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Isolation of Highly Purified γ-Glutamylcysteine Synthetase from Rat Kidney*

Marian Orlowski and Alton Meister

ABSTRACT: The presence in mammalian kidney of substantial amounts of γ-glutamyl transpeptidase and γ-glutamyl cyclotransferase, enzymes which catalyze the degradation of glutathione and γ-glutamylamino acids, suggested that kidney might also possess catalytic activity capable of synthesizing γ-glutamylamino acids such as γ-glutamylcysteine. However, the determination of γ-glutamylcysteine synthetase in crude tissue preparations which contain enzymes that catalyze the degradation of γ-glutamylamino acids presents a difficult analytical problem. A reliable procedure for the determination of γ-glutamylcysteine synthetase activity in crude tissue preparations was developed and is described here. In the new method, the tissue preparation is incubated with [^{14}C]L-glutamate, adenosine 5′-triphosphate (ATP), magnesium ions, L-α-aminobutyrate, and an excess of purified brain γ-glutamyl cyclotransferase; under these conditions, the [^{14}C]glutamyl moiety of the [^{14}C]γ-glutamylamino acid formed is quantitatively converted into [^{14}C]pyrrolidonecarboxylate, which is separated from other ^{14}C compounds and determined. Application of this procedure indicates that a number of rat tissues contain γ-glutamylcysteine synthetase activity and that the most active tissue is kidney (specific activity, about 10 units (micromoles per hour) per milligram of protein). The enzyme has been purified in about 40% yield from rat kidney leading to an apparently homogeneous (by analytical ultracentrifugation ($s_{20,w}$ = 5.6 S) and acrylamide gel electrophoresis) protein exhibiting a specific activity of 540 units/mg. The present findings indicate that γ-glutamylcysteine synthetase constitutes 2–3% of the protein in the soluble fraction of rat kidney homogenates. The rat kidney preparation described here is about 100 times more active than previously reported preparations of γ-glutamylcysteine synthetase (from hog liver and bovine lens). The pH optimum and the apparent K_m values for the substrates have been determined. The purified rat kidney enzyme loses activity progressively when stored at 0° and may be reactivated by exposure to dithiothreitol. The enzyme is markedly inhibited by *p*-mercuribenzoate, *p*-mercuribenzenesulfonate, and iodoacetamide. The enzyme is active when α-methylglutamate and β-methylglutamate are substituted for glutamate; it interacts with D-glutamate as indicated by the formation of inorganic phosphate at about 2% of the rate observed with L-glutamate. L-α-Aminobutyrate can be replaced by L-cysteine and several other amino acids; replacement with hydroxylamine leads to formation of an hydroxamic acid. The possibility is suggested that γ-glutamylamino acids are involved in amino acid transport.

Earlier work in this laboratory on enzymes that catalyze reactions involving γ-glutamylamino acids led to the isolation of highly purified preparations of γ-glutamyl transpeptidase (Orlowski and Meister, 1963, 1965) and γ-glutamyl cyclotransferase (Orlowski *et al.*, 1969). Since substantial amounts of these enzymes are present in mammalian kidney (Orlowski, 1963; Orlowski and Meister, 1970a,b), it became of interest to us to determine whether the kidney also possesses catalytic activity capable of synthesizing γ-glutamylamino acids. The synthesis of γ-glutamylcysteine, the first of the two steps involved in the enzymatic synthesis of glutathione, was initially studied by Bloch and his collaborators (Bloch, 1949; Johnston and Bloch, 1949, 1951) in pigeon liver preparations. In this work, liver preparations from a number of animals were examined and found to be rather poor sources of the enzyme; no activity was detected in extracts of acetone dried rabbit kidney. Later, Mandeles and Bloch (1955) and Strumeyer (1959) succeeded in obtaining a partially purified preparation of the enzyme from hog liver. A purified preparation of γ-glutamylcysteine synthetase has also been obtained from bovine lens (Rathbun, 1967). γ-Glutamylcysteine synthetase catalyzes the following reaction: L-glutamate + L-cysteine + ATP → L-γ-glutamyl-L-cysteine + ADP + P_i. When L-cysteine is replaced by L-α-aminobutyric acid, L-γ-glutamyl-L-α-aminobutyrate is formed at about the same rate as found for the synthesis of γ-glutamylcysteine. With relatively purified enzyme preparations the reactions may be followed quantitatively by determination of the inorganic phosphate released. However, this method is not applicable to determination of the enzyme in crude tissue extracts because of the presence of other systems that catalyze the dephosphorylation of ATP. For this reason, an alternative procedure was devised by Mandeles and Bloch (1955) in which the incorporation of [^{14}C]glycine or of [^{14}C]glutamate into glutathione was determined in the presence of an excess of glutathione synthetase, the enzyme that catalyzes the

* From the Department of Biochemistry, Cornell University Medical College, New York, New York 10021. *Received August 7, 1970.* Supported by a grant from the National Science Foundation.

synthesis of gluuathione from γ-glutamylcysteine and glycine. While this method has been usefully applied to the determination of γ-glutamylcysteine synthetase, we have found that it does not yield valid results when applied to tissue preparations that contain substantial amounts of γ-glutamyl transpeptidase and γ-glutamyl cyclotransferase activity. This is particularly true of crude kidney preparations which are capable of catalyzing the cleavage of glutathione rapidly. We have therefore sought a new approach to the determination of γ-glutamylcysteine synthetase. In the assay method described here, an excess of γ-glutamyl cyclotransferase is added to convert the γ-glutamyl moiety of the enzymatically synthesized γ-glutamylamino acid into pyrrolidonecarboxylic acid. Under the conditions employed, such conversion is quantitative and thus reflects an accurate measure of the amount of γ-glutamylamino acid that has been synthesized. Thus, we have used [^{14}C]L-glutamate as substrate for the synthetase reaction and have quantitatively determined the formation of [^{14}C]pyrrolidonecarboxylate.

In the present work we have found that γ-glutamylcysteine synthetase activity is present to a much greater extent in rat tissues than previously reported; in addition, the activity of rat kidney extracts is more than 20 times higher than that of a number of other rat tissues examined. We have isolated a homogeneous preparation of the enzyme from rat kidney by a relatively simple procedure. The present communication describes the new assay procedure, the isolation of the enzyme from rat kidney, and gives data on the specificity and other properties of the enzyme.

Experimental Section

Materials. Generally labeled [^{14}C]L-glutamic acid was obtained from New England Nuclear Corp. This product was purified by passing a solution of it through a column (1 × 2.5 cm) of Dowex 50-X2 (H^+); after washing the column with water, the [^{14}C]glutamic acid was eluted with 2 N ammonium hydroxide. The eluate was taken to dryness *in vacuo* and the residue was dissolved in a small amount of water. The solution was diluted with unlabeled sodium L-glutamate to obtain a 0.1 M [^{14}C]L-glutamate solution containing about 50,000 cpm/μmole.

γ-Glutamyl cyclotransferase was obtained from human brain as previously described (Orlowski *et al.*, 1969); the preparation was carried through step 8 of the isolation procedure.

Creatine phosphokinase (rabbit muscle), phosphocreatine, L-glutamate, L-α-aminobutyrate, sodium ATP, *S*-methyl-L-cysteine, L-cysteine, L-alanine, L-serine, glycine, β-alanine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-methionine sulfone, γ-aminobutyric acid, DL-*C*-allylglycine, *threo*-β-methyl-DL-aspartic acid, α-methyl-DL-aspartic acid, β-chloro-L-alanine, glutaric acid, α-ketoglutarate, iodoacetamide, *N*-ethylmaleimide, *p*-mercuribenzoate, and *p*-mercuribenzenesulfonic acid were obtained from Sigma Chemical Co. Glycylglycine, glycinamide, L-leucinamide, L-ornithine, L-threonine, L-arginine, putrescine, L-histidine, *S*-carbamyl-L-cysteine, DL-homocysteine, and D-glutamic acid were obtained from Nutritional Biochemical Corp. L-Glutamine, L-2,4-diaminobutyric acid, L-methionine *SR*-sulfoximine, L-aspartic acid, D-alanine, DL-homoserine, L-valine, and dithiothreitol were obtained from Calbiochem. Ammonium sulfate (ultrapure), hydroxy-L-proline, L-tryptophan, L-methionine, cadaverine, and L-asparagine were obtained from Mann Research Laboratories. DL-β-Methylglutamic acid (Meister *et al.*, 1955) and L-norvaline, L-norleucine, L-α-aminoheptylic acid, and L-α-aminoadipic acid (Greenstein, 1954) were prepared in this laboratory.

γ-L-Glutamylglycine, γ-L-glutamyl-β-alanine, γ-glutamyl-L-leucine, and γ-glutamyl-L-α-aminobutyric acid were prepared according to LeQuesne and Young (1950). γ-L-Glutamyl-L-alanine, γ-L-glutamyl-L-valine, and γ-L-glutamyl-*S*-methyl-L-cysteine were generously provided by Dr. John F. Thompson. γ-L-Glutamyl-L-serine was prepared by the action of hog kidney γ-glutamyl transpeptidase (Orlowski and Meister, 1965) on glutathione in the presence of L-serine as described previously for the preparation of γ-L-glutamyl-L-glutamine (Orlowski *et al.*, 1969).

Analytical grade Dowex 50 (AG 50W-X2) was obtained from Bio-Rad Laboratories. The resin was purified by washing in sequence with acetone, petroleum ether (bp 40–60°), acetone, 2 N HCl, 2 N NaOH, 2 N HCl, and water.

Methods

Determination of Enzyme Activity. In crude tissue preparations, the synthesis of γ-glutamyl-α-aminobutyrate was carried out in the presence of excess of γ-glutamyl cyclotransferase. Thus, the following enzymatic reactions form the basis of this procedure.

$$[^{14}\text{C}]\text{L-glutamate} + \text{L-}\alpha\text{-aminobutyrate} + \text{ATP} \xrightarrow{\text{synthetase}} [^{14}\text{C}]\text{L-}\gamma\text{-glutamyl-L-}\alpha\text{-aminobutyrate} + \text{ADP} + P_i$$

$$[^{14}\text{C}]\text{L-}\gamma\text{-glutamyl-L-}\alpha\text{-aminobutyrate} \xrightarrow{\text{cyclotransferase}} [^{14}\text{C}]\text{pyrrolidonecarboxylate} + \text{L-}\alpha\text{-aminobutyrate}$$

In the presence of an excess of the cyclotransferase the γ-glutamyl moiety of the product of the reaction catalyzed by the synthetase is quantitatively converted into pyrrolidonecarboxylate. The [^{14}C]pyrrolidonecarboxylate is separated from unreacted [^{14}C]L-glutamate on a small column of Dowex 50 (H^+) and the radioactivity of the eluate is determined.

The assay reaction mixtures (final volume, 0.5 ml) contained 0.01 M sodium [^{14}C]L-glutamate (250,000 cpm), 0.01 M L-α-aminobutyrate, 0.02 M magnesium chloride, 0.01 M sodium ATP, 0.01 M phosphocreatine, 0.1 M Tris-HCl buffer (pH 8.2), γ-glutamyl cyclotransferase (5 units), creatine phosphokinase (0.03 mg), and enzyme solution. The mixtures were incubated for 10–20 min at 37°. The reaction was terminated by placing the mixture at 100° for 2 min; after cooling in ice, the protein was removed by centrifugation. An aliquot (0.25 ml) of the supernatant solution was applied to the top of a small column (1 × 2.5 cm) equipped with a sintered glass bottom and filled with about 1 ml of packed Dowex 50 (H^+) AG 50W-X2 resin. A separate column was used for each determination. Each column was washed with water until a total of 2.5 ml of effluent was collected. Blanks in which ATP and enzyme were separately omitted were carried out. The radioactivity of the effluent ([^{14}C]-pyrrolidonecarboxylate) was determined on a 0.4-ml aliquot after mixing with 10 ml of Bray's (1960) solution using a Nuclear Chicago scintillation counter.

A unit of enzyme activity is defined as the amount that catalyzes the synthesis of 1 μmole of γ-glutamylamino acid (pyrrolidonecarboxylate) per hour under the conditions described above. Specific activity is expressed in terms of

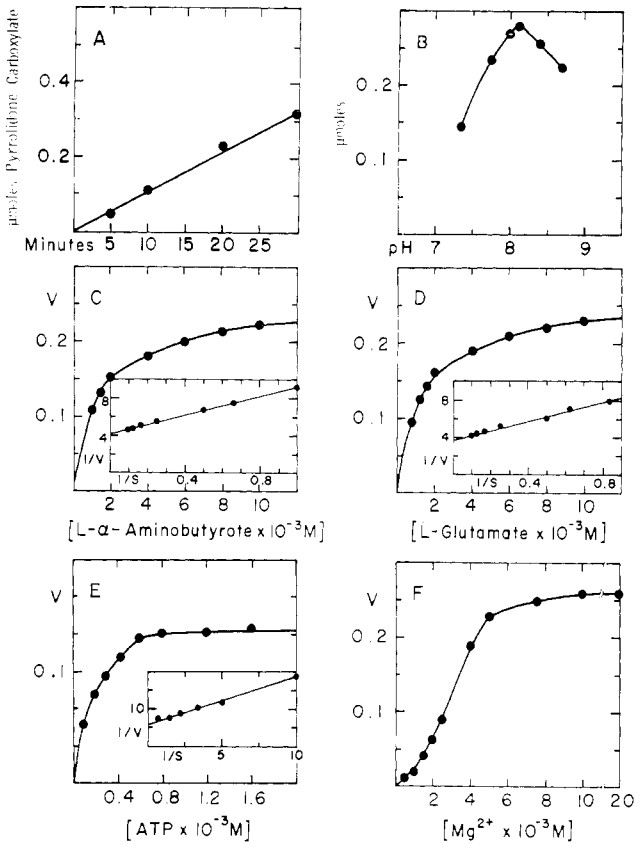

FIGURE 1: (A) Time course of the formation of pyrrolidonecarboxylate. The reaction mixtures contained the components of the coupled assay system (see Methods) and 0.1 ml of mouse liver extract (1.5 mg of protein). (B) pH dependence of the reaction. The reaction mixtures contained Tris-HCl buffer (0.1 M), L-glutamate (0.01 M), L-α-aminobutyrate (0.01 M), sodium ATP (0.005 M), magnesium chloride (0.02 M), and purified rat kidney enzyme (1.5 units of enzyme) in a final volume of 0.5 ml. The formation of inorganic phosphate was determined at 37°. (C) Effect of L-α-aminobutyrate concentration on the rate of the reaction. The reaction mixtures consisted of Tris-HCl buffer (pH 8.2; 0.1 M), L-glutamate (0.01 M), sodium ATP (0.005 M), magnesium chloride (0.02 M), and purified rat kidney enzyme (1.5 units) in a final volume of 0.5 ml. The formation of inorganic phosphate was determined at 37°. The inset gives the double reciprocal plot of the data. (D) Effect of L-glutamate concentration on the rate of the reaction. The reaction mixtures contained Tris-HCl buffer (pH 8.2; 0.1 M), L-α-aminobutyrate (0.01 M), sodium ATP (0.005 M), magnesium chloride (0.02 M), L-glutamate as indicated, and purified rat kidney enzyme (1.5 units) in a final volume of 0.5 ml. The formation of inorganic phosphate was determined at 37°. The inset gives the double reciprocal plot of the data. (E) Effect of ATP concentration on the rate of the reaction. The reaction mixtures contained Tris-HCl buffer (pH 8.2, 0.1 M), L-glutamate (0.01 M), L-α-aminobutyrate (0.01 M), magnesium chloride (0.02 M), ATP as indicated, and purified rat kidney enzyme (0.75 unit) in a final volume of 0.5 ml. The formation of inorganic phosphate was followed at 37°; the inset gives the double reciprocal plot of the data. (F) Effect of magnesium chloride concentration on the rate of the reaction. The reaction mixtures contained Tris-HCl buffer (pH 8.2; 0.1 M), L-glutamate (0.01 M), L-α-aminobutyrate (0.01 M), sodium ATP (0.005 M), magnesium chloride as indicated, and purified rat kidney enzyme (1.5 units), in a final volume of 0.5 ml. The formation of inorganic phosphate was determined at 37°.

units per milligram of protein, determined as described by Lowry *et al.* (1951) using bovine serum albumin as standard.

Under the assay conditions described above, the formation of product was proportional to the amount of enzyme and to time of incubation during the first 30 min of the reaction

TABLE I: Dependence of [^{14}C]Pyrrolidonecarboxylate Formation on Various Components of the Coupled Assay System.[a]

Reaction Mixture	Pyrrolidonecarboxylate (μmoles/ml per hr)
Complete system	6.4
Minus tissue extract	0
Minus α-aminobutyrate	0.90
Minus γ-glutamyl cyclotransferase	0.96
Minus ATP and ATP-generating system	0.20
Minus ATP-generating system	6.1

[a] The reaction mixture described in the text was used; the reaction was started by addition of 0.1 ml (1.5 mg of protein) of mouse liver extract; incubated for 10 min at 37°.

(Figure 1A). As indicated in Table I omission of ATP led to marked decrease in product formation. The reaction required the presence of α-aminobutyrate and γ-glutamyl cyclotransferase; the small amount of product formed in their absence may probably be ascribed to the presence of amino acids and some cyclotransferase activity in the crude tissue homogenate. An ATP-generating system (phosphocreatine and creatine phosphokinase) was included in the assay mixture although in mouse liver extracts this gave only a slight increase in product formation. It should be noted that a more commonly employed ATP-generating system consisting of phosphoenolpyruvate and pyruvate kinase is not suitable for the present assay. In crude tissue preparations, the pyruvate formed from phosphoenolpyruvate transaminates with glutamate, thus reducing the concentration of glutamate; furthermore the [^{14}C]α-ketoglutarate formed passes through the Dowex 50 column and may erroneously be counted as [^{14}C]pyrrolidonecarboxylate. Studies were carried out in order to determine the amount of γ-glutamyl cyclotransferase required to obtain maximal conversion of γ-glutamyl-α-aminobutyrate into pyrrolidonecarboxylate. The addition of two units of cyclotransferase was sufficient for all of the tissue extracts examined except for rat kidney. The very high γ-glutamylamino acid synthetase activity of this tissue required the addition of at least 5 units of the cyclotransferase when the amount of kidney extract used was reduced to 0.025 ml. After step 2 (ammonium sulfate fractionation) of the purification procedure, the activity of the synthetase was followed by determining the formation of inorganic phosphate. This procedure gave results identical with those obtained with the assay described above when purified enzyme preparations were employed. The assay reaction mixtures were the same as described above except that [^{12}C]L-glutamate was used and phosphocreatine and creatine phosphokinase were omitted. The reaction was terminated by adding 0.5 ml of 10% trichloroacetic acid and the inorganic phosphate formed was determined by the method of Fiske and Subbarow (1925). Removal of protein prior to determination of phosphate was not

TABLE II: γ-Glutamyl-α-aminobutyrate Synthetase Activity of Several Animal Tissues.[a]

Tissue	No. of Determinations	Activity (Units per Gram of Tissue)		Specific Activity (Units per Milligram of Protein)	
		Mean	Range	Mean	Range
Rat kidney	8	612	522–700	10.1	8.7–12.0
Rat liver	4	33.5	26.9–38.7	0.40	0.27–0.53
Mouse liver	2	70.0	64.0, 76.0	0.50	0.48, 0.52
Hog liver	2	17.6	15.1, 20.2	0.19	0.18, 0.20
Rat brain	4	4.35	2.9–4.5	0.15	0.09–0.25
Rat spleen	4	4.5	3.9–5.4	0.07	0.05–0.09
Rat testis	4	5.7	5.4–5.9	0.15	0.11–0.17
Rat lung	4	5.2	4.2–6.2	0.08	0.07–0.10
Sheep brain acetone powder extract	3	8.7	8.2–9.6	0.067	0.063–0.074

[a] Extracts were prepared as described in the text.

always necessary because the amount of protein was exceedingly small and did not interfere with the determination.

The tissue extracts were prepared by homogenizing the fresh tissues at 0° with 9 volumes of a solution containing 0.15 M potassium chloride, 0.005 M 2-mercaptoethanol, and 0.001 M magnesium chloride in a Potter-Elvehjem glass homogenizer equipped with motor-driven Teflon pestle. Homogenization was carried out for 2 min at 1600 rpm. The homogenates were centrifuged at 10,000*g* for 15 min and the supernatant solutions were used in the studies described here.

Enzyme Activity of Several Tissues. The γ-glutamyl-α-aminobutyrate synthetase activity of several animal tissues was examined using the coupled enzyme assay described above. The extracts were prepared by centrifugation of the tissue homogenates as described under Methods. Of the several tissues examined the highest activity observed was found with rat kidney (Table II). It is notable that the specific activity of the unfractionated rat kidney extract was 2 to 3 times higher than the most purified preparations of γ-glutamylcysteine synthetase that have been obtained from hog liver (Strumeyer, 1959) and bovine lens (Rathbun, 1967). The specific activity of rat liver homogenates was less than 5% of that of rat kidney. Somewhat lower though significant activity was found in rat brain, spleen, testes, and lung and also in extracts of sheep brain acetone powder that had been stored for 3 years at −15°. It is notable that the values for the specific activity of hog liver extracts as determined by the present assay method are about 100 times greater than those reported by Mandeles and Bloch (1955) and by Strumeyer (1959), who used assays based on the incorporation of [^{14}C]glycine into glutathione.

Purification of the Enzyme from Rat Kidney. Sprague–Dawley rats (250–300 g) were used; unless otherwise stated all steps were carried out at 4°.

Step 1. The rats were decapitated and exsanguinated; the kidneys were immediately excised and weighed. The kidneys were homogenized with 9 volumes of a cold solution containing 0.15 M potassium chloride, 0.005 M 2-mercaptoethanol, and 0.001 M magnesium chloride. An ice-cooled Potter-Elvehjem homogenizer with motor-driven Teflon pestle was used; homogenization was carried out for 2 min at 1600 rpm. The homogenate was centrifuged at 0° for 15 min at 10,000*g*. The supernatant solutions thus obtained from the kidneys of 13 rats were pooled and immediately processed as described below.

Step 2 (Ammonium Sulfate Fractionation). The solution obtained in step 1 was placed in a flask immersed in ice over a magnetic stirrer. Solid ammonium sulfate (31.3 g/100 ml) was added with stirring over a 30-min period. After stirring the solution for 15 min it was allowed to stand for an additional 15 min and the suspension was centrifuged at 13,000*g* for 15 min. The precipitated protein was discarded and additional ammonium sulfate (9.4 g/100 ml) was added to the supernatant solution. The resulting precipitate was collected by centrifugation and dissolved in about 6 ml of a solution containing 0.05 M Tris-HCl buffer (pH 7.4), 0.005 M L-glutamate, and 0.005 M magnesium chloride.

Step 3 (Gel Filtration). The solution obtained in step 2 was applied through a flow adapter to the top of a column of Sephadex G-100 (2.8 × 100 cm) which was previously equilibrated with a solution containing 0.05 M Tris-HCl buffer (pH 7.4) containing 5 mM L-glutamate and 5 mM $MgCl_2$. The column was eluted overnight with the same buffer at constant pressure at a flow rate of about 30 ml/hr. Fractions of about 5 ml were collected. The concentration of protein was monitored by determining the absorbance at 280 mμ, and the activity of the enzyme was determined by following the release of inorganic phosphate. The enzyme emerges from the column as a single peak of activity located within the main peak of protein (Figure 2). The fractions exhibiting a ratio of activity to absorbance at 280 mμ of greater than 50 were pooled and immediately processed as described below.

Step 4 (Chromatography on DEAE-Cellulose). The solution obtained in step 3 was added to the top of a column of DEAE-cellulose (Whatman DE-52, microgranular, preswollen, 1.5 × 15 cm) which was previously equilibrated with a buffer mixture (pH 7.4) consisting of 0.05 M Tris-HCl, 0.005 M L-glutamate, and 0.005 M magnesium chloride. The column was washed with 50 ml of buffer and elution was begun with a linear gradient established between 220 ml of 0.05 M Tris-HCl buffer (pH 7.4) containing 0.005 M L-glutamate and 0.005 M $MgCl_2$, and 220 ml of the same buffer containing

TABLE III: Summary of Purification of the Enzyme from Rat Kidney.[a]

Step	Volume (ml)	Protein		Activity		Yield (%)
		Total (mg)	Concn (mg/ml)	Total (units)	Specific (units/mg)	
1. Rat kidney homogenate	255	1660	6.51	21,800	13.1	[100]
2. Ammonium sulfate fractionation	6.9	427	62.0	21,100	49.4	97
3. Gel filtration	57.5	169	2.94	13,700	81.0	63
4. DEAE-cellulose chromatography	1.9	14.4	7.6	7,780	540	36

[a] From about 30 g of rat kidney; see the text.

0.2 M sodium chloride in addition to L-glutamate and $MgCl_2$. The buffer was pumped into the column at a constant rate of 20 ml/hr using a Varioperpex peristaltic pump (LKB Producter, Sweden). Fractions of 5 ml were collected. More than half of the protein does not bind to the column and the activity emerged at the end of the gradient as a distinct protein peak which coincided closely with the enzyme activity (Figure 3); the fractions in the central portion of this peak were pooled and the protein was precipitated by adding solid ammonium sulfate (47.2 g/100 ml). The suspension was centrifuged at 17,000*g* for 15 min and the precipitated protein was dissolved in a small amount of 0.1 M Tris-HCl buffer (pH 8.2) containing 0.15 M potassium chloride, 0.005 M 2-mercaptoethanol, and 0.001 M magnesium chloride.

The entire purification procedure may be carried out in 2 days. A summary of the purification is given in Table III. As indicated in the table a preparation exhibiting a specific activity of over 500 units/mg was obtained indicating a 40-fold purification.

Properties of the Enzyme. When the enzyme was examined by acrylamide gel electrophoresis at pH 8.2 a single band of protein was observed (Figure 4). Occasionally in some enzyme preparations, a very faint protein band moving faster than the enzyme was seen. Electrophoresis of the enzyme on acrylamide gel at pH 7.4 also gave a single band. The location of enzymatic activity within the acrylamide gels corresponded exactly to the location of protein.

Enzyme activity was located in the acrylamide gels by incubating them at 25° in a solution (pH 9.0) containing L-glutamate (10 mM), L-α-aminobutyrate (10 mM), magnesium chloride (20 mM), sodium ATP (5 mM), calcium chloride (18 mM), and Tris-HCl buffer (pH 9.0; 30 mM) for 0.5–6 hr (depending on the amount of enzyme present). Under these conditions the inorganic phosphate released during the reaction is precipitated within the gel as calcium phosphate and becomes visible as a distinct white band, which thus indicates the location of the enzyme. The position of the white band is compared with that of the protein band seen in a parallel gel stained in the usual manner for protein. With purified γ-glutamylcysteine synthetase, no reaction occurred when either glutamate or α-aminobutyrate was omitted from the incubation mixture. Following incubation the gels were rinsed with a 2% calcium chloride solution (pH 9.0) and stored in this solution in stoppered test tubes.

Examination of the enzyme by ultracentrifugation was carried out on two separate preparations and in both instances a single moving boundary was observed. Representative sedimentation patterns are shown in Figure 5. The sedimentation coefficient calculated from the maximum ordinate movement was 5.6 S.

The activity of the enzyme was determined in Tris buffers over the pH range 7.35–8.70; maximal activity was observed in the range 8.0–8.4 (Figure 1B). The effect of varying concentrations of L-α-aminobutyrate (Figure 1C), L-glutamate (Figure 1D), and ATP (Figure 1E) was determined. The following respective apparent K_m values were obtained by extrapolation of the corresponding double reciprocal plots: 1.25×10^{-3} M, 1.59×10^{-3} M, and 2×10^{-4} M. The curve describing the effect of Mg^{2+} concentration on activity (Figure 1F) is concave upward at concentrations less than 0.003 M $MgCl_2$. It is possible that the Mg–ATP complex is the

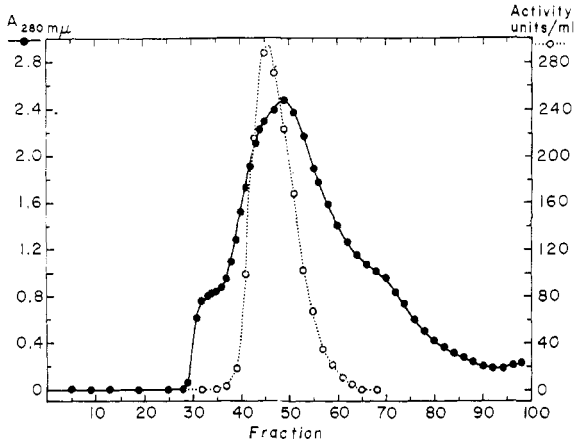

FIGURE 2: Chromatography of the enzyme on Sephadex G-100 (see the text; step 3 of the purification procedure).

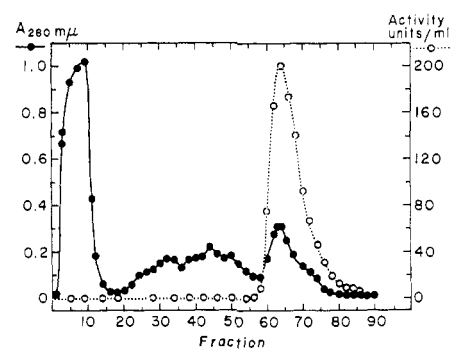

FIGURE 3: Chromatography of the enzyme on DEAE-cellulose (see the text; step 4 of the purification procedure).

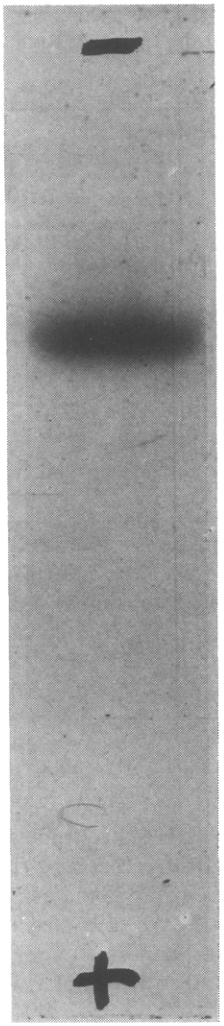

FIGURE 4: Polyacrylamide gel electrophoresis of the purified rat kidney enzyme. The procedure was carried out essentially as described by Davis (1964); a continuous buffer system was used consisting of 0.05 M Tris adjusted to pH 8.2 by addition of acetic acid, and electrophoresis was carried out in 6% gels at 25°. The enzyme sample (0.01–0.025 ml) containing 10% sucrose was layered on top of the gel column (0.4 × 5.0 cm). Bromophenol blue in a 10% sucrose solution was layered on a separate gel column and electrophoresis was stopped when the dye reached the bottom of the gel.

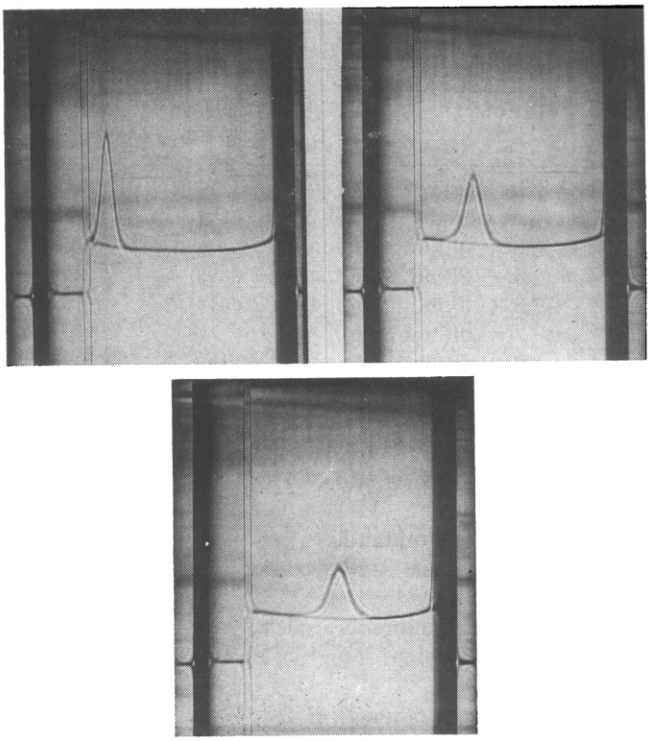

FIGURE 5: Sedimentation velocity pattern of the purified rat kidney enzyme. The protein concentration was 0.76% in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.005 M L-glutamate, 0.005 M magnesium chloride, 0.005 M 2-mercaptoethanol, and 0.15 M potassium chloride. Sedimentation was carried out in a Model E Spinco analytical ultracentrifuge at 60,000 rpm at 4°. Sedimentation is from left to right; the photographs were taken at 25, 65, and 105 min, respectively, after final speed was achieved. The bar angle was 65°.

active reactant; however, further study of this point is necessary.

When the purified enzyme was stored at 0° a rapid and progressive loss of activity was observed. Thus, as indicated in Figure 6 about 50% of the initial activity was lost after about 5 days and only 5% of the initial activity remained after 1 month. However, the enzyme could be reactivated to within 80% or more of the initial value by exposure to dithiothreitol (0.002–0.005 M) (Table IV, expt 3). Such reactivation appears to be virtually instantaneous; however, the enzyme thus reactivated was found to lose all of its activity after 2 days (expt 4) and additional treatment with dithiothreitol did not restore activity (expt 5). When reactivation was carried out with dithiothreitol in the presence of Tris buffer (expt 6), only half of the activity had disappeared after 2 days (expt 7). Reactivation in the presence of Tris, glutamate, and Mg^{2+} (expt 9–11) and of Tris, glutamate, Mg^{2+}, and ATP (expt 12–15) led to more stable enzyme preparations. These findings strongly suggest that the enzyme requires sulfhydryl groups for activity. Additional evidence consistent with this conclusion was obtained. Thus, addition of 2×10^{-6} and 10^{-5} M *p*-chloromercuribenzoate to the assay mixture led to 12 and 47% inhibition, respectively; *p*-chloromercuribenzenesulfonate at these concentrations gave 20 and 53% inhibition, respectively. Preincubation of the enzyme with 5 mM iodoacetamide for 5 min led to 50% inactivation.

Specificity of the Enzyme. Studies in which L-glutamate was replaced by a number of other compounds are summarized in Table V. Very little activity was observed with L-α-aminoadipate and no activity was found with L-aspartate. Significant activity was observed with α-methylglutamate and β-methylglutamate; additional studies are required in order to investigate the stereospecificity of these reactions.

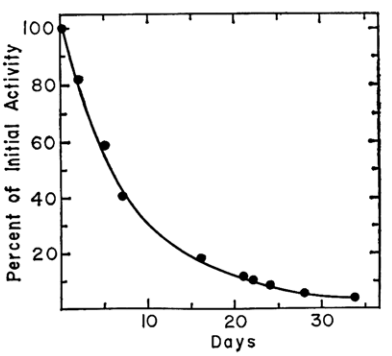

FIGURE 6: Decrease in the activity of the purified enzyme on storage at 0°. (See the text).

TABLE IV: Reactivation of the Enzyme by Dithiothreitol.[a]

Expt	Treatment of Enzyme	Activity (units/ml)
1	Isolated enzyme (A)	860
2	A, stored at 0°, for 20 days (B)	91
3	B + dithiothreitol, after 5 min	685
4	B + dithiothreitol, after 2 days	12
5	B + dithiothreitol, after 2 days + dithiothreitol	12
6	B + dithiothreitol + Tris, after 5 min	749
7	B + dithiothreitol + Tris, after 2 days	350
8	B + dithiothreitol + Tris, after 13 days	25
9	B + dithiothreitol + Tris + glutamate + Mg^{2+}, after 5 min	675
10	B + dithiothreitol + Tris + glutamate + Mg^{2+}, after 2 days	582
11	B + dithiothreitol + Tris + glutamate + Mg^{2+}, after 13 days	461
12	B + dithiothreitol + Tris + glutamate + Mg^{2+} + ATP, after 5 min	736
13	B + dithiothreitol + Tris + glutamate + Mg^{2+} + ATP, after 1 day	803
14	B + dithiothreitol + Tris + glutamate + Mg^{2+} + ATP, after 2 days	765
15	B + dithiothreitol + Tris + glutamate + Mg^{2+} + ATP, after 13 days	435

[a] Purified rat kidney enzyme (1.6 mg/ml) was used. Reactivation was carried out by adding 0.05 ml of enzyme solution to 0.95 ml of a solution containing (as indicated in the table) dithiothreitol (5 μmoles), Tris-HCl buffer (50 μmoles pH 8.1), Na glutamate (5 μmoles), $MgCl_2$ (5 μmoles), and NaATP (5 μmoles). After 5 min, 1 day, 2 days, or 13 days (as indicated in the table), assay was carried out on an aliquot of 0.05 ml.

TABLE V: Specificity of the Enzyme; Replacement of Glutamate by Analogs.[a]

Glutamate Analog	Relative Activity
None	0
L-Glutamate	100
D-Glutamate	1.4
L-α-Aminoadipate	0.6
L-Aspartate	0
DL-α-Methylglutamate	13.9
DL-β-Methylglutamate[b]	3.2
DL-α-Methylaspartate	0
DL-β-Methylaspartate (threo)	0
L-Pyrrolidonecarboxylate	0
α-Ketoglutarate	0
Glutarate	0

[a] The reaction mixtures contained L-α-aminobutyrate (0.01 M), ATP (0.005 M), $MgCl_2$ (0.02 M), Tris-HCl buffer (pH 8.2; 0.1 M), purified rat kidney enzyme (2–8 units), and L-glutamate or analog (0.01 M) in a final volume of 0.5 ml; incubated for 10–120 min at 37°. The formation of inorganic phosphate was determined. [b] Mixture of the 4 isomers.

The enzyme interacts with D-glutamate; however, the rate of inorganic phosphate formation is less than 2% of that observed with L-glutamate.

The specificity of the enzyme was also examined by replacing L-α-aminobutyrate with various amino acids, amines, amino acid amines, and peptides using the standard assay system. The results are summarized in Table VI. L-Cysteine and L-α-aminobutyrate were about equally active. The activity observed with *S*-carbamyl-L-cysteine may be at least partially due to its conversion to cysteine under these conditions. It is of interest that substantial activity was observed with *S*-methyl-L-cysteine, *C*-allylglycine, L-norvaline, β-chloro-L-alanine, homocysteine, threonine, alanine, and homoserine. Somewhat lower activity values were obtained with other amino acids as indicated in Table VI and no activity was detected with ammonia and with a number of additional amino acids, amines, and amides. Replacement of L-α-aminobutyrate by NH_2OH led to some activity, and application of the ferric chloride procedure (Lipmann and Tuttle, 1945) indicated that a hydroxamic acid was formed. Paper chromatographic studies of the enzymatically synthesized γ-glutamylamino acids confirmed the synthesis of several of these for which authentic samples were available (Table VII).

Discussion

A significant observation reported here is that rat kidney contains unexpectedly high concentrations of γ-glutamylcysteine synthetase. Thus, even the specific activity of the enzyme in centrifuged rat kidney homogenates is several times higher than the most highly purified preparations of the enzyme that have been obtained from other sources. Indeed, the specific activity of the purified rat kidney enzyme is about 100 times greater than previously reported purified preparations. On the basis of the specific activity of the purified rat kidney enzyme it may be calculated that this enzyme constitutes 2–3% of the total protein present in the soluble fraction of rat kidney homogenate. The assay method described here makes it possible to determine the enzyme reliably even in the presence of γ-glutamyl transpeptidase. Our finding that the specific activity of crude hog liver preparations is 0.18 unit/mg of protein as compared to a value of about 1% of this as determined by measurement of [^{14}C]-glycine incorporation into glutathione indicates that the latter assay procedure may determine only a fraction of the activity present in crude tissue preparations.

The presence of such large amounts of this enzyme in kidney raises the interesting question as to the nature of the function of this catalytic entity. We have considered previously (Orlowski *et al.*, 1969) the possibility that γ-glutamylamino acids are involved in the transport of amino acids in the kidney. The presence of substantial amounts of γ-glutamylcysteine synthetase in kidney would seem to provide an

TABLE VI: Specificity of the Enzyme; Replacement of L-α-Aminobutyrate by Other Amino Acids.[a]

Amino Acid	Relative Activity
L-α-Aminobutyrate	100
L-Cysteine	96.6
S-Carbamyl-L-cysteine	89.5
S-Methyl-L-cysteine	68.4
DL-*C*-Allylglycine	67.2
β-Chloro-L-alanine	62.9
L-Norvaline	50.1
L-Norleucine	2.8
L-α-Aminoheptylic acid	1.4
DL-Homocysteine	25.3
L-Threonine	20.2
L-Alanine	9.9
DL-Homoserine	6.5
Glycine	2.9
L-Valine	2.9
L-Serine	2.2
L-Methionine	1.2
L-Leucine	1.2
L-Asparagine	1.1
β-Alanine	1.0
L-Isoleucine	0.4
L-Citrulline	0.3
Hydroxylamine	1.1

[a] The reaction mixtures contained L-glutamate (0.01 M), ATP (0.005 M), $MgCl_2$ (0.02 M), Tris-HCl buffer (pH 8.2; 0.1 M), amino acid (0.01 M) or hydroxylamine (0.2 M), and purified rat kidney enzyme (2–8 units) in a final volume of 0.5 ml; incubated 10–120 min at 37°. The formation of inorganic phosphate was determined. No activity was detected with the following compounds (0.01 M); L-lysine, L-phenylalanine, L-glutamine, L-aspartate, L-proline, hydroxy-L-proline, L-ornithine, L-arginine, L-tryptophan, L-hisitdine, L-2,4-diaminobutyrate, L-methionine sulfone, L-methionine *SR*-sulfoximine, D-alanine, glycyglycine, glycinamide, L-leucinamide, γ-aminobutyric acid, cadaverine, putrescine, or ammonium sulfate (0.2 M).

TABLE VII: Chromatographic Studies of the Enzymatically Synthesized γ-Glutamylamino Acids.[a]

Amino Acid and Its γ-Glutamyl Derivative	Solvent A R_F	Solvent B R_F
L-Glutamate	0.18	0.28
L-α-Aminobutyrate	0.43	0.45
L-γ-Glutamyl-L-α-aminobutyrate[b]	0.28	0.44
S-Methyl-L-cysteine	0.49	0.41
L-γ-Glutamyl-*S*-methyl-L-cysteine[b]	0.31	0.33
L-Alanine	0.39	0.29
L-γ-Glutamyl-L-alanine[b]	0.24	[c]
Glycine	0.23	0.22
L-γ-Glutamylglycine[b]	[c]	0.19
L-Serine	0.29	0.20
L-γ-Glutamyl-L-serine[b]	[c]	0.17
L-Isoleucine	0.64	0.72
L-γ-Glutamyl-L-isoleucine	0.37	0.64
L-Leucine	0.66	0.72
L-γ-Glutamyl-L-leucine[b]	0.37	0.65
L-Asparagine	0.17	0.14
L-γ-Glutamyl-L-asparagine	0.12	0.13
L-Threonine	0.31	0.28
L-γ-Glutamyl-L-threonine	[c]	0.24
L-Methionine	0.53	0.55
L-γ-Glutamyl-L-methionine	0.31	0.44
DL-Homoserine	0.32	0.26
L-γ-Glutamylhomoserine	[c]	0.20
L-Valine	0.52	0.56
L-γ-Glutamyl-L-valine[b]	0.30	0.52
DL-*C*-Allylglycine	0.43	0.51
γ-Glutamyl-*C*-allylglycine	0.28	0.45

[a] Enzymatic formation of γ-glutamyl amino acids was studied by paper chromatography in two solvent systems: solvent A consisted of 1-butanol–pyridine–water (1:1:1), solvent B consisted of 1-butanol–acetic acid–water (60:15:25). Incubation mixtures were prepared containing enzyme (2–8 units), glutamic acid (5 μmoles), amino acid (5 μmoles), ATP (2.5 μmoles), $MgCl_2$ (10 μmoles), and Tris-HCl buffer (50 μmoles; pH 8.2) in a final volume of 0.5 ml. After incubation at 37° for 0, 60- and 120-min aliquots (10 μl) of the reaction mixtures were removed, spotted on a sheet of Whatman No. 1 paper, and developed by the descending technique for 16 hr. The products were located by treating the dried chromatograms with a 0.3% ninhydrin solution in acetone. In all experiments an increase in the amount of the enzymatically synthesized γ-glutamylamino acids with time was demonstrated. The R_F values of the enzymatically synthesized compounds were compared with the R_F values of available authenic γ-glutamylamino acids as standards and shown to be identical in both solvent mixtures. [b] The R_F values of these compounds were verified by comparing their migration with the authentic compounds. [c] The product could not be separated from the reactants.

enzymatic mechanism for the formation of γ-glutamylamino acids (and of glutathione) which could serve as substrates for γ-glutamyl transpeptidase and γ-glutamyl cyclotransferase, enzymes which are also present in considerable amounts in the kidney. γ-Glutamyl transpeptidase, which is known to be associated with the particulate fraction of kidney, is probably membrane bound and might function in the binding and transfer of amino acids across the cell membrane. One may also envision that the energy-requiring aspect of amino acid transport is explained by the need for ATP for the synthesis of γ-glutamylcysteine and glutathione; these reactions, which are catalyzed by soluble enzymes within the cell, would then provide substrate for γ-glutamyl transpeptidase, *i.e.*, carrier for the amino acids to be transported. γ-Glutamyl cyclotransferase, also a soluble enzyme, might function in the release of amino acid from γ-glutamyl linkage within the cell. Further elaboration of this hypothesis and additional evidence for it is dealt with elsewhere (Orlowski and Meister, 1970).

γ-Glutamylcysteine synthetase is evidently a "sulfhydryl enzyme" and the data indicate that one or more SH groups are either directly involved in the catalytic reaction or affect in some way the conformation of the active site of the enzyme. The remarkable loss of activity on standing at 0° and the prompt reactivation of the enzyme by dithiothreitol require additional investigation, which work is now in progress.

The reaction catalyzed by this enzyme is similar to that catalyzed by glutamine synthetase. However, purified glutamine synthetase does not catalyze the formation of γ-glutamylcysteine nor is the present enzyme active with ammonia. The slight but definite activity observed with hydroxylamine is consistent with the formation of an activated glutamate intermediate. It is pertinent to note that ^{18}O studies have shown that there is a transfer of oxygen from glutamate to inorganic phosphate in the course of γ-glutamylcysteine synthesis (Strumeyer and Bloch, 1960). There is now substantial evidence that enzyme-bound γ-glutamyl phosphate is an intermediate in the reaction catalyzed by glutamine synthetase and it seems probable that a similar intermediate is involved in the synthesis of γ-glutamylcysteine. The availability of large amounts of homogeneous γ-glutamylcysteine synthetase from rat kidney now makes possible a variety of studies on the mechanism of the reaction and on the structure of the enzyme.

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Conformational Isomers of Alkaline Phosphatase in the Mechanism of Hydrolysis*

Ted W. Reid† and Irwin B. Wilson‡

ABSTRACT: The level of phosphoryl-enzyