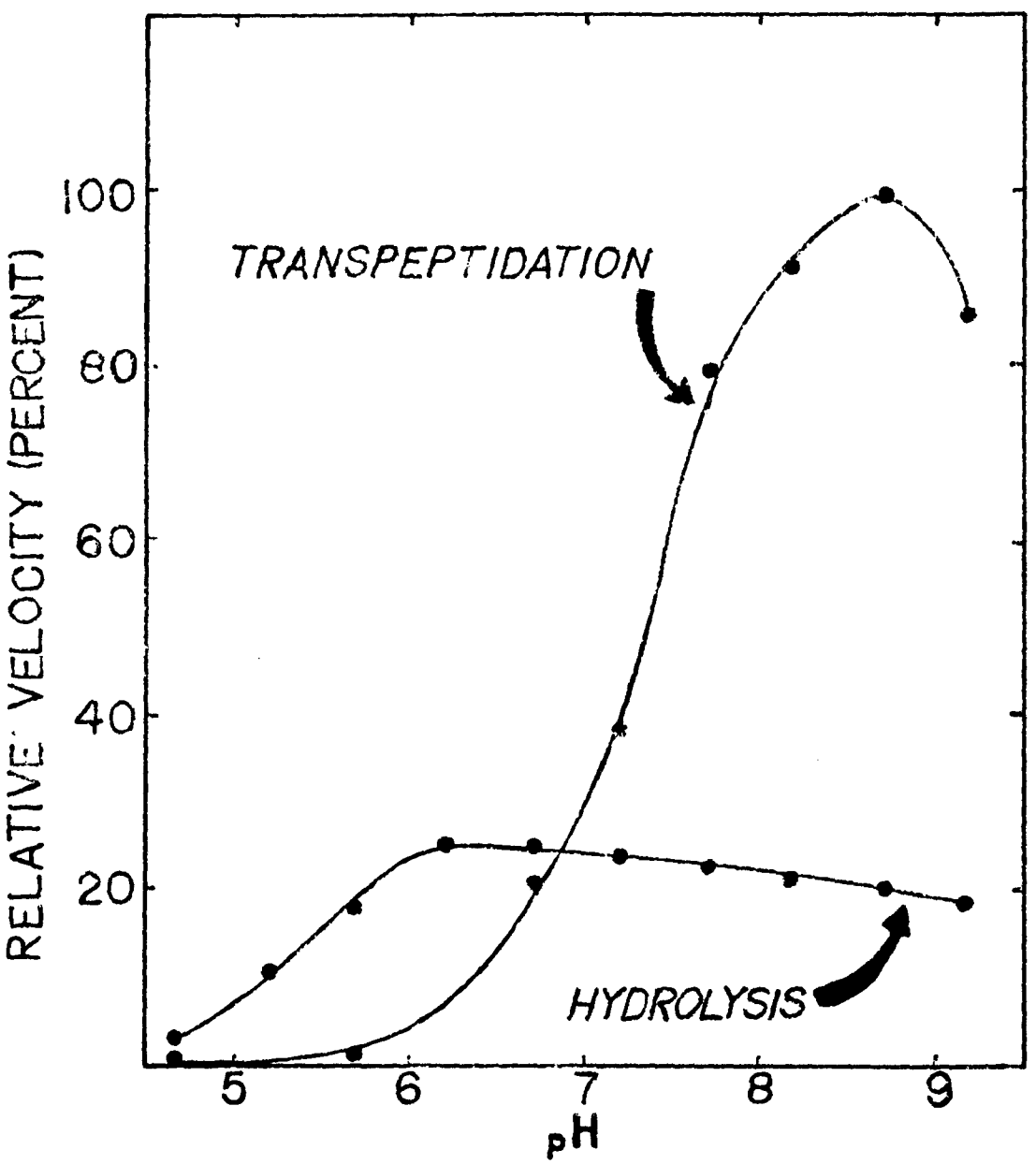


Fig. 3. Effect of L-α-methyl-γ-glutamyl-L-α-amino-butyrate on p-nitroaniline release from L-γ-glutamyl-p-nitro-anilide. The incubation mixture (final volume, 0.5 ml) contained 100 mM Tris-HCl buffer (pH 9.0) 80 mM glycylglycine and 0.03 µg of enzyme. The concentration of the substrate (S) was varied in the range 0.5 - 3 mM and the inhibitor held constant at 0 (curve 1), 10 mM (curve 2) and 20 mM (curve 3).

Fig. 4. pH dependence of the hydrolytic and transpeptidation reactions. The incubation mixtures (final volume, 0.2 ml) contained 100 mM Tris-HCl buffer (pH 7.2 - 9.2) or 100 mM ammonium phosphate-acetate buffer, (pH 4.7 - 7.8), 10 mM L-α-methyl-γ-glutamyl-L-[^{14}C]-α-aminobutyrate and enzyme (0.02 μg protein). The hydrolytic reaction was followed by measuring the formation of L-[^{14}C]-α-aminobutyrate. The incubation mixture for measuring the transpeptidation reaction was the same as above except that unlabeled L-α-methyl-γ-glutamyl-L-α-aminobutyrate was used and L-[^{14}C]methionine (10 mM, 250,000 cpm) was included as the acceptor. The formation of L-α-methyl-γ-glutamyl-L-[^{14}C]methionine was determined as described under "Methods". The velocity is expressed relative to that obtained in the transpeptidation reaction at optimal pH.


RELATIVE VELOCITY (PERCENT)
100
80
60
40
20
TRANSPEPTIDATION
HYDROLYSIS
5
6
7
8
9
pH

transpeptidation reaction, in which the formation of L-α-methyl-γ-glutamyl-L-[^{14}C]methionine was measured has a sharp maximum at about pH 8.8. This last pH optimum is similar to that obtained with γ-glutamyl-p-nitroanilide as substrate (Orlowski and Meister, 1965).

The K_m for L-α-methyl-γ-glutamyl-L-α-aminobutyrate in the hydrolytic reaction is 2.9 mM.

Several papers have appeared describing the effects of cations on γ-glutamyl transpeptidase activity. Mg^{+2} has been shown to increase the activity of this enzyme when the enzyme was purified from hog kidney (Orlowski and Meister, 1965), but not from beef kidney (Szewczuk and Baranowksi, 1963). Monovalent cations (Na^+, K^+, Li^+, Cs^+ and tetraethylammonium) have been shown to enhance the γ-glutamyl transpeptidase activity when the activity from any of several different tissues in several different species was assayed (Orlowski et al., 1973). Other preparations of γ-glutamyl transpeptidase, however, were not activated, or only slightly activated, in the presence of monovalent cations (Taniguchi et al., 1975). The activating effects of added cations on the γ-glutamyl transpeptidase purified from rat kidney was dependent on the anion of the salt added. Cationic chlorides activated the enzyme much stronger than acetates or sulfates (Elce et al., 1974).

The activating effects of cations on sheep kidney γ-glutamyl transpeptidase was shown to be dependent on the γ-glutamyl donor. A greater enhancement of activity by ca-

tions was observed when glutathione was the donor as compared to either D-, or L-, γ-glutamyl-p-nitroanilide. When glutathione was the γ-glutamyl donor, in the absence of added amino acids, activation by metal cations, seemed to occur primarily through an increase in the autotranspeptidation reaction (Zelazo and Orlowski, 1976).

Using the same sheep kidney enzyme and L-α-methyl-γ-glutamyl-L-α-aminobutyrate as the donor, the effects of cations on the rate of the hydrolytic reaction, in the absence of any other reaction, was studied (Table III). The results indicate that at pH 9.0, monovalent and divalent cations had little effect on the rate of hydrolysis of the substrate. In addition, other studies (data not shown), at pH 7.8 with various concentrations of Na^{+} (10-150 mM), K^{+} (10-150 mM), Mg^{+2} (1-10 mM), and Ca^{+2} (1-10 mM), showed that, at lower pH, the hydrolytic reaction was also insensitive to the presence of cations.

In the presence of an added amino acid or peptide acceptor, both transpeptidation to the acceptor, and hydrolysis occurs. The activating effects of cations on the reactions occurring when L-α-methyl-γ-glutamyl-L-α-aminobutyrate and either methionine, alanine or glycylglycine are incubated with γ-glutamyl transpeptidase, as measured by L-α-aminobutyrate formation, are shown in Table IV. In general, the effects of cations are small; calcium, however, seems to have a small inhibitory effect on these reactions. It seems possible that the activating effects of cations ob-

TABLE III

EFFECT OF METAL IONS ON THE HYDROLYTIC REACTION OF γ-GLUTAMYL TRANSPEPTIDASE WITH L-α-METHYL-γ-GLUTAMYL-L-α AMINOBUTYRATE AS SUBSTRATE

The reaction mixture (final volume, 0.2 ml) contained 10 mM L-α-methyl-γ-glutamyl-L-[^{14}C]-α-aminobutyrate as substrate, 13 ng enzyme and the metal ion chlorides as indicated, in a 0.1 M Tris-HCl buffer pH 9.0. The reaction mixtures were incubated for 75 min. The values in parenthesis are the velocities relative to an incubation mixture containing no cations.

Metal Ion	Formation of L-[^{14}C]-α-aminobutyrate	
	n moles/hr	
150 mM Na^{+}	24.9	(100)
150 mM K^{+}	26.5	(106)
10 mM Mg^{+2}	24.9	(100)
10 mM Ca^{+2}	22.0	(88)
1 mM Ni^{+2}	25.1	(101)
1 mM Cu^{+2}	25.0	(101)
1 mM Co^{+2}	19.7	(80)

TABLE IV

EFFECTS OF CATIONS ON THE FORMATION OF L-[^{14}C]-α-AMINOBUTYRATE FROM L-α-METHYL-γ-GLUTAMYL-L-[^{14}C]-α-AMINOBUTYRATE IN THE PRESENCE OF AMINO ACIDS CATALYZED BY γ-GLUTAMYL TRANSPEPTIDASE

The reaction mixture (final volume, 0.2 ml) contained 5 mM L-α-methyl-γ-glutamyl-L-[^{14}C]-α-aminobutyrate, 100 mM Tris-HCl buffer (pH 9.0), 16 ng enzyme, 20 mM amino acid and the metal ion chlorides as indicated. Incubations were for 120 min. Relative activities, in parenthesis, are expressed relative to those obtained for the amino acid in the absence of metal ions.

Metal Ion	Formation of L-[^{14}C]-α-Aminobutyrate with Amino Acids					
	Methionine		Glycylglycine		Alanine	
	nmole/hr					
None	15.9	(100)	22.0	(100)	4.71	(100)
150 mM Na^{+}	14.0	(88)	19.9	(91)	3.08	(85)
150 mM K^{+}	13.2	(83)	14.3	(65)	4.16	(88)
10 mM Mg^{+2}	16.2	(102)	20.7	(94)	4.83	(103)
10 mM Ca^{+2}	10.0	(63)	14.4	(65)	4.43	(94)

served with other γ-glutamyl donors, may be due to the specific activation of the transpeptidation reaction (Zelazo and Orlowski, 1976). An autotranspeptidation reaction doesn't occur with L-α-methyl-γ-glutamyl-L-α-aminobutyrate, so that no activation by cations is observed.

Studies of the effects of sulfhydryl reagents (sulfhydryl blocking or disulfide reducing) on the hydrolytic reaction are shown in Table V. Various degrees of inhibition were observed with sulfhydryl blocking reagents. The strongest inhibition (about 40%), was obtained with iodoacetamide. Preincubation with iodoacetamide completely inactivated the enzyme. Preincubation with several other sulfhydryl blocking reagents, however, did not significantly enhance their inhibition. On the other hand, the thiols, dithiothreitol and 2-mercaptoethanol which would be expected to reduce disulfides did not have any effect on the activity of the enzyme.

γ-Glutamyl transpeptidase from various other sources are inhibited by sulfhydryl reagents. The degree of this inhibition varies greatly with different enzymes and different inhibitors (Orlowski, 1963; Szewczuk and Connell, 1965; Richter, 1969). Some enzyme preparations are little affected by these inhibitors (Taniguchi, 1974). It, therefore, seems unlikely that the sulfhydryl group is directly involved in the catalytic process.

The effects of several acids on the rate of the hydrolytic reaction are also shown in Table V. Of the acids

TABLE V

EFFECT OF VARIOUS ADDITIONS ON THE RATE OF L-α-METHYL-γ-GLUTAMYL-L-[^{14}C]-α-AMINOBUTYRATE HYDROLYSIS

The reaction mixtures (final volume, 0.2 ml) contained 10 mM substrate, 100 mM Tris-HCl (pH 8.2), enzyme (0.02-0.08 μg) and 15 mM of the listed additions. The incubation periods were 60 - 120 min. All reactions were initiated by the addition of enzyme. Preincubation experiments were carried out with the enzyme for 60 min at 37° with a complete reaction mixture except for the substrate. Activities are expressed relative to a control mixture, containing no additions, arbitrarily set as 100.

Addition	Relative Activity
	% control
None	100
Iodoacetamide	59
Iodoacetamide(preincubated)	0
Iodoacetate	102
Iodoacetate(preincubated)	83
N-Ethylmaleamide	63
N-Ethylmaleamide (preincubated)	76
p-Chloromercuribenzoate	65
p-Chloromercuribenzoate (preincubated)	69
5,5'-Dithiobis-(2-nitrobenzoate)	69
5,5'-Dithiobis-(2-nitrobenzoate) (preincubated)	79
Maleic Acid	164
EDTA	93
Pyruvate	103
Phthalate	97
Fumarate	96
Salicylate	78

tested, maleate significantly activated the hydrolytic reaction. Maleate has also been shown to increase the hydrolytic action of γ-glutamyl transpeptidase at the expense of the transpeptidation reaction with rat kidney enzyme (Tate and Meister, 1974b).

Table VI shows the effect of various amino acids and peptides on L-α-aminobutyrate release from L-α-methyl-γ-glutamyl-L-α-aminobutyrate. In general, the effect of the amino acids on the rate of reaction was similar to their effects which have been observed with other substrates, such as γ-glutamyl-p-nitroanilide and glutathione (Zelazo and Orlowski, 1976). Among the common amino acids, L-methionine activates most strongly. It is of interest that glutamine activated the reaction with L-α-methyl-γ-glutamyl-L-α-aminobutyrate much less than was observed in reactions with glutathione or L-γ-glutamyl-p-nitroanilide. This effect is probably due to the competition between glutamine and the substrate for the γ-glutamyl binding site of the enzyme. Glutamine itself was shown to be a weak substrate capable of acting both as a γ-glutamyl donor and acceptor (Orlowski and Meister, 1965). The low activity of glutamine in the reaction with L-α-methyl-γ-glutamyl-L-α-aminobutyrate may indicate a much lower affinity of the latter as a γ-glutamyl donor for the enzyme as compared with glutathione or L-γ-glutamyl-p-nitroanilide.

As with other substrates the dipeptide glycylglycine was the strongest activator while the tripeptide glycylgly-

TABLE VI

THE EFFECT OF AMINO ACIDS AND PEPTIDES ON THE RATE OF RELEASE OF L-[^{14}C]-α-AMINOBUTYRATE FROM L-α-METHYL-γ-GLUTAMYL-L-[^{14}C]-α-AMINOBUTYRATE

The reaction mixtures (final volume, 0.2 ml) contained 5 mM L-α-methyl-γ-glutamyl-L-[^{14}C]-α-aminobutyrate, 100 mM Tris-HCl (pH 9.0), 0.015 μg enzyme, and 20 mM amino acid. Incubations were for 120 min. Relative activity is expressed relative to the amount of L-[^{14}C]-α-aminobutyrate liberated in the absence of added amino acids, arbitrarily set as 100.

Amino Acid	Relative Activity
	% control
None	100
Glycine	63
D-Alanine	67
L-Alanine	76
L-Valine	91
L-Aspartate	94
L-Glutamine	120
L-Isoleucine	121
D-Methionine	149
L-Histidine	154
L-Glutamate	183
Glycylglycylglycine	184
L-Arginine	217
L-Asparagine	222
L-Leucine	239
L-Lysine	241
L-Phenylalanine	256
L-Glutamylglycine	280
L-Methionine	292
Glycyl-L-proline	337
Glyclyglycine	378

cylglycine was much less effective in this respect. Among the neutral amino acids containing a hydrophobic side group the activating effect seemed to be related to the size of the hydrophobic group or to the molecular weight. Thus, the rate of the reaction increased in the order: glycine < alanine < valine < leucine < phenylalanine.

D. Kinetics

1. Kinetics of γ-Glutamyl Transpeptidase with L-α-Methyl-γ-glutamyl-L-α-aminobutyrate

The initial velocity patterns for the formation of L-α-methyl-γ-glutamyl-L-[^{14}C]methionine with α-methyl-γ-glutamyl-L-α-aminobutyrate as the variable substrate at different fixed concentrations of L-[^{14}C]methionine is shown in Fig. 5. A series of straight intersecting lines was obtained.

When the initial velocities for the formation of L-[^{14}C]-α-aminobutyrate were measured at different fixed concentrations of methionine as a function of various concentrations of L-α-methyl-γ-glutamyl-L-[^{14}C]-α-aminobutyrate a series of parallel lines was obtained in a double reciprocal plot (Fig. 6).

These kinetic patterns are consistent with a modified ping-pong mechanism as shown in Mechanism I. Similar mechanisms were described by Arion and Nordlie (1964) for liver microsomal glucose-6-phosphatase, and also by Folk (1969) and Chung and Folk (1972) for various transglutaminases.

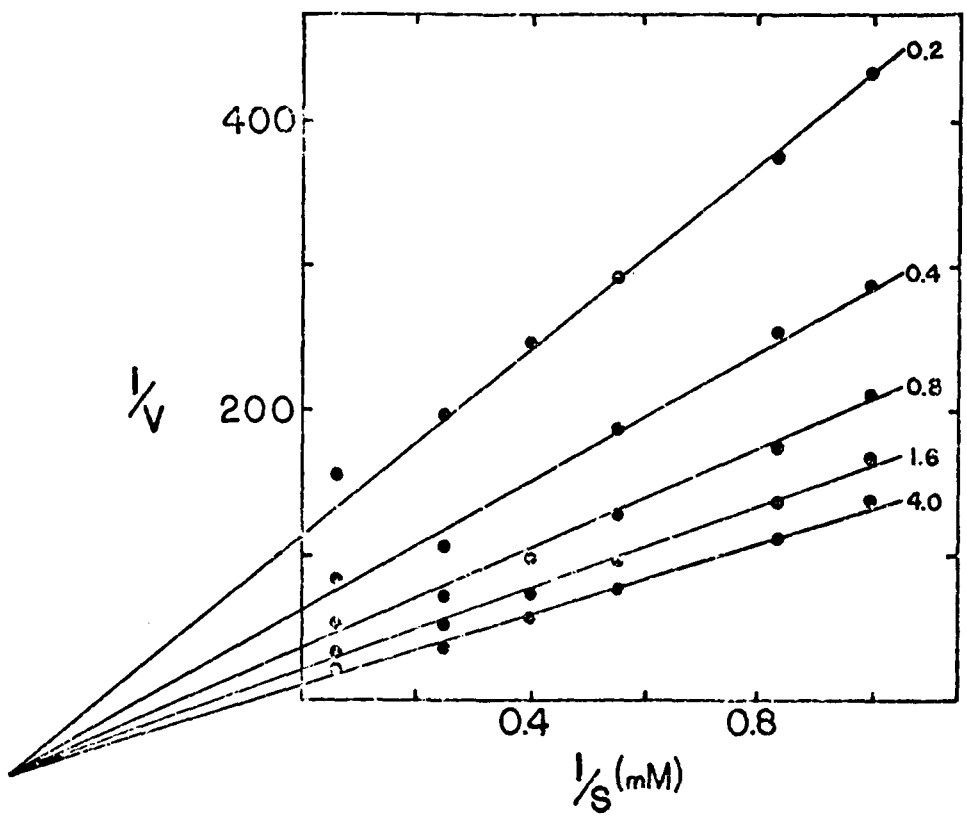


Fig. 5. Initial velocity patterns for the formation of L-α-methyl-γ-glutamyl-L-[^{14}C]methionine catalyzed by γ-glutamyl transpeptidase. The concentration of L-α-methyl-γ-glutamyl-L-α- aminobutyrate was varied at different fixed concentrations of L-[^{14}C]methionine (mM) indicated at the ends of the lines. Initial velocities are expressed as μmol of product formed per h per 0.04 μg enzyme. Incubation time was 60 min. Other conditions are given under "Methods".

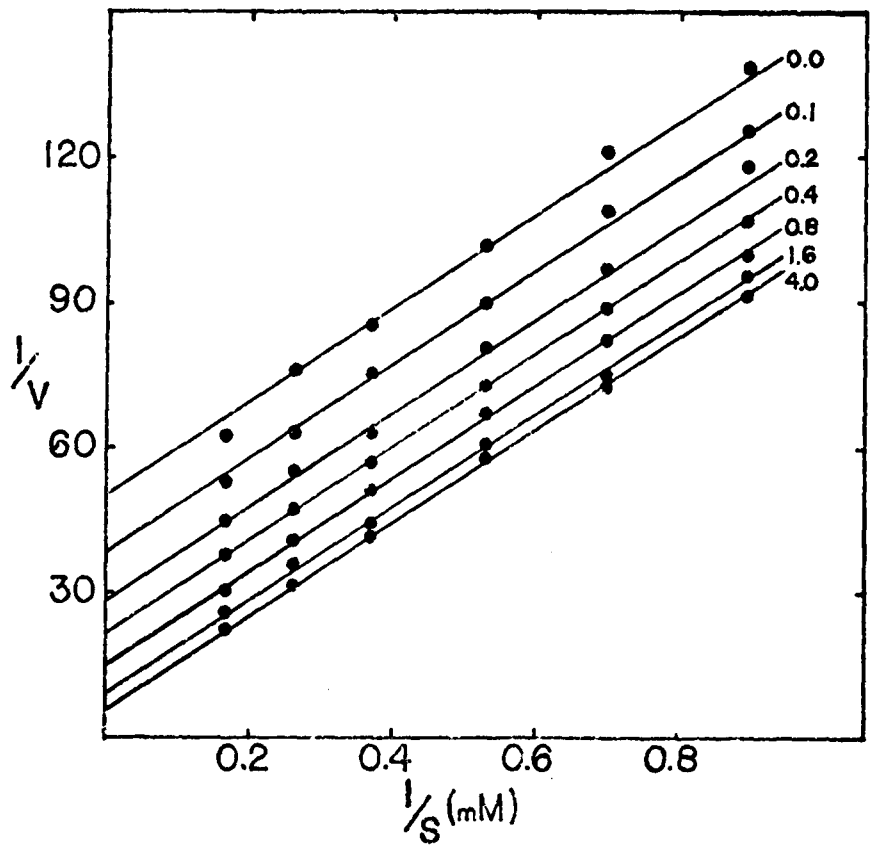
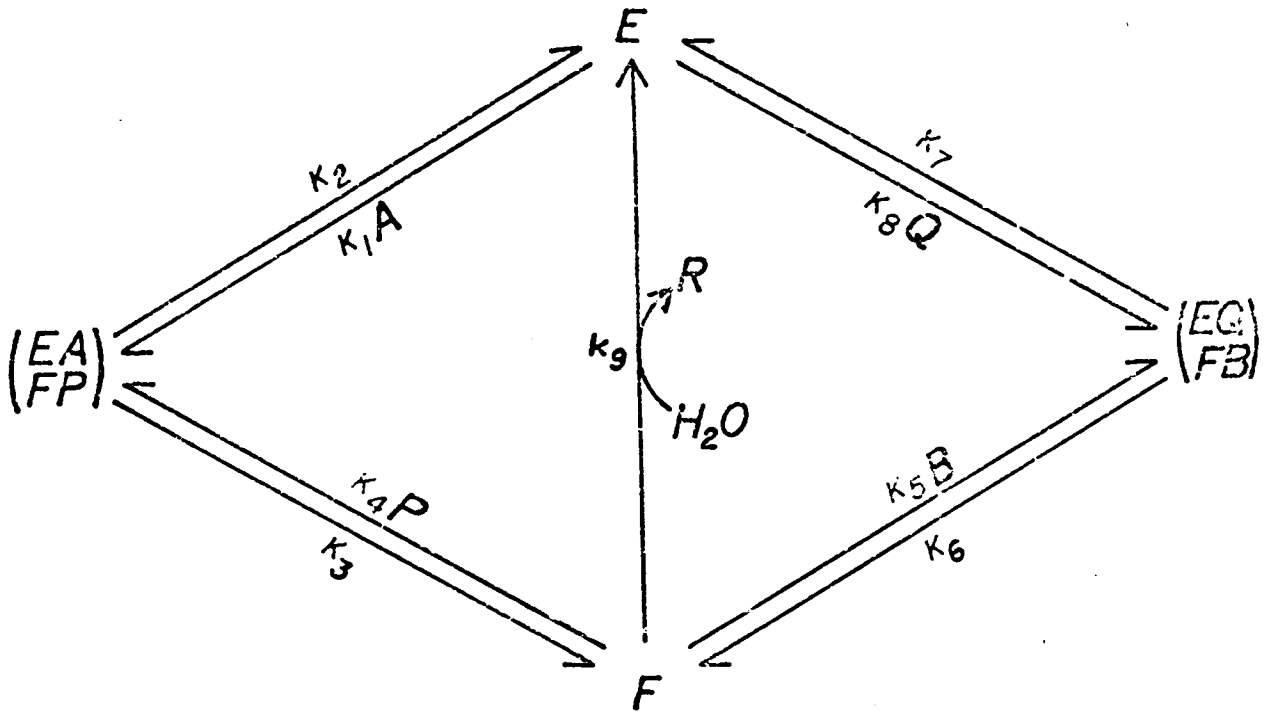


Fig. 6. Initial velocity pattern for the formation of L-[^{14}C]-α-aminobutyrate from L-α-methyl-γ-glutamyl-L-[^{14}C]-α-aminobutyrate and L-methionine catalyzed by γ-glutamyl transpeptidase. The molar concentration of the substrate, L-α-methyl-γ-glutamyl-L-[^{14}C]-α-aminobutyrate (S), was varied while the concentration of L-methionine was constant at the mM level indicated by the numbers at the ends of the lines. Initial velocities are expressed as μmol of product formed per h per 0.04 μg enzyme. Incubation time was 90 min. Other conditions of the assay are given under "Methods".



Mechanism I

This mechanism describes the binding of a substrate (A), to an enzyme (E), with the subsequent release of the first product (P), and the formation of an activated enzyme intermediate (F). The second substrate (B) then binds to this activated enzyme intermediate subsequently forming the second product (Q), and concurrently regenerating the native enzyme (E). In the absence of, or in the presence of low concentrations of B, water competes for the activated enzyme intermediate, forming the hydrolysis product (R), and regenerating the native enzyme.

The initial velocity plots obtained (Fig. 5 and 6) are consistent with such a mechanism (Mechanism I) with A, as L-α-methyl-γ-glutamyl-L-α-aminobutyrate; P, as L-α-aminobutyrate; B, as methionine; Q, as L-α-methyl-γ-glutamyl methionine, the transpeptidation product; R, as L-α-methyl glutamate; and F as an L-α-methyl-γ-glutamyl-enzyme intermediate.

The initial velocity equations which describe the formation of transpeptidation product (v_q) and the formation of L-α-aminobutyrate (v_p) on the basis of this modified ping-pong mechanism are (Folk, 1969):

$$v_q = \frac{V_{ab} \; AB}{K_{ah}K_{bt} + K_{at}B + K_{bt}A + AB} \qquad (22)$$

$$v_p = \frac{V_a \; A}{K_{ah} + \frac{A\left(1 + \frac{B}{K_{bt}}\right)}{\left(1 + \frac{B}{K_{ibb}}\right)}} \tag{23}$$

where the constants of these equations are defined as

$$K_{ah} = \frac{k_9(k_2 + k_3)}{k_1\,(k_3 + k_9)} \tag{23a}$$

$$K_{bt} = \frac{(k_6 + k_7)\;\;(k_3 + k_9)}{k_5\,(k_3 + k_7)} \tag{23b}$$

$$K_{at} = \frac{k_7(k_2 + k_3)}{k_1(k_3 + k_7)} \tag{23c}$$

$$K_{ibb} = \frac{k_9(k_6 + k_7)}{k_5 k_7} \tag{23d}$$

$$V_{ab} = \frac{k_3 k_7 E_o}{k_3 + k_7} \tag{23e}$$

$$V_a = \frac{k_3 k_9 E_o}{k_3 + k_9} \tag{23f}$$

A computer analysis of the data of the experiments shown in Fig. 5 and 6 is shown in fig. 7.

2. Kinetics of γ-Glutamyl Transpeptidase with L-γ-Glutamyl-p-nitroanilide

Most of the previous studies on γ-glutamyl transpeptidase used glutathione or another γ-glutamyl compound as the substrate. Substrates containing a chromogenic group

Fig. 7. Computer simulation of the initial velocity patterns predicted on the basis of mechanism I. The points represent experimental observations. The curves represent the best fit calculated by the computer on the basis of equations 22 and 23. The best fit parameters are listed below. The initial velocities are expressed as μmol of products formed per h per 0.04 μg of enzyme. A, Initial velocity for the production of L-[^{14}C]-α-aminobutyrate from L-α-methyl-γ-glutamyl-α-amino[^{14}C]butyrate, as substrate, in the presence of different fixed levels of methionine. The mM concentration of L-methionine is indicated at the ends of the curves. B, Initial velocity for the production of L-α-methyl-γ-glutamyl-L-[^{14}C]methionine from L-α-methyl-γ-glutamyl-L-α-aminobutyrate, as substrate, at different fixed levels of L-[^{14}C]methionine. The concentration of L-methionine (mM) is given at the ends of the curves.

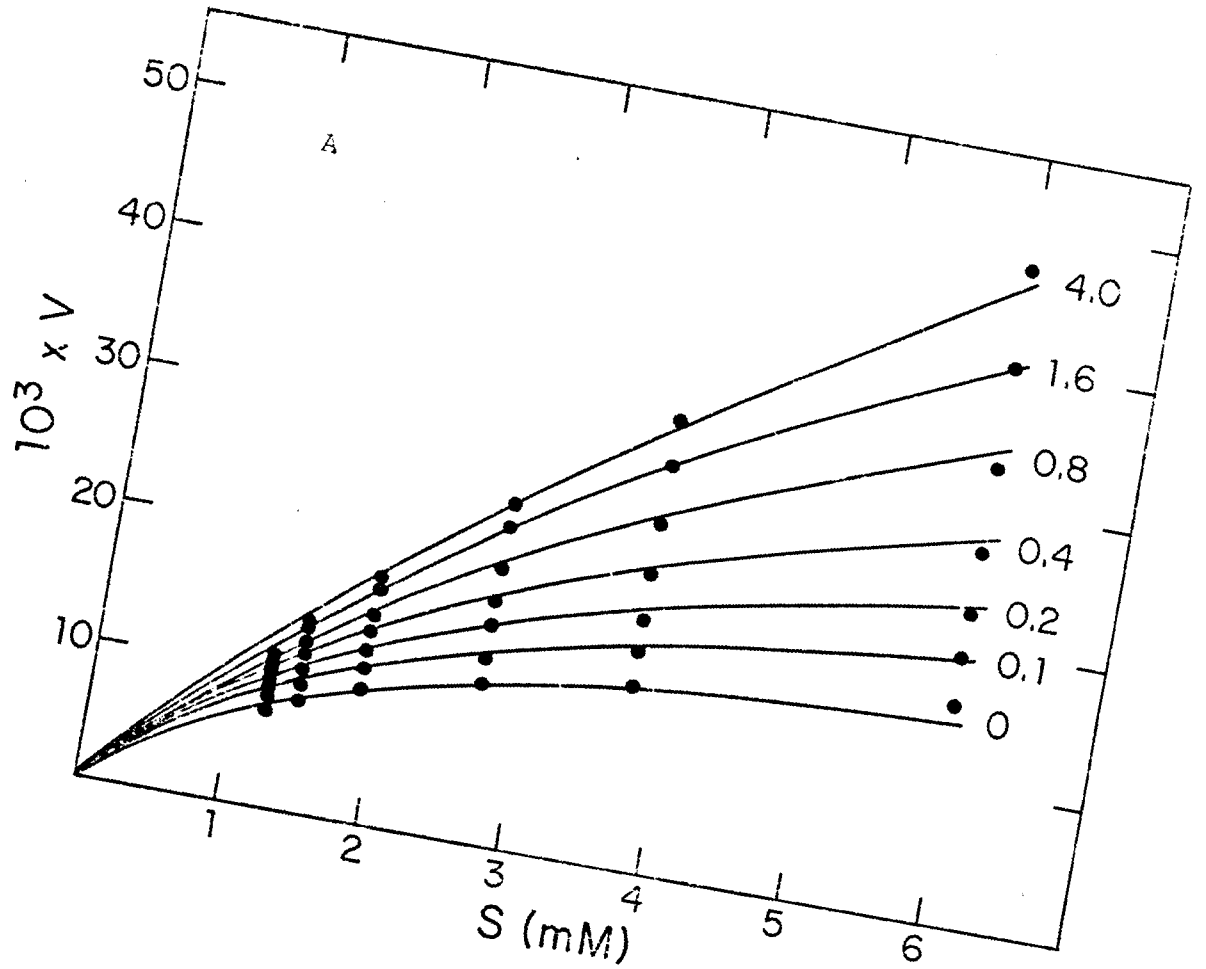
The best values for the parameters of equations 22 and 23 are:

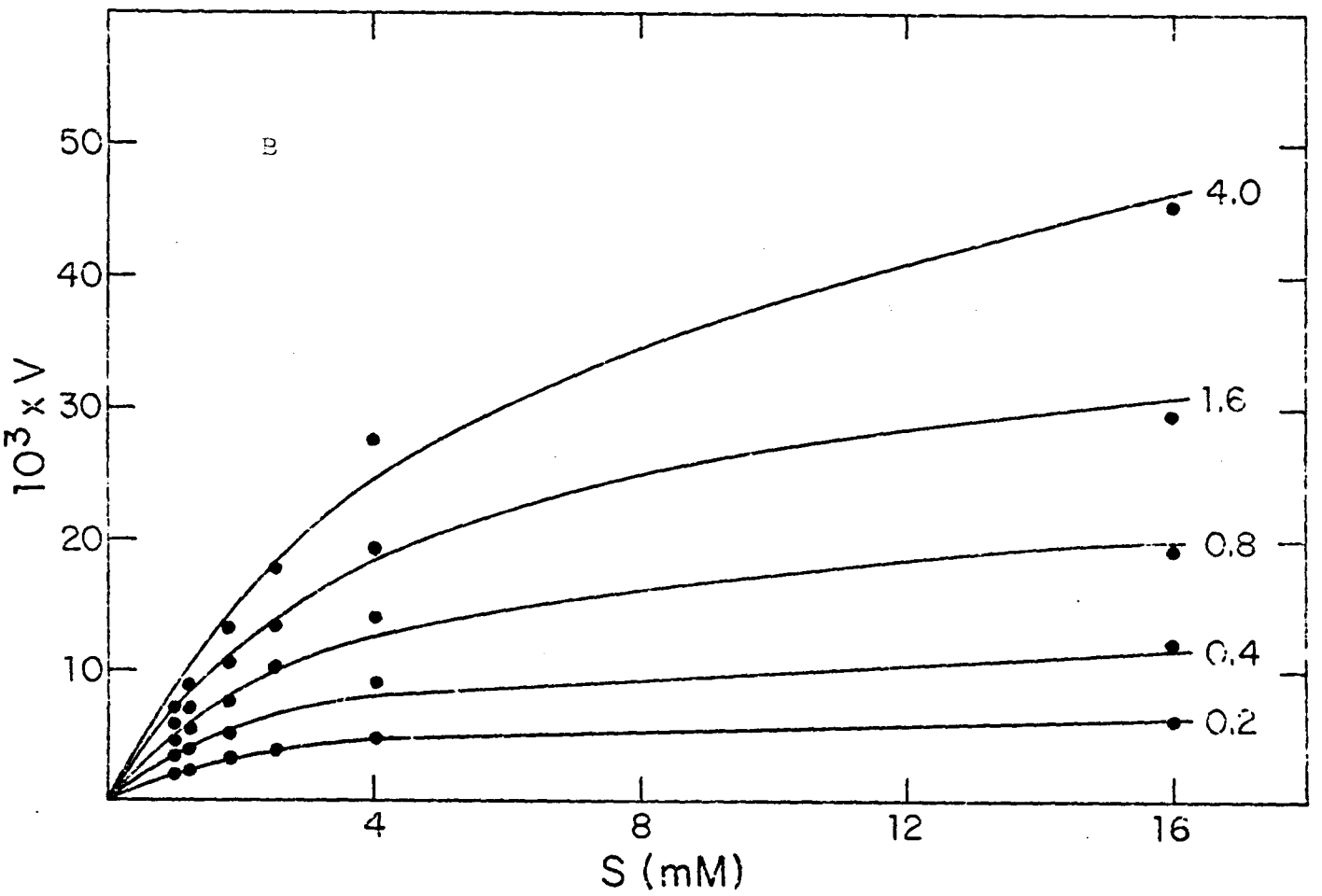
K_{ah}= 1.80 ± 0.08 mM; K_{bt}= 2.93 ± 0.23 mM;

K_{at}= 9.94 ± 0.79 mM; K_{ibb} = 0.203 ± 0.016 mM;

V_{ab}= 0.112 ± 0.006 μmol h^{-1} per 0.04 μg enzyme;

V_a= 0.0190 ±0.0007 μmol h^{-1} per 0.04 μg enzyme.


A
50
40
30
20
10
$10^3 \times V$
4.0
1.6
0.8
0.4
0.2
0.1
0
1
2
3
4
5
6
S (mM)


B
50
40
30
20
10
$10^3 \times V$
4.0
1.6
0.8
0.4
0.2
4
8
12
16
S (mM)

attached to the γ-glutamyl residue (Orlowski and Szewczuk, 1962; Orlowski and Meister, 1963), have been increasingly used in both experimental and clinical studies of the enzyme. We have studied the kinetics of L-γ-glutamyl-p-nitroanilide one of the substrates most commonly used for the determination of γ-glutamyl transpeptidase activity. In the absence of added amino acids γ-glutamyl transpeptidase catalyzes the following two reactions:

$$\text{L-}\gamma\text{-Glutamyl-p-nitroanilide} + H_2O \longrightarrow \text{L-glutamate} + \text{p-nitroaniline} \quad (24)$$

$$\text{L-}\gamma\text{-Glutamyl-p-nitroanilide} + \text{L-}\gamma\text{-glutamyl-p-nitroanilide} \longrightarrow \text{L-}\gamma\text{-glutamyl-L-}\gamma\text{-glutamyl-p-nitroanilide} + \text{p-nitroaniline} \quad (25)$$

These two reactions are similar to the reaction of γ-glutamyl transpeptidase with L-α-methyl-γ-glutamyl-L-α-aminobutyrate in the presence of added amino acid (reactions 20 and 21). The first reaction in each case describes the hydrolysis of each substrate. The second reaction describes the transpeptidation. The difference between these two sets of reactions is that the amino acid acceptor in the case of L-γ-glutamyl-p-nitroanilide is not an added amino acid but the substrate itself (autotranspeptidation).

It is therefore, possible to describe the kinetic behavior of L-γ-glutamyl-p-nitroanilide in the presence of γ-glutamyl transpeptidase in terms of the equations (equations 22 and 23) used to describe the kinetics of the enzyme

with L-α-methyl-γ-glutamyl-L-α-aminobutyrate in the presence of an added amino acid. The difference between these two sets of equations is that in this case L-γ-glutamyl-p-nitroanilide (A'), is now both an amino acid acceptor (B) and a γ-glutamyl donor (A).

Substitution of A' for both A and B in equation 23 and transformation to its reciprocal form yields the following equation:

$$\frac{1}{v_p} = \frac{K_{ah}}{V_a}\left(\frac{1}{A'}\right) + \frac{1}{V_{ab}}\left(\frac{K_{bt} + A'}{K_{ibb} + A'}\right) \qquad (26)$$

where $1/V_{ab} = K_{ibb}/K_{bt} \cdot V_a$ (26a)

based on the previously given definitions of the constants.

When K_{ibb} is much smaller than A'; that is, when the rate of hydrolysis is much slower than the rate of both transpeptidation and release of the transpeptidation product, equation 26 will yield linear double reciprocal plots of $1/v_p$ versus $1/A'$. Under these conditions V_{max} will be equal to V_{ab} and K_m will be equal to $(V_{ab} \cdot K_{ah}/V_a) + K_{bt}$. When K_{ibb} is of the same order of magnitude as A', that is, when hydrolysis is significant compared to the formation and release of the transpeptidation product, then double reciprocal plots for the formation of p-nitroaniline would be expected to be non-linear. The double reciprocal plots for the formation of p-nitroaniline at several different pH values are shown in Figure 8. These results are consistent with the proposed kinetic mechanism.

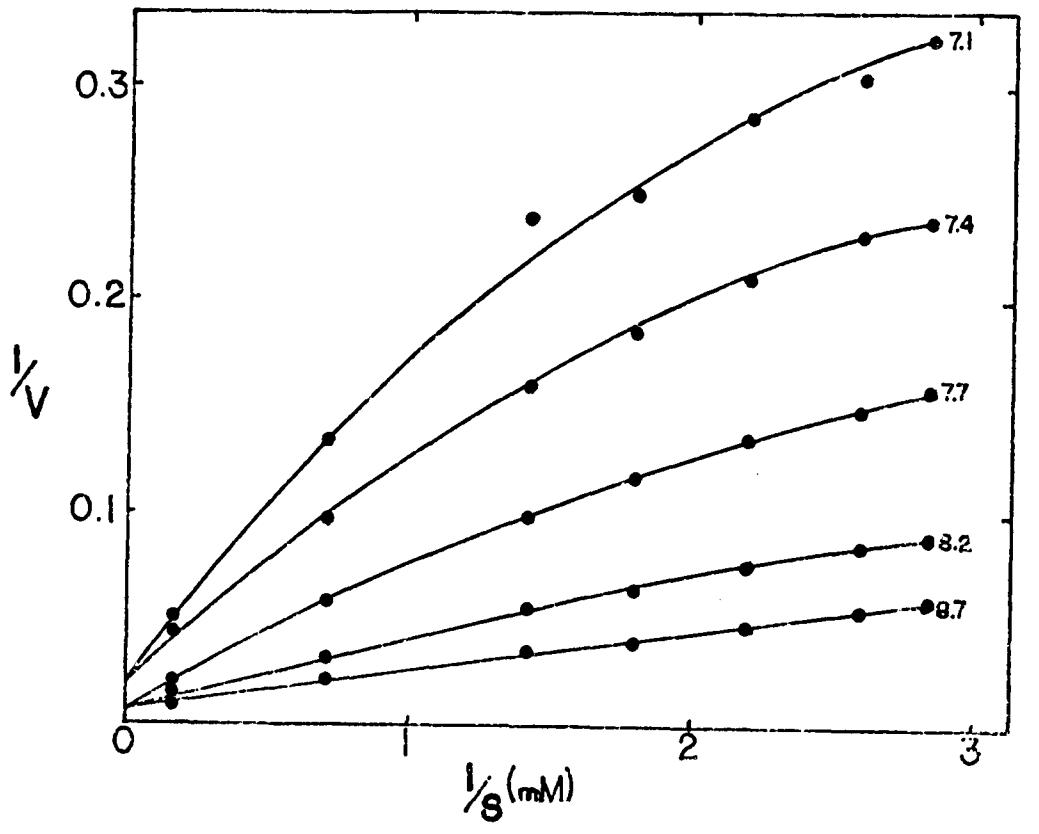


Fig. 8. Effect of pH on the initial velocity of p-nitroaniline formation from L-γ-glutamyl-p-nitroanilide. The reaction mixtures (final volume, 1 ml) contained 100 mM Tris-HCl at the pH values indicated at the end of each curve, γ-glutamyl-p-nitroanilide as substrate (S), and 0.05 µg of enzyme. The initial velocity is expressed as µmol of p-nitroaniline formed per h per 0.05 µg enzyme. Incubation time was between 9 and 45 min.

At low pH values and at low L-γ-glutamyl-p-nitroanilide concentrations the predominant reaction is hydrolysis yielding glutamate and free p-nitroaniline. With increasing concentrations of L-γ-glutamyl-p-nitroanilide the rate of transpeptidation increases disproportionately because the substrate, in addition to acting as a γ-glutamyl donor, now competes effectively with water as an acceptor of the γ-glutamyl-enzyme intermediate. This dual role for L-γ-glutamyl-p-nitroanilide results in a marked acceleration of the reaction as the L-γ-glutamyl-p-nitroanilide concentration increases. A consequence of this is an increase in the slope of the reciprocal velocity plots.

At high pH values, no disproportionate velocity increase of p-nitroaniline formation is observed. This is due to the fact that at the concentration of L-γ-glutamyl-p-nitroanilide used, hydrolysis is overshadowed by transpeptidation. These last conditions describe the situation where $K_{ibb} << A'$.

3. Kinetics of γ-Glutamyl Transpeptidase With L-γ-Glutamyl-p-nitroanilide and an Added Amino Acid

In the presence of an amino acid and L-γ-glutamyl-p-nitroanilide, γ-glutamyl transpeptidase catalyzes reaction 27 in addition to reactions 24 and 25.

L-γ-glutamyl-p-nitroanilide + amino acid ⟶

L-γ-glutamyl amino acid + p-nitroaniline (27)

The velocity of the formation of p-nitroaniline can be calculated from the equation which describes the release of the first product in Mechanism II. The scheme shown in Mechanism II is similar to that shown in Mechanism I in that Mechanism II is also a modified ping-pong mechanism. In this case, however, there are two amino acids competing for the activated enzyme intermediate (F).

The initial velocity equation for the formation of the first product (P) is:

$$v_p = \frac{V_a A}{K_{ah} + A\left(\frac{1 + \frac{B}{K_{bt}} + \frac{C}{K_{ct}}}{1 + \frac{B}{K_{ibb}} + \frac{C}{K_{icc}}}\right)} \qquad (28)$$

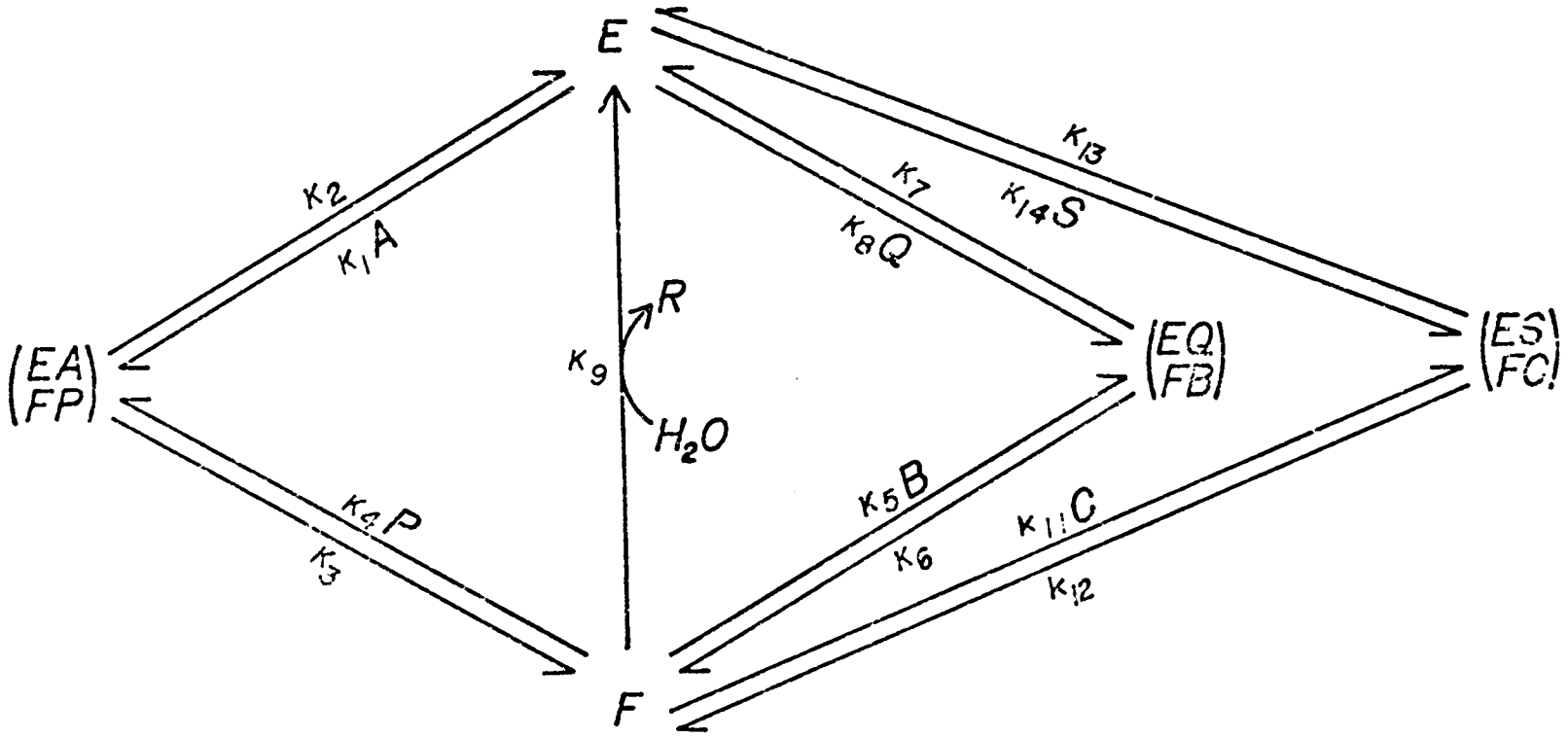
where C is the second amino acid and the new constants are defined as

$$K_{ct} = \frac{(k_{12} + k_{13})\ (k_3 + k_9)}{k_{11}\ (k_3 + k_{13})} \qquad (28a)$$

$$K_{icc} = \frac{k_9(k_{12} + k_{13})}{k_{11}k_{13}} \qquad (28b)$$

The new constants K_{ct} and K_{icc} correspond to the constants K_{bt} and K_{ibb} respectively.

In the presence of an amino acid, L-γ-glutamyl-p-nitroanilide, (A') acts both as first substrate, (A), and also as one of the amino acids, (B) competing for the active enzyme intermediate.



Mechanism II

The reciprocal form of the velocity equation for the appearance of p-nitroaniline under these circumstances is:

$$\frac{1}{v_p} = \frac{K_{ah}}{V_a}\left(\frac{1}{A'}\right) + \frac{1}{V_{ab}}\left(\frac{K_{bt} + A' + \frac{K_{bt}}{K_{ct}}C}{K_{ibb} + A' + \frac{K_{ibb}}{K_{icc}}C}\right) \qquad (29)$$

This equation predicts that the double reciprocal plots of $1/v_p$ versus $1/A'$ will be non-linear for p-nitroaniline release. The experimental results shown in Figure 9 are consistent with this prediction. At elevated pH, when K_{ibb} has already been shown to be much less than the concentration of A' used in these experiments, three patterns of p-nitroaniline formation can be distinguished.

When $(K_{ibb}/K_{icc})C$ is much less than A', the reciprocal velocity plots are linear with V_{max} equal to V_{ab} and K_m equal to $[(K_{ah}V_{ab})/V_a + K_{bt} + (K_{bt}/K_{ct})C]$.

When $(K_{ibb}/K_{icc})C$ is greater than A' the reciprocal velocity plots are also linear with V_{max} equal to $V_{ab}K_{ibb}K_{ct}C/K_{bt}K_{icc}(K_{ct} + C)$ and a K_m equal to $(K_{ct}K_{ab}C)/(K_{icc}(K_{ct} + C))$. When $(K_{ibb}/K_{icc})C$ is approximately equal to A', the reciprocal velocity plots are non-linear having apparent values of K_m and V_{max} between these two extremes.

The results obtained in Figure 9 can be interpreted as follows. (K_{ibb}.methionine)/(K_{icc}.L-γ-glutamyl-p-nitroanilide) is the ratio of the transpeptidation product formed with methionine when compared to that formed with γ-glutamyl-p-nitroanilide. At a constant concentration of methionine

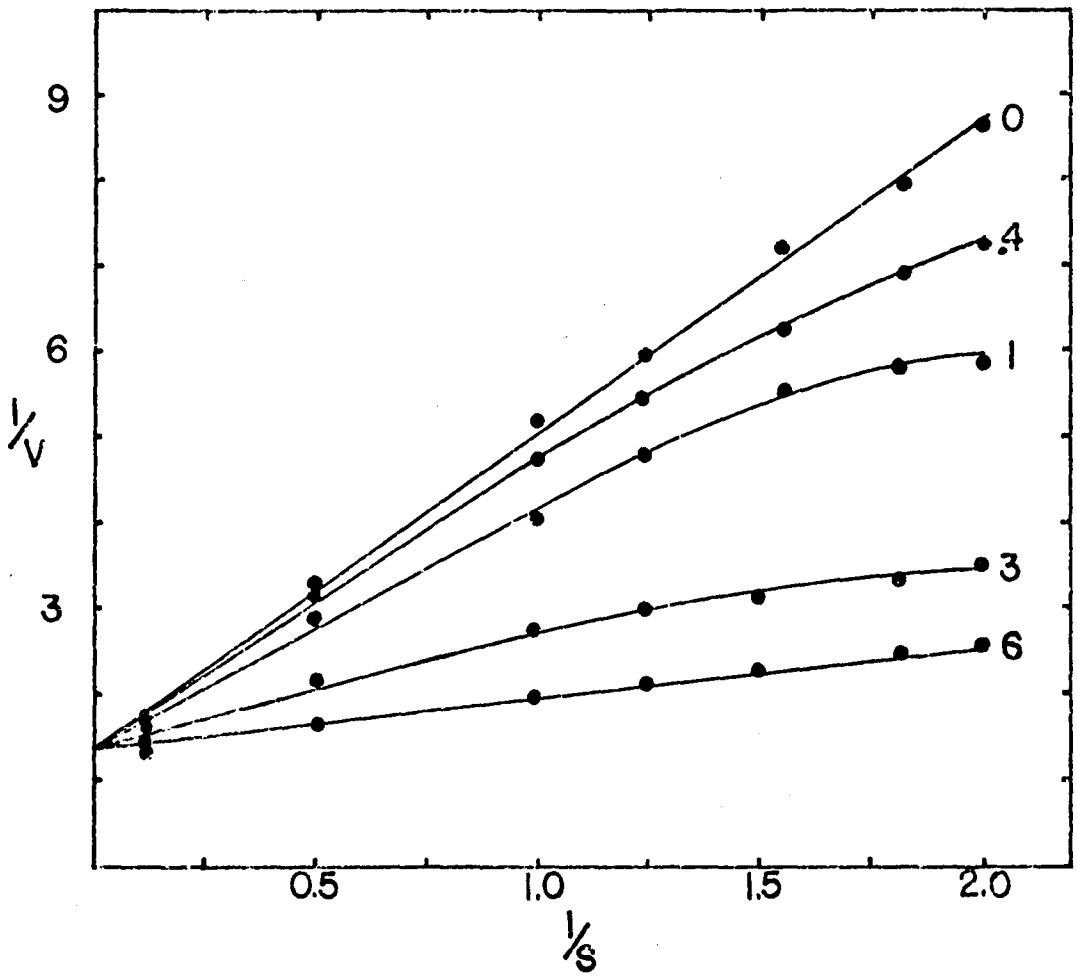


Fig. 9. Effect of L-methionine on the initial velocity pattern of p-nitroaniline formation from L-γ-glutamyl-p-nitroanilide. The reaction mixture (final volume, 1 ml) contained 100 mM Tris-HCl (pH 9.0), γ-glutamyl-p-nitroanilide as substrate (S), L-methionine and 0.032 µg of enzyme. The concentrations of L-methionine (mM) are listed at the end of each curve. Initial velocity is expressed as µmol of p-nitroaniline formed per h per 0.032 µg enzyme. Incubation times were between 25 and 50 min.

and at low concentrations of L-γ-glutamyl-p-nitroanilide, the ratio (K_{ibb}.methionine)/K_{icc}.L-γ-glutamyl-p-nitroanilide) is high implying that the predominant mode for the formation of p-nitroaniline is through the transpeptidation reaction to methionine. This situation is analogous to the case described before with the formation of L-α-aminobutyrate from L-α-methyl-γ-glutamyl-L-α-aminobutyrate in the presence of methionine which yielded intersecting initial velocity patterns. As the concentrations of L-γ-glutamyl-p-nitroanilide is increased, autotranspeptidation to L-γ-glutamyl-p-nitroanilide becomes significant and deviations from linearity occur. With increasing concentrations of methionine higher concentrations of L-γ-glutamyl-p-nitroanilide are needed to cause deviations from linearity. At very high concentrations of L-γ-glutamyl-p-nitroanilide the predominant mode for the formation of p-nitroaniline is through autotranspeptidation and all curves converge independent of the concentration of methionine.

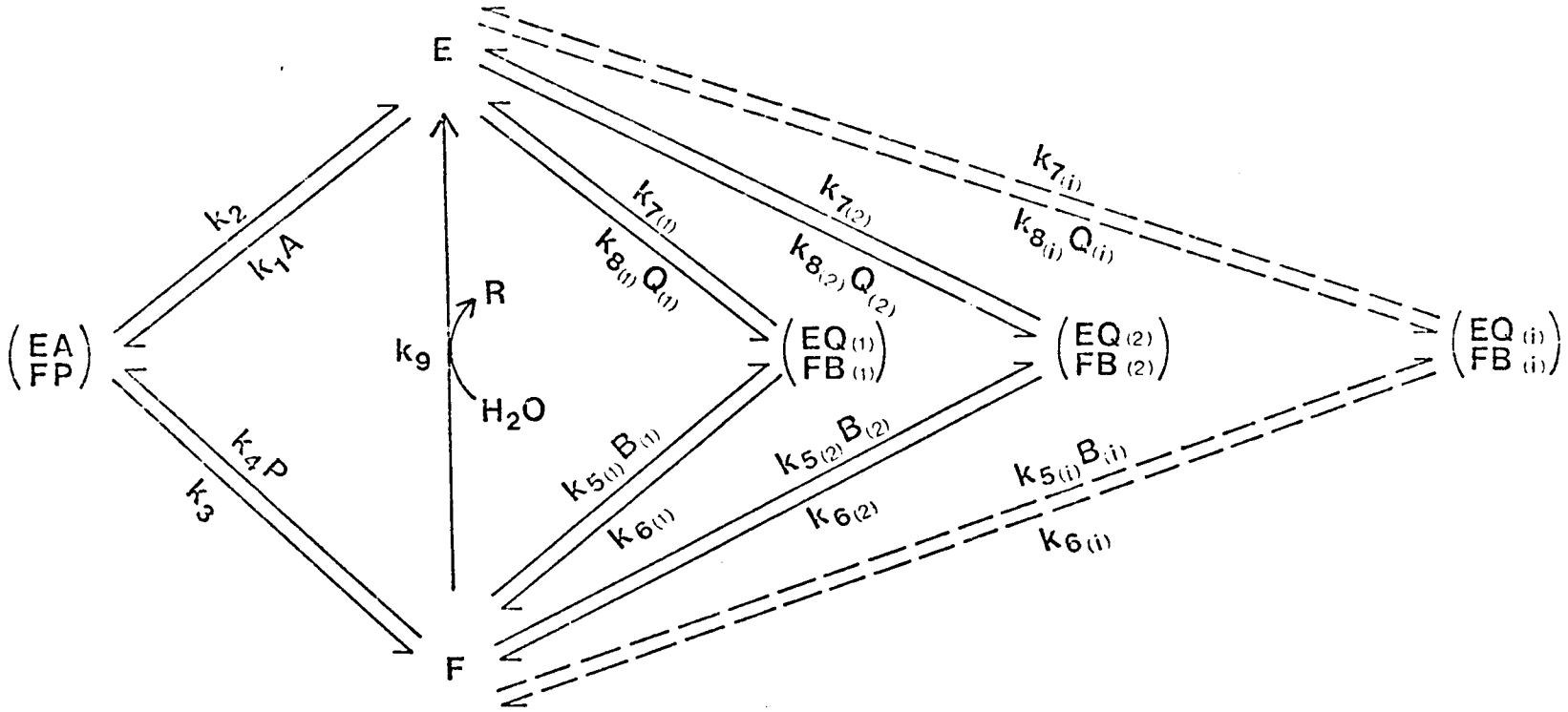
4. Kinetics of γ-Glutamyl Transpeptidase in the Presence of Several Amino Acids

Previous studies of the interactions of amino acids with γ-glutamyl transpeptidase have been limited to experiments in which optimal concentrations of a single amino acid or peptide acceptor have been used. This type of experiment does not reflect the in vivo situation, where a complex mixture of amino acids would be expected to interact with the enzyme.

In order to better understand the interactions which occur between γ-glutamyl transpeptidase in the simultaneous presence of several amino acids, we studied the kinetics of the scheme shown as Mechanism III. This mechanism is probably more representative of the interactions which occur with γ-glutamyl transpeptidase in vivo then the scheme shown as Mechanism I, since Mechanism III also considers the competition between the amino acids for the binding site of the enzyme. Mechanism III is a generalized model of the ping-pong mechanism shown as Mechanism I. The only difference between the two mechanisms is that in Mechanism III several amino acids $[B_{(1)}, B_{(2)} \cdots\cdots B_{(n)}]$, instead of one, are capable of reacting with the γ-glutamyl-enzyme intermediate to form the corresponding transpeptidation product $[Q_{(1)}, Q_{(2)} \cdots\cdots Q_{(n)}]$.

The initial velocity equations for Mechanism III which describe the formation of the first product, $v_{(p)}$ (equation 30) and the individual transpeptidation products (equation 31) are:

$$v_{(p)} = \frac{V_a A}{K_{ah} + A \left(\dfrac{\sum_i \dfrac{B_{(i)}}{K_{bt_{(i)}}} + 1}{\sum_i \dfrac{B_{(i)}}{K_{ibb_{(i)}}} + 1} \right)} \qquad (30)$$



Mechanism III

$$V_{q(i)} = \frac{\frac{V_{ab(i)}}{K_{bt(i)}} B_{(i)} A}{K_{ah}\left(\sum_i \frac{B_{(i)}}{K_{ibb(i)}} + 1\right) + A\left(\sum_i \frac{B_{(i)}}{K_{bt(i)}} + 1\right)} \quad (31)$$

where V_a (equation 23f), K_{ah} (equation 23a) and E_o are as previously defined. The subscript in parenthesis denotes the amino acid to which the constant of Mechanism III applies and:

$$V_{ab(i)} = \frac{k_3 k_{7(i)} E_o}{k_3 + k_{7(i)}} \quad (31a)$$

$$K_{ibb(i)} = \frac{k_9(k_{6(i)} + k_{7(i)})}{k_{5(i)} \cdot k_{7(i)}} \quad (31b)$$

$$K_{bt(i)} = \frac{(k_{6(i)} + k_{7(i)})(k_3 + k_9)}{k_{5(i)}(k_3 + k_{7(i)})} \quad (31c)$$

An analysis of these expressions shows that the initial velocity plots display complex behavior. If the γ-glutamyl compound is capable of acting both as a γ-glutamyl donor and also as an amino acid acceptor, and the concentration of all amino acids in the incubation medium are kept constant, then the reciprocal velocity plots for the formation of the first product, or of the transpeptidation product, versus the concentration of the γ-glutamyl donor ($1/v_{(p)}$ or $1/v_{q(i)}$ vs. 1/A) are non-linear. When the γ-glutamyl donor is not concurrently capable of acting as an acceptor, these same initial velocity plots are linear.

The K_m and V_{max} values though are dependent on the L-γ-glutamyl donor, its concentration and also on the concentration of the individual amino acids of the solution.

At constant γ-glutamyl donor concentrations, the reciprocal velocity plot for the formation of the first product versus the concentration of any one of the amino acids of the solution ($1/v_{(p)}$ vs $1/B_{(i)}$) is non-linear. Under these same conditions, the reciprocal velocity plot for the formation of the transpeptidation product of an amino acid, versus its precursor amino acid concentration ($1/v_{q(i)}$ vs. $1/B_{(i)}$) is linear. The K_m and V_{max} values obtained, however, are dependent on the structure and concentration of the γ-glutamyl donor and the concentration of the amino acids of the solution.

The relationship between the rates of formation of the individual transpeptidation products (e.g. $v_{q(1)}$ and $v_{q(2)}$) can conveniently be expressed by equation 32.

$$\frac{v_{q(1)}}{v_{q(2)}} = \frac{\dfrac{V_{ab(1)}B_{(1)}A}{K_{bt(1)}}}{\dfrac{V_{ab(2)}.B_{(2)}A}{K_{bt(2)}}} \qquad (32)$$

upon substituting the definitions of $V_{ab(i)}$ (equation 31a) and $K_{bt(i)}$ (equation 31c) the following expression is obtained (equation 33):

$$\frac{v_{q(1)}}{v_{q(2)}} = \frac{k_{5(1)}\ k_{7(1)}}{k_{6(1)} + k_{7(1)}} \cdot \frac{k_{6(2)} + k_{7(2)}}{k_{5(2)}\ k_{7(2)}} \cdot \frac{B_{(1)}}{B_{(2)}} \qquad (33)$$

Equation 33 can alternatively be written as:

$$\frac{v_{q(1)}}{v_{q(2)}} = \frac{k_{(1)}}{k_{(2)}} \cdot \frac{B_{(1)}}{B_{(2)}} = \frac{\text{amino acid}_{(1)}}{\text{amino acid}_{(2)}} \text{ reactivity ratio} \qquad (34)$$

where,

$$k_{(1)} = \frac{k_{5(1)}k_{7(1)}}{k_{6(1)} + k_{7(1)}} \qquad (34a)$$

and

$$k_{(2)} = \frac{k_{5(2)}K_{7(2)}}{k_{6(2)} + k_{7(2)}} \qquad (34b)$$

Equations 33 and 34 show that the relative velocities of formation of the transpeptidation products are linearly dependent on the relative concentrations of the precursor amino acids. The constant of proportionality $k_{(1)}/k_{(2)}$, is related to the interactions of the amino acids with the γ-glutamyl-enzyme intermediate. The interactions are: (1) the relative ability of amino acids to bind to the intermediate $k_{5(1)}/k_{5(2)}$; and (2) the relative abilities of the bound amino acids to consummate the transpeptidation reaction and allow release of the transpeptidation product :

$$\frac{k_{7(1)}}{k_{6(1)} + k_{7(1)}} \cdot \frac{k_{6(2)} + k_{7(2)}}{k_{7(2)}}$$

The constant $k_{(1)}/k_{(2)}$ as defined in equation 34 is independent of terms related to the binding of the L-γ-glutamyl donor. In view of the fact that this constant reflects the relative interactions which occur during, or

after, the binding of the amino acid to the enzyme we have defined this constant as the reactivity ratio of the amino acid.

The reactivity ratio is a useful parameter to assess the interactions of amino acids with γ-glutamyl transpeptidase. As can be seen from equation 34 all that is necessary to determine the reactivity ratio of amino acids is a knowledge of the concentrations of the amino acids, and the velocity of formation of the corresponding transpeptidation products. The reactivity ratio is, thus, independent of most other conditions of the incubation mixture such as the concentration of the enzyme and both the concentration of amino acid acceptors and γ-glutamyl donors. Evidence that the amino acid reactivity ratios for γ-glutamyl transpeptidase are independent of these conditions will be shown below.

The reactivity ratio, furthermore, is a mathematical expression for a readily understandable physical process. The ratio describes the relative abilities of amino acids to compete for reaction with the intermediate generated in the transpeptidase reaction. As such, reactivity ratios can be used to study the specificity of γ-glutamyl transpeptidase for amino acids when several amino acids are present simultaneously in the incubation mixture. Such studies will also be described below.

5. Reactivity Ratios

a) Independence of Reactivity Ratios on Enzyme Concentrations

The reactivity ratios of amino acids are independent of the concentration of the enzyme. This independence of enzyme concentration can be seen from the data summarized in Table VII. When the concentration of the solublized γ-glutamyl transpeptidase was varied over a 30-fold range (3.6-108 *ng* protein) in the presence of methionine, alanine and the γ-glutamyl donor, no change in the methionine/alanine reactivity ratio was detected. A crude, particle-bound enzyme preparation gave a methionine/alanine reactivity ratio identical to that found in experiments in which the purified enzyme was used. Since the reactivity ratio does not change during the process of solublization of the particle-bound enzyme (which includes deoxycholate extraction and trypsin treatment) (Zelazo and Orlowski, 1976), this procedure apparently does not modify the structure of the amino acid binding site of γ-glutamyl transpeptidase.

b) Independence of the Reactivity Ratio on the Concentration of the Amino Acids Used to Calculate the Ratio

Although the velocities of formation of the individual transpeptidation products markedly changes as the concentration of one of the amino acids is varied, the reactivity ratios remained unaffected. This independence

TABLE VII

EFFECT OF DIFFERENT CONCENTRATIONS OF SHEEP KIDNEY γ-GLUTAMYL TRANSPEPTIDASE ON THE METHIONINE/ALANINE REACTIVITY RATIOS

The incubation mixtures (final volume, 0.2 ml) contained 5 mM methionine, 15 mM alanine and 7 mM N^2-γ-L-glutamyl-N^6-benzyloxycarbonyl-L-lysine as the γ-glutamyl donor in a Tris-HCl buffer (pH 8.7, 140 mM). Tracer quantities of either L-[^{14}C]methionine or L-[^{14}C]alanine were added to the incubation mixtures in order to determine the rate of formation of the γ-glutamyl amino acids (for details see "Methods"). The incubation times varied between 4 and 120 minutes.

γ-Glutamyl Transpeptidase	Methionine/Alanine Reactivity Ratio
ng protein	
3.6	2.26
10.8	2.37
36	2.27
108	2.38
21[a]	2.29

[a]Particle bound γ-glutamyl transpeptidase

can be seen from the data presented in Tables VIII and IX. In Table VIII the methionine concentration was varied while the concentrations of glycylglycine, α-aminobutyrate and glutathione were held constant. The effect of changing the concentration of methionine on the velocity of formation of γ-glutamylglycylglycine, γ-glutamyl-α-aminobutyrate, γ-glutamylmethionine and γ-glutamylglutathione are tabulated. As the concentration of methionine is increased the rate of formation of γ-glutamylmethionine is increased. At the same time, the rate of formation of the other γ-glutamyl compounds is decreased. The various amino acid reactivity ratios, however, remain unchanged.

Table IX describes a similar set of experiments. The concentration of methionine was varied in the presence of constant concentrations of alanine and N^2-γ-L-glutamyl-N^6-benzyloxycarbonyl-L-lysine, as the γ-glutamyl donor. The experiments show that a 10-fold increase in the concentration of methionine results in a 590 percent increase in the rate of formation of γ-glutamylmethionine. Concurrently, the rate of formation of γ-glutamylalanine drops 43 percent. The methionine/alanine reactivity ratios, however, remain unchanged. It is, thus, apparent that the amino acid reactivity ratios are independent of the concentration of the amino acids considered in determining this ratio.

c) Independence of the Reactivity Ratios on the Concentration of Amino Acids Other Than Those Used to Calculate the Ratio

TABLE VIII

EFFECT OF VARYING THE CONCENTRATION OF METHIONINE ON THE RATE OF FORMATION OF TRANSPEPTIDATION PRODUCTS AND ON REACTIVITY RATIOS

The reaction mixture (final volume, 1 ml) contained 1.8 mM glycylglycine, 18 mM L-α-aminobutyrate (αAB), 6 mM glutathione, 7.5 mM dithiothreitol, 10 mM $MgCl_2$, 0 to 24 mM methionine, 11 ng enzyme in a Tris-HCl buffer (100 mM, pH 8.6). Incubation time was 12 min. The formation of the transpeptidation product was measured on an amino acid analyzer (see Method 1 under "Methods"). The data represent the mean of 3 experiments.

Concentration of Met	Rate of Formation of γ-Glutamyl Derivatives of			
	GlyGly	Met	αAB	GSH
mM	nmoles/117 ng protein/12 min			
0	250	0	307	94
1.8	233	118	275	90
3.6	209	238	261	91
6.0	204	353	244	83
12.0	172	552	191	67
24.0	127	796	140	44

TABLE VIII - Continued

Amino Acid Reactivity Ratios					
GlyGly/Met	GlyGly/αAB	GlyGly/GSH	Met/αAB	Met/GSH	GSH/αAB
...	8.86	8.83	...	...	1.01
1.96	8.95	8.65	4.42	4.29	1.03
1.81	8.45	7.68	4.68	4.24	1.11
1.98	8.78	8.07	4.43	4.08	1.09
2.13	9.39	8.29	4.42	4.13	1.13
2.18	9.35	9.12	4.30	4.19	1.03

TABLE IX

EFFECT OF METHIONINE CONCENTRATIONS ON THE METHIONINE/ALANINE REACTIVITY RATIOS OF SHEEP KIDNEY γ-GLUTAMYL TRANSPEPTIDASE

The incubation mixtures (final volume, 0.2 ml) contained 7 mM N^2-γ-L-glutamyl-N^6-benzyloxycarbonyl-L-lysine, 35 ng of enzyme, 15 mM alanine and methionine as listed in the table in a Tris-HCl buffer (pH 8.7, 140 mM). Tracer quantities of either L-[^{14}C]alanine or L-[^{14}C]methionine were added to the incubation mixtures in order to determine the rate of formation of the γ-glutamyl amino acids (for details see "Methods").

Concentration of Methionine	γ-Glutamyl-alanine Formed	γ-Glutamyl-methionine Formed	Methionine/Alanine Reactivity Ratio
mM	μmoles/10 min/35 ng enzyme		
1.0	.1023	.0148	2.21
2.0	.0911	.0264	2.22
3.5	.0870	.0437	2.19
5.0	.0765	.0585	2.33
7.0	.0706	.0737	2.27
10.0	.0586	.0868	2.25

Several experiments have confirmed the independence of reactivity ratio on the number and concentration of the amino acids of the incubation medium. In this case the amino acids which were varied were not those used to determine the amino acid reactivity ratios. For example, Table X shows the effect of various concentrations of glycylglycine on the rate of formation of γ-glutamylmethionine and γ-glutamylalanine in a solution containing alanine, methionine and N^2-γ-L-glutamyl-N^6-benzyloxycarbonyl-L-lysine. Increasing the concentration of glycylglycine causes a concommittant decrease (32%) in the rates of formation of γ-glutamylmethionine and γ-glutamylalanine. This velocity decrease is to be expected since glycylglycine competes with alanine and methionine for reaction with the γ-glutamyl-enzyme intermediate. The methionine/alanine reactivity ratios, however, remain constant and are independent of the presence and concentration of glycylglycine. Furthermore, when in addition to glycylglycine (2mM) other amino acids such as tryptophan, phenylalanine and α-aminobutyrate (each 5 mM) (data not shown), were added to the incubation mixture, a 25% decrease was observed in the rate of formation of the transpeptidation products. There was still, however, no change in the methionine/alanine reactivity ratio. Amino acid reactivity ratios are thus, independent of the presence, and concentration of other amino acids in the solution.

TABLE X

EFFECT OF GLYCYLGLYCINE ON THE METHIONINE/ALANINE REACTIVITY RATIOS OF SHEEP KIDNEY γ-GLUTAMYL TRANSPEPTIDASE

The incubation mixtures (final volume, 0.2 ml) contained 7 mM N^2-γ-L-glutamyl-N^6-benzyloxycarbonyl-L-lysine, 15 mM alanine, 5 mM methionine, glycylglycine at the concentrations listed, and 35 ng of enzyme in a Tris-HCl buffer (pH 8.7, 140 mM) Tracer quantities of either L-[^{14}C]methionine or L-[^{14}C]alanine were added to the incubation mixtures in order to determine the rate of formation of the γ-glutamyl amino acids (for details see "Methods").

Concentration of Glycylglycine	γ-Glutamyl-alanine Formed	γ-Glutamyl-methionine Formed	Methionine/Alanine Reactivity Ratio
mM	nmoles/10 min/35 ng enzyme		
0	7.64	5.85	2.33
1	7.39	5.38	2.22
2	7.12	5.14	2.20
4	6.18	4.80	2.37
8	5.24	3.86	2.23

d) Independence of the Reactivity Ratios on the Concentration of the γ-Glutamyl Donor

That the reactivity ratios are independent of the concentration of the L-γ-glutamyl donor is shown in Figure 10. When glycylglycine, α-aminobutyrate and methionine were incubated with increasing concentrations of glutathione, and the initial velocity for the formation of the three γ-glutamyl products was measured, an increase in the velocity of formation of the three γ-glutamyl derivatives with the increase in the concentration of glutathione was observed. At the highest concentration of glutathione used in these experiments, however, some decrease in the formation of the transpeptidation products may have occurred. This decrease was probably due to interference caused by the autotranspeptidation reaction of glutathione and the formation of γ-glutamylglutathione. The various amino acid reactivity ratios (glycylglycine/methionine, glycylglycine/α-aminobutyrate, methionine/α-aminobutyrate) are, however, unaffected by these glutathione concentration changes. In a similar experiment summarized in Table IX, methionine and alanine were incubated witha broader range of glutathione concentrations (2.8-21 mM) the resultant methionine/alanine reactivity ratios were here also found to be independent of the concentration of the γ-glutamyl donor.

e) Independence of Reactivity Ratios on the Structure of the L-γ-Glutamyl Donor

Fig. 10. Effect of varying the concentration of GSH on the transpeptidation product formation and on the reactivity ratios of the amino acids. The reaction mixtures (final volume, 1.0 ml) contained 1.5 mM glycylglycine, 5 mM methionine, 15 mM α-aminobutyrate, 10 mM $MgCl_2$, 11.2 mM dithiothreitol, 32 ng of enzyme, 100 mM Tris-HCl (pH 8.7) and 1.2 to 7.8 mM glutathione. The incubation time was 10 minutes. The velocities for the formation of the transpeptidation product were measured on an amino acid analyzer (see Method 1 under "Methods"). Five experiments were performed and the values shown represent the mean ± S.E. The initial velocities for the formation of γ-glutamylmethionine (■), γ-glutamyl-α-aminobutyrate (▲), and γ-glutamylglycylglycine (●), are shown as the concentration of glutathione was varied from 1.2 to 7.8 mM. The insert shows the reactivity ratios of glycylglycine/methionine (k_1/k_3), glycylglycine/α-aminobutyrate (k_1/k_2), and methionine/α-aminobutyrate (k_3/k_2) as the concentration of glutathione was varied from 1.2 to 7.8 mM.

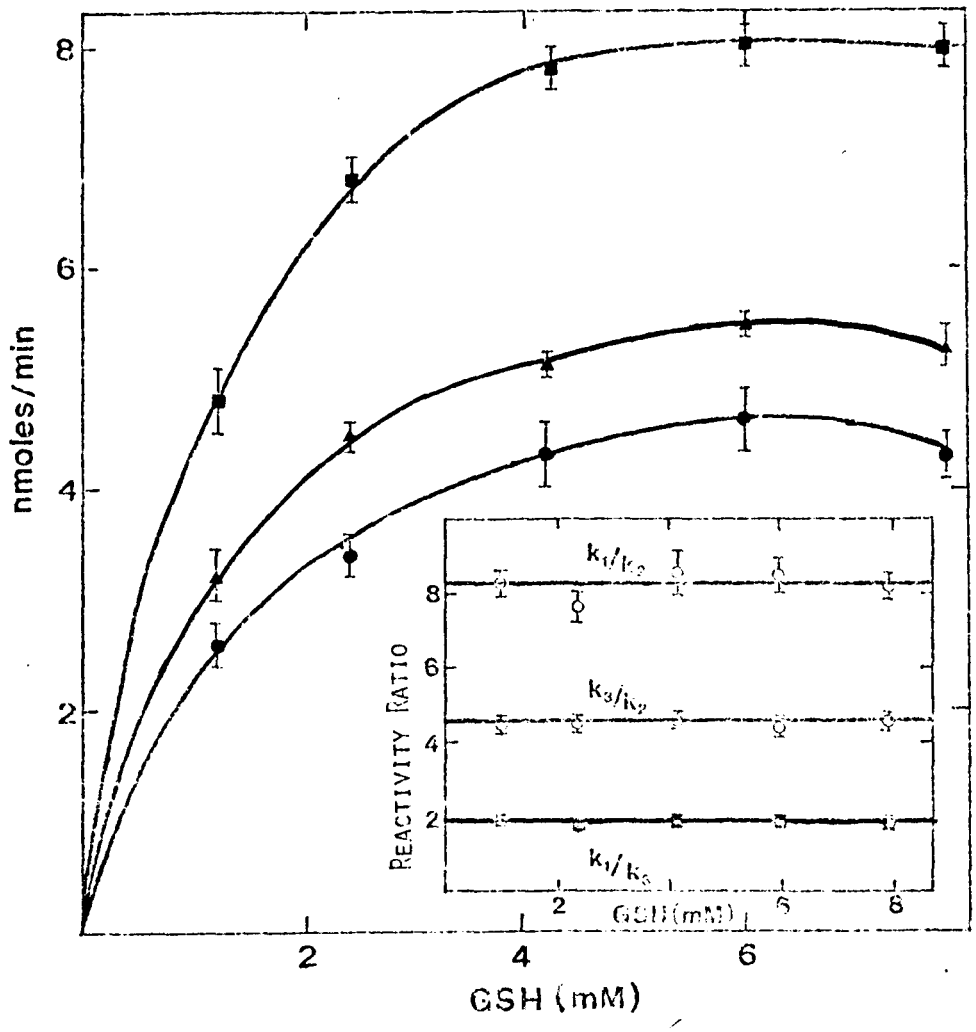

nmoles/min
GSH (mM)
REACTIVITY RATIO
GSH(mM)

TABLE XI

EFFECTS OF DIFFERENT GLUTATHIONE CONCENTRATIONS ON THE METHIONINE/ALANINE REACTIVITY RATIO

The incubation mixtures (final volume, 0.2 ml) contained 5 mM methionine, 15 mM alanine, glutathione at the concentrations listed and 50 ng protein in a Tris-HCl buffer (pH 8.7, 140 mM). Tracer quantities of either L-[^{14}C]-methionine or L-[^{14}C]alanine were added to the incubation mixtures in order to determine the rate of formation of the γ-glutamyl amino acids (for details see "Methods"). The incubation time was 6 minutes.

Concentration of Glutathione	Methionine/Alanine Reactivity Ratio
mM	
2.8	2.21
7.0	2.28
14.0	2.08
21.0	2.17

According to the mechanism of action of γ-glutamyl transpeptidase (Mechanism III), the same γ-glutamyl-enzyme intermediate would be expected to be formed from any L-γ-glutamyl donor. The structure of such an intermediate should be independent of the other part of the peptide (i.e. the leaving group). This intermediate would, furthermore, be predicted to display the same pattern of reaction with the amino acid acceptors of the incubation medium, independent of the structure of the parent donor.

To test this hypothesis we incubated γ-glutamyl transpeptidase with L-methionine and L-alanine and a number of γ-glutamyl donors (Table XII). Subsequent to this incubation, the reactivity ratio of the amino acids was determined as described under "Methods". As predicted, the reactivity ratio obtained with each of the L-γ-glutamyl compounds was almost identical (2.21-2.45). This finding is consistent with and supports the view that all L-γ-glutamyl donors yield the same L-γ-glutamyl-enzyme intermediate. A similar conclusion can be drawn from the data summarized in Table XII. In this set of experiments the various reactivity ratios were determined when glycylglycine, α-aminobutyrate and methionine were incubated with any of three different L-γ-glutamyl donors. Here too, there were no significant differences in the various amino acid reactivity ratios when the L-γ-glutamyl donor was changed.

f. Dependence of the Amino Acid Reactivity Ratios on the Structure of the γ-Glutamyl Portion of the Donor

TABLE XII

EFFECT OF THE γ-GLUTAMYL DONOR STRUCTURE ON THE METHIONINE/ALANINE REACTIVITY RATIO

The reaction mixtures (final volume, 0.2 ml) contained 5 mM L-methionine, 15 mM L-alanine, 32-97 ng of sheep kidney γ-glutamyl transpeptidase, 140 mM Tris-HCl buffer (pH 8.7) and a tracer amount of either L-[^{14}C]methionine or L-[^{14}C]alanine and one of the γ-glutamyl donors (7mM) listed in the table. When glutathione was the γ-glutamyl donor 7.5 mM dithiothreitol was included in the incubation mixtures. Data are mean values ± S.E. The number of experiments is given in parentheses. For details of the determination of the labeled γ-glutamyl product see under "Methods".

γ-Glutamyl Donor	Methionine/Alanine Reactivity Ratio	
L-γ-Glutamyl-O-benzyl ester	2.36	(3)
L-γ-Glutamyl-L-α-aminobutyrate	2.45	(3)
N^2-γ-L-Glutamyl-N^6-benzyloxycarbonyl-L-lysine	2.33 ± .02	(7)
GSH	2.31 ± .05	(10)
L-γ-Glutamyl-p-nitroanilide	2.21	(4)
D-γ-Glutamyl-p-nitroanilide	1.70 ± .02	(8)
D-γ-Glutamyl-L-phenylalanine	1.76	(2)
L-α-Methyl-γ-glutamyl-L-α-aminobutyrate	1.89 ± .03	(7)

TABLE XIII

EFFECT OF THE STRUCTURE OF THE L-γ-GLUTAMYL DONOR ON AMINO ACID REACTIVITY RATIOS

The reaction mixture (final volume, 1 ml) contained 15 mM glycylglycine, 15 mM α-aminobutyrate(αAB), 5 mM methionine, 7 mM γ-glutamyl donor and 110 ng enzyme in a Tris-HCl buffer (pH 8.7). When glutathione was the γ-glutamyl donor 7.5 mM dithiothreitol, was included. The incubation time was between 20 and 45 minutes depending on the donor. The formation of transpeptidation products was measured on an amino acid analyzer (see Method 1 under "Methods"). The data represent the mean (± S.E.M.) of 4 experiments.

Donor	Amino Acid Reactivity Ratios		
	GlyGly/Met	GlyGly/αAB	Met/αAB
Glutathione	2.21(±.07)	9.20(±.3)	4.18(±.07)
L-γ-Glutamyl-L-phenylalanine	2.14(±.12)	8.82(±.4)	4.11(±.07)
L-γ-Glutamyl-O-benzyl ester	2.33(±.11)	9.49(±.4)	4.10(±.07)

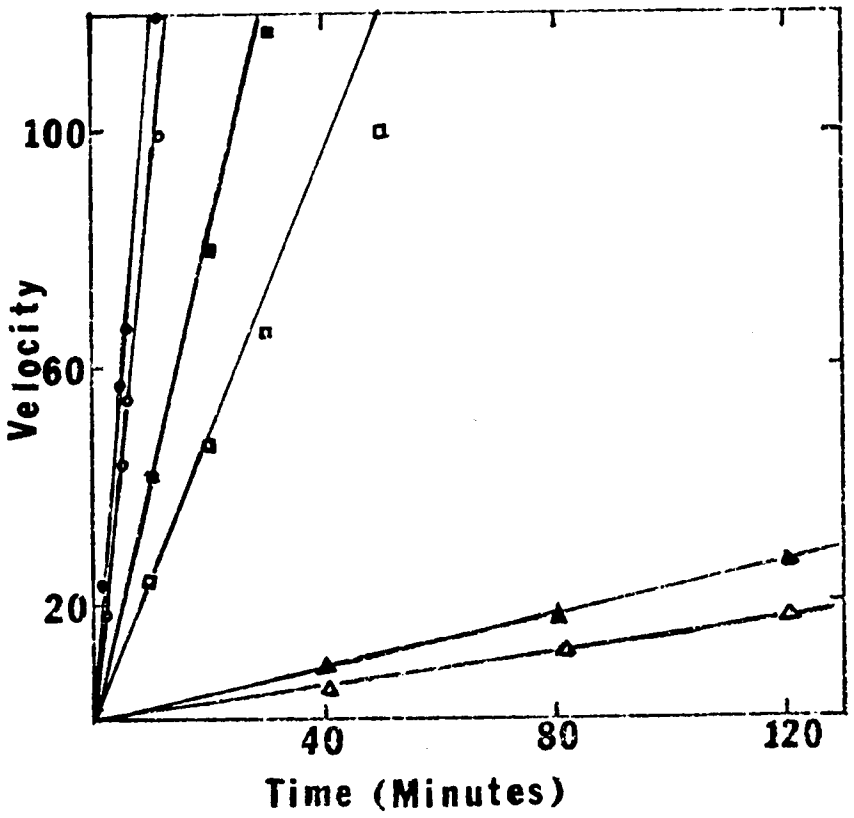
Previous studies have shown that the specificity of γ-glutamyl transpeptidase is not limited to L-γ-glutamyl compounds as donors of the γ-glutamyl group. The enzyme can interact with D-γ-glutamyl compounds (Orlowski and Meister, 1965; Thompson and Meister, 1976). The enzyme can also interact with L-α-methyl-γ-glutamyl derivatives although at a significantly reduced rate (Orlowski and Meister, 1965; Karkowsky et al., 1976). The mechanism of action of the enzyme with these substrates is identical to that postulated for the L-γ-glutamyl donors. An activated γ-glutamyl-enzyme is formed as an intermediate in each of these reactions. The structure of the D-γ-glutamyl-, and L-α-methyl-γ-glutamyl-, enzyme intermediates, however, would be expected to be somewhat different from that formed with L-γ-glutamyl derivatives. It was therefore of interest to determine whether a change in the structure of the γ-glutamyl-enzyme intermediate would affect the interactions of this complex with amino acids. Table XII shows that when D-γ-glutamyl compounds were used as the γ-glutamyl donors (i.e. D-γ-glutamyl-p-nitroanilide or D-γ-glutamyl-L-phenylalanine) the resulting methionine/alanine reactivity ratios were approximately the same for the two D- donors. The two D-compounds, however, displayed methionine/alanine reactivity ratios which were significantly different from those observed with the L-donors (Table XII). The similarity in the reactivity ratios for the two D-compounds apparently results from the identical intermediate generated by both

D-donors. This D-γ-glutamyl-enzyme intermediate is as with the L-intermediate divided at a consistent rate ratio, between methionine and alanine to form the corresponding transpeptidation products.

In a similar experiment, when L-α-methyl-γ-glutamyl-L-α-aminobutyrate was used as the γ-glutamyl donor the methionine/alanine reactivity ratio differed from those obtained with the L-γ-glutamyl-, and D-γ-glutamyl-, donors.

These results suggest that modification of the structure of the glutamyl donor influences its interaction with the enzyme and thus, the amino acid specificity of the reaction. The changes in specificity do not seem to be due to artifacts caused by the interference of secondary reactions. This can be seen from the fact that the rate of formation of the various γ-glutamyl derivatives of alanine and methionine were initially linear with time, when glutathione, D-γ-glutamyl-p-nitroanilide or L-α-methyl-γ-glutamyl-L-α-aminobutyrate were the γ-glutamyl donors (Figure 11). At longer incubation times, however, the initial velocities for the formation of the various γ-glutamyl derivatives of glutathione, and more noticeably, D-γ-glutamyl-p-nitroanilide begin to deviate from linearity. The reason for this deviation is probably due to the increasing prevalence of secondary reactions. Such reactions probably include the re-utilization of the γ-glutamyl derivatives, which were formed as products, as acceptors with the formation of

Fig. 11. Initial velocities for the formation of various γ-glutamyl derivatives of methionine and alanine. The incubation medium (final volume, 0.2 ml) contained 5 mM methionine, 15 mM alanine, 7 mM γ-glutamyl donor (either GSH, D-γ-glutamyl-p-nitroanilide or L-α-methyl-γ-glutamyl-L-α-aminobutyrate), 49 ng of sheep kidney γ-glutamyl transpeptidase and 140 mM Tris-HCl (pH 8.7). When GSH was the γ-glutamyl donor 7.5 mM dithiothreitol was also included. Tracer quantities of [^{14}C]alanine were included in the incubation medium when the product measured was γ-glutamyl-[^{14}C]alanine derivatives (filled symbols). Tracer quantities of [^{14}C]methionine were included in the incubation medium when the product measured was γ-glutamyl [^{14}C]methionine derivatives (open symbols). The concentration of the L-γ-glutamyl derivatives (circles), D-γ-glutamyl derivatives (squares) and α-methyl-γ-glutamyl derivatives (triangles) of alanine and methionine were all isolated and counted as described in Method 2 under "Methods". The velocity is expressed as nmoles formed per 48 ng enzyme.


100
60
20
Velocity
40
80
120
Time (Minutes)

higher order transpeptidation products. It is also possible that the γ-glutamyl derivatives formed as products could be reutilized as competitive donor substrates.

g) Reactivity Ratios of Serum Amino Acids

Though the amino acid reactivity ratios are independent of the concentration of the enzyme, the concentration of the serum amino acids, and the concentration of the L-γ-glutamyl donor, they may still be dependent on other factors such as the concentration of cations and the pH of the medium. In order to obtain a better understanding of the amino acids which are primarily involved in the degradation of glutathione under physiological conditions, the reactivity ratios of several serum amino acids were measured.

When N^2-γ-L-glutamyl-N^6-benzyloxycarbonyl-L-lysine and sheep kidney γ-glutamyl transpeptidase were incubated with pooled normal serum (pH 7.3), the reactivity ratios relative to alanine listed in Table XIV were obtained. The methionine/alanine reactivity ratios under these conditions (2.76) are somewhat different than those observed at pH 8.7 in Tris-HCl buffer (2.31).

Of the amino acids in serum which were tested, the reactivity ratios of glutamine, cystine and methionine were the highest. The small, neutral amino acids: alanine, serine, and glycine were good substrates, but had reactivity ratios of approximately 20% of the previously mentioned amino acids. The aromatic, branched chain, acidic (glutamate) and basic (arginine) amino acids which were tested

TABLE XIV

REACTIVITY RATIOS OF SERUM AMINO ACIDS TOWARD γ-GLUTAMYL TRANSPEPTIDASE

The incubations were carried out as described under "Methods", with 57-70 ng of sheep kidney γ-glutamyl transpeptidase. The serum was preincubated 1 hour with a mixture of 95% O_2-5% CO_2, and the pH adjusted to 7.3. The incubation time was 10 to 25 minutes. Data are mean values ± S.E.

Amino Acid	Serum Concentration	Reactivity Ratio	Relative Utilization of Amino Acid
	mM		
Cystine	0.041	3.66 ± 0.54	0.32
Glutamine	0.405	3.66 ± 0.76	3.15
Methionine	0.358[a]	2.76 ± 0.30	0.06[b]
Alanine	0.470	1.00	1.00
Serine	0.185	0.66 ± 0.07	0.26
Histidine	0.078	0.53 ± 0.06	0.09
Glycine	0.386	0.42 ± 0.03	0.34
Leucine	0.149	0.32 ± 0.02	0.10
Tyrosine	0.075	0.32 ± 0.02	0.05
Arginine	0.121	0.31 ± 0.07	0.08
Phenylalanine	0.129	0.30 ± 0.03	0.08
Glutamate	0.276	0.14 ± 0.02	0.08
Valine	0.221	0.04 ± 0.02	0.02

[a]Methionine was added to the incubation mixtures measuring the reactivity ratio of this amino acid. The final concentration of this amino acid is given.

[b]Normal serum methionine concentrations were approximately .010 mM. This value was used to calculate the relative utilization of this amino acid.

had low reactivity ratios when compared with cystine or glutamine.

In order to determine the most probable substrate for γ-glutamyl transpeptidase, the concentration of the amino acid, in addition to the reactivity ratio must also be considered. The product of the reactivity ratio and the amino acid concentration is the relative (in this experiment relative to alanine) amount of that particular amino acid which serves as the substrate in the transpeptidation reaction. The data in Table XIV clearly suggest that under conditions that prevail in serum, glutamine is the amino acid most expected to react with γ-glutamyl transpeptidase. Alanine, and to a lesser extent, glycine and cystine would also seem likely to account for a significant portion of glutathione degradation via γ-glutamyl transpeptidase.

E. Studies of Glutathione Metabolism in Choroid Plexus

Preliminary investigations were also undertaken to study the metabolism of glutathione in rabbit choroid plexus. When rabbit choroid plexuses were incubated for various time periods in Hepes-buffered medium containing glucose, a rapid decline in the intracellular concentrations of total glutathione (GSH + GSSG) was observed (Figure 12). The decline in the concentration of this tripeptide was apparently due to its degradation. Only small (approximately 5% of the initial glutathione concentration) amounts of glutathione were found in the incubation medium, and this small amount did not appreciably change with the time of

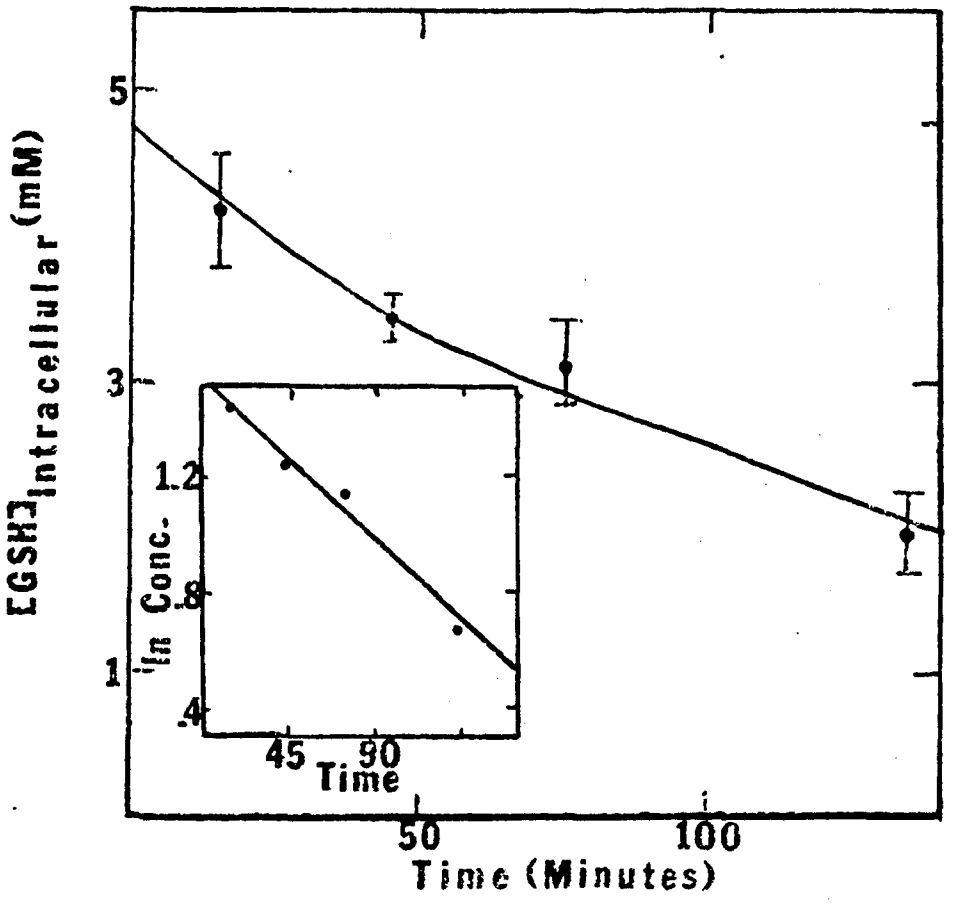


Fig. 12. Concentration of glutathione in rabbit choroid plexus as a function of the duration of incubation. Intracellular concentrations of glutathione were determined as described under "Methods". Time denotes time after decapitation. The inset is the semi-logarithmic plot of ln concentration of glutathione versus time.

incubation. Thus, secretion of oxidized glutathione as observed with red blood cells (Srivastava and Beutler, 1969) and porcine liver (Sies et al., in Flohe et al., 1974), could not totally account for decreases in the intracellular concentration of glutathione. The glutathione found in the incubation medium was more probably due to some tissue damage, or to the hemolysis of red blood cells found in the vasculature of the choroid plexus.

The half-time for the degradation of glutathione in choroid plexus is approximately 110 minutes. Since the value of the half-time of the degradation of glutathione does not compensate for the amount of glutathione that is resynthesized during the time of the incubation, the value for the half-time of turnover of glutathione, under these conditions, may be somewhat smaller. Although it is not known if the in vivo degradation of glutathione occurs as rapidly as was observed in these in vitro experiments, the results (shown in Figure 12) suggest that the choroid plexus has, at least, the capabilities for the rapid degradation of glutathione.

The semi-logarithmic plot of the concentration of glutathione versus time of incubation is linear (Figure 12). The linearity of this plot indicates that the glutathione in the choroid plexus occurs as a single pool, with respect to its ability to be degraded. If glutathione degradation would occur as the result of more than one pool

(i.e. a pool of glutathione which is degraded rapidly and one which is degraded slowly) such a plot would be expected to be non-linear.

V. DISCUSSION

A. Comments on the Use of L-α-Methyl-γ-glutamyl-L-α-aminobutyrate as the γ-Glutamyl Donor

Studies of the kinetics and mechanism of action of γ-glutamyl transpeptidase have been complicated by the occurrence of several competing reactions when glutathione, or any one of a number of synthetic substrates are used (reactions 4,6 and 7). It was for this reason that we synthesized L-α-methyl-γ-glutamyl-L-α-aminobutyrate, a substrate which we anticipated would be a γ-glutamyl donor but not an amino acid acceptor. This approach was stimulated by the following two observations. Firstly, it has been noted that Lα-methyl glutamine is slowly hydrolyzed by the enzyme and no transpeptidation products are detected with this substrate (Orlowski and Meister, 1965). Secondly, α-methyl amino acids have been shown by us (Karkowsky et al., 1976) and others (Tate and Meister,1974) not to act as amino acid acceptors for the γ-glutamyl group when either glutathione or other γ-glutamy. compounds are used as the γ-glutamyl donor. As expected L-α-methyl-γ-glutamyl-L-α-aminobutyrate was shown to have the desirable donor and acceptor properties and was therefore used in kinetic studies of this enzyme.

The use of this substrate eliminates the interference derived from the autotranspeptidation reaction, and allows the separate determination of the hydrolytic reaction catalyzed by γ-glutamyl transpeptidase. Another advantage in using this substrate is that the transpeptidation products formed in the reaction with amino acid acceptors similarly contain a methyl group on the α-carbon and thus are not capable of acting as γ-glutamyl acceptors.

B. Kinetics

The results of our kinetic studies with sheep kidney γ-glutamyl transpeptidase are consistent with Mechanism III. This mechanism predicts that an activated enzyme is formed as an intermediate in the reaction catalyzed by this enzyme. This intermediate can then react with an amino acid (or peptide) acceptor to form the transpeptidation product, or with water to form the hydrolytic product. The most probable structure for this intermediate is a L-γ-glutamyl-enzyme. Such an intermediate is consistent with our observations that when any of several L-γ-glutamyl donors are incubated with γ-glutamyl transpeptidase and two or more amino acids, the ratio of the resultant transpeptidation products does not depend on the structure of the L-γ-glutamyl donor (i.e. the leaving group). These observations indicate that a common intermediate (i.e. a L-γ-glutamyl-enzyme) is formed from all L-γ-glutamyl donors. That this common intermediate is related to the attachment of the L-γ-glutamyl portion of the donor to the enzyme is consistent with our observations on

these transpeptidation product ratios when the γ-glutamyl portion of the donor is modified. When either D-γ-glutamyl-, or L-α-methyl-γ-glutamyl-, donors were incubated under identical conditions as the L-γ-glutamyl donors, the ratio of the resultant transpeptidation products were different than those observed with the L-γ-glutamyl donors. These results suggest that the incubation of D-, or L-α-methyl-γ-glutamyl-, donors with γ-glutamyl transpeptidase results in intermediates (i.e. a D-γ-glutamyl-enzyme or L-α-methyl-γ-glutamyl-enzyme respectively) which are not the same as the L-γ-glutamyl-enzyme intermediate generated by any of the L-γ-glutamyl donors. It should be noted that the two D-γ-glutamyl donors had nearly identical transpeptidation product ratios. The fact that these ratios were similar is consistent with the formation of the same D-γ-glutamyl-enzyme intermediate from both of these donors.

Additional supportive evidence for the formation of a γ-glutamyl-enzyme, was the observation that when [^{14}C]-glutathione and [Gly-H^{3}]glutathione were incubated with rat kidney γ-glutamyl transpeptidase carbon-14 was more effecient-ly bound to the protein than was tritium (Elce and Broxmeyer, 1976). These results suggest that a portion of the glutathione molecule not containing tritium (probably the glutamyl residue) is bound to the enzyme.

A similar γ-glutamyl-enzyme intermediate has been postulated in the reaction catalyzed by transglutaminase (Folk and Chung, 1973) and several other enzymes (Buchanan, 1973).

The conclusion that sheep kidney γ-glutamyl transpeptidase reacts by a ping-pong mechanism modified by a hydrolytic shunt is in agreement with the conclusion concurrently arrived at by other investigators for the γ-glutamyl transpeptidase obtained from rat kidney (Elce and Broxmeyer, 1976), and hog kidney (London et al., 1976). It would thus seem reasonable to suggest that all γ-glutamyl transpeptidases react by this modified ping-pong mechanism.

When L-γ-glutamyl-p-nitroanilide is used as the substrate at various pH values, and the formation of p-nitroaniline is measured as a function of the concentration of the L-γ-glutamyl donor, double reciprocal plots are non-linear. Non-linear double reciprocal plots are also obtained, at optimal pH values, when the rate of the reaction is measured at several fixed methionine concentrations as a function of the concentration of L-γ-glutamyl-p-nitroanilide.

It is therefore of interest that using L-γ-glutamyl-p-nitroanilide as the donor and glycylglycine as the acceptor, with serum γ-glutamyl transpeptidase Stromme and Theodorsen (1976) obtained parallel double reciprocal plots when either the concentration of glycylglycine was varied at several fixed γ-glutamyl-p-nitroanilide concentrations, or when the concentration of γ-glutamyl-p-nitroanilide was varied at several fixed glycylglycine concentrations. Similar parallel double reciprocal plots had previously been described for rat kidney γ-glutamyl transpeptidase when glutathione (which would be expected to act in an analogous

manner to γ-glutamyl-p-nitroanilide) was used as the γ-glutamyl donor and methionine as the γ-glutamyl acceptor (Tate and Meister, 1974a). In both cases ping-pong mechanisms were postulated on the basis of these results. The model presented to explain the kinetic behavior of both rat kidney and serum γ-glutamyl transpeptidase did not take into account the hydrolytic component of the reaction, or the effects of the autotranspeptidation reaction on the kinetics of the reaction. Intersecting or non-linear plots would have thus been predicted. It is possible that under the conditions prevailing in these cases (i.e. relatively high concentrations of acceptor, and relatively low concentrations of γ-glutamyl donor) the slope of the curves may not have been sufficiently influenced by the hydrolytic and autotranspeptidation components of the reaction to show deviations from ping-pong behavior.

Our results which show the kinetics of p-nitroaniline formation from γ-glutamyl-p-nitroanilide at different pH values, clearly indicate that the hydrolytic component of the transpeptidase reaction is of major importance particularly at pH values close to the physiological range. Thus, at pH 7.1 and 7.4 the double reciprocal plots show distinct curvature, indicating that two reactions, autotranspeptidation and hydrolysis, both contribute significantly to p-nitroaniline formation at the concentration of γ-glutamyl-p-nitroanilide used in these experiments. The conclusion that at physiological pH the hydrolytic component is of

significance is consistent with the observation that at lower pH values glutamate is the predominant product when γ-glutamylanilide is incubated with an enzyme isolated from kidney beans (Goore and Thompson, 1967). Glutamate was also shown to be the predominant product when glutathione is incubated with kidney homogenates at pH values below 7.0 (Woodward and Reinhart, 1942). These observations together with the results of our kinetic studies suggest that the hydrolytic reaction catalyzed by the enzyme is of a much greater significance at physiological pH values than is commonly accepted. The significance of this reaction needs, therefore, more consideration in studies of the physiological function of the enzyme .

C. Amino Acid Specificity of γ-Glutamyl Transpeptidase

Understanding of the physiological role of γ-glutamyl transpeptidase requires knowledge of its specificity and mechanism of action.

At pH 8.0-9.0 the main reaction catalyzed by the enzyme is the transfer of the γ-glutamyl group of glutathione to appropriate acceptors. Lowering the pH toward more physiological values, however, decreases the rate of this reaction and increases the relative significance of the hydrolytic reaction. This observation has led some investigators to postulate that the main function of the enzyme is the hydrolysis of extracellular glutathione (Elce and Broxmeyer, 1976; Schulman et al., 1975). Recent studies,

however, in which the in vivo metabolism of γ-glutamyl derivatives was studied have clearly indicated that the transfer reaction catalyzed by γ-glutamyl transpeptidase is a significant part of the metabolism of these compounds (Orlowski and Wilk, 1975; 1976). Since the transfer reaction of γ-glutamyl transpeptidase can be demonstrated in vivo, it seems relevant to obtain a clearer understanding of the amino acid specificity of this reaction.

The amino acid specificity of this enzyme has been usually studied under conditions in which an optimal concentration of a single amino acid acceptor (usually 10-30 mM) (Ball et al., 1956; Revel and Ball, 1959), was incubated with the enzyme and a γ-glutamyl donor such as glutathione (Zelazo and Orlowski, 1976) or a model substrate such as γ-glutamyl-p-nitroanilide (Orlowski and Meister, 1965) at a pH optimal for the transfer reaction (usually 8.2-9.0). The specificity of the enzyme has been evaluated from the ability of an amino acid to accelerate the release of the first product (i.e., cysteinylglycine from glutathione, or p-nitroaniline from γ-glutamyl-p-nitroanilide). These studies have led to a general consensus that the enzyme has a broad amino acid specificity and that it can transfer the γ-glutamyl group to almost any amino acid, although methionine, glutamine and cystine activated the reaction to a greater extent than other amino acids (Orlowski and Meister, 1965; Orlowski and Zelazo, 1976; Thompson and Meister, 1975).

There are several drawbacks in such an approach. The results are dependent on the concentration of the amino acid acceptor and the concentration of the γ-glutamyl donor and do not provide any quantitative information concerning the ability of an amino acid to compete with other amino acid acceptors for the γ-glutamyl-enzyme intermediate. Furthermore, such experiments do not reflect the conditions in vivo where some 20 or more amino acids with widely differing concentrations are expected to compete in the reaction for the γ-glutamyl-enzyme intermediate, in an ionic environment greatly different from a simple buffer solution.

The method used in this thesis to study the specificity of the enzyme is based on kinetic considerations. It determines reactivity ratios which express the ability of any amino acid relative to a reference amino acid (in our case alanine) to react with the γ-glutamyl-enzyme intermediate. By using this ratio the efficiency of any amino acid to function as an acceptor for the γ-glutamyl group can be compared with any other amino acid. Furthermore, the reactivity ratio is independent of the concentration of the enzyme and both the concentration of the amino acid acceptor and the γ-glutamyl donor. It provides therefore a true measure of the ability of a particular amino acid to bind to the enzyme, react with the γ-glutamyl-enzyme intermediate and form the γ-glutamyl product.

This approach provides new insight into the speci-

ficity of γ-glutamyl transpeptidase. Thus although the enzyme can interact with almost any amino acid, its specificity is by no means as broad as was previously suggested. Thus, our determination of the reactivity ratios under conditions found in normal human serum show (Table XIV) that although at equimolar concentrations of amino acids the enzyme would be expected to react approximately equally well with glutamine, cystine and methionine, the reaction with phenylalanine, tyrosine, leucine and arginine would be ten times less favored, and the reaction with glutamate and valine would be even less significant. In addition, knowledge of the reactivity ratios and the concentration of amino acids in serums can be used to calculate the likelihood of a reaction between glutathione and any of the serum amino acids as catalyzed by γ-glutamyl transpeptidase. These calculations show that since glutamine is one of the most predominant serum amino acids, this amino acid would be 30-50 times more favored as substrate in the transpeptidation reaction than the aromatic, branched chain, acidic and basic amino acids. Of the other amino acids, only alanine, and to a lesser extent glycine, cystine and serine would be expected to significantly interact with the enzyme. Although these reactivity ratios were obtained with an enzyme isolated from sheep kidney, the amino acid acceptor specificities as determined by activation of first product release, seem to be similar for enzymes isolated from various sources.

It would, therefore, seem that the observations made here should also be valid for other γ-glutamyl transpeptidase preparations.

The specificity of γ-glutamyl transpeptidase towards glutamine is consistent with the finding of significant γ-glutamylglutamine concentrations in mammalian tissues including brain, intestine and kidney (Kanazawa and Sano, 1967). The high γ-glutamylglutamine concentrations found are of interest when it is noted that γ-glutamylglutamine is an excellent substrate (Orlowski and Meister, 1973) for γ-glutamyl cyclotransferase (Connell and Hanes, 1956). This enzyme catalyzes conversion of γ-glutamyl amino acids to pyrrolidine carboxylate and free amino acids. The rate of formation of γ-glutamylglutamine would, therefore, have to be significant in order to maintain such concentrations of this compound in the presence of an enzyme which readily causes its degradation. These findings suggest that glutamine is the preferred acceptor in vivo in the reaction between glutathione and amino acids.

D. Relationship of the Structure of the γ-Glutamyl-Enzyme Intermediate to the Amino Acid Specificity of γ-Glutamyl Transpeptidase

The structure of the γ-glutamyl-enzyme intermediate apparently influences the specificity of the enzyme towards amino acids. This conclusion is derived from the following two observations. First, reactivity ratios of amino acids remained unchanged when any of several L-γ-glutamyl donors

were used as the substrate in the transpeptidase reaction. Second, reactivity ratios were found to be different from those observed with the L-γ-glutamyl donors when D-γ-glutamyl-, or L-α-methyl-γ-glutamyl-, donors were substituted as substrates in the reaction. Since all L-γ-glutamyl donors yield a common intermediate which differs in structure from the intermediate formed when either D-γ-glutamyl-, or L-α-methyl-γ-glutamyl-, donors are used as substrates, a change in the structure of the intermediate apparently influenced the amino acid specificity of the reaction, as manifest by a change in the amino acid reactivity ratios. Such a structural change is probably due to changes in the orientation of the γ-glutamyl "type" group in the active site of the enzyme. These orientation differences are apparently sufficient to alter either the binding, or the reaction of the amino acid with the γ-glutamyl-enzyme intermediate.

Since the nature of the active site of γ-glutamyl transpeptidase is not yet known, any explanation as to how modification of the γ-glutamyl "type" group in the active site of the enzyme affects the various rate constants is totally conjectural. It is possible, however, to speculate that a change in the structure of the acyl-enzyme intermediate from an L-, to a D-, γ-glutamyl intermediate could slightly alter the orientation of the acyl moiety in the active site of the enzyme. The change in orientation could

possibly make the acyl-enzyme bond more sensitive to steric effects of the approach of the incoming amino acid nucleophile. Since the size of the alkyl groups on methionine and alanine are different, the change in orientation would be expected to decrease the reactivity of methionine relative to alanine and, thus, result in a decrease in the methionine/alanine reactivity ratio.

That the rate constants for binding and reactivity of the second substrate, (i.e. substrate B), are sensitive to modifications in the structure of the acyl-enzyme intermediate has also been observed with guinea pig liver transglutaminase (Chung et al., 1970). Based on initial velocity data the K_{ibb} values were determined for two different B substrates (D-, and L-, alanine ethyl ester) in the presence of each of three different A substrates (p-nitrophenyl acetate, p-nitrophenyl propionate and Z-α-L-glutamyl(γ-p-nitrophenyl ester)glycine). The acyl-enzyme intermediate differed with each of the A substrates. It should be noted that with each of the A substrates the ratio of the K_{ibb} values for the two B substrates is the inverse of the reactivity ratio. It is thus possible to calculate the D-alanine ethyl ester/L-alanine ethyl ester reactivity ratio based on the K_{ibb} values. The ratios were 4.2 for the acetyl-, 1.9 for the propionyl-, and 0.004 for the Z-α-L-glutamylglycine γ-, enzyme intermediates. Evidently small changes in the structure of the intermediate (e.g. from an

acetyl to a propionyl) can result in striking changes in the reactivity ratio. The modest changes which we were able to make in the structure of the acyl-enzyme intermediate of the γ-glutamyl transpeptidase reaction is, therefore, consistent with the relatively small changes in the reactivity ratio which we observed (2.3 to 1.7).

Our conclusion would suggest that a change in the structure of the L-γ-glutamyl donor in vivo would not alter the specificity of the transpeptidase reaction. This conclusion is consistent with the observation that no aminoaciduria was found in patients with pyroglutamic aciduria (Eldjarn et al., 1972). In this disease it is believed that γ-glutamylcysteine, or some other γ-glutamyl derivative, substitutes for glutathione in the transpeptidase reaction of the γ-glutamyl cycle (Wellner et al., 1974). Substitution of different L-γ-glutamyl donors for glutathione would not be expected to alter the specificity of the transpeptidase reaction, the γ-glutamyl cycle and, therefore, amino acid transport in these individuals. It is of interest to note that the Norwegian patient developed a massive aminoaciduria upon injection of a mixture of amino acids (Eldjarn et al., 1972). It could thus be suggested that although the specificity of the transpeptidase reaction may not have been altered the velocity of the reaction could have been markedly reduced. A reduction in the velocity of this reaction would decrease the velocity of the γ-glutamyl cycle

and hence the capacity of the cycle to handle amino acids. Aminoaciduria would thus be a reasonable expectation in the presence of loading doses of amino acids. Different velocities for the formation of various γ-glutamyl derivatives were indeed observed in vitro when different γ-glutamyl donors were used as substrates.

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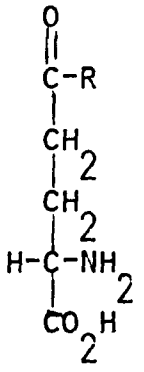
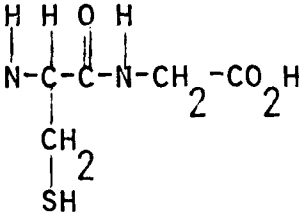
VII APPENDIX

Structures of Various γ-Glutamyl Compounds



Glutathione: R=



γ-Glutamyl-p-nitroanilide: R=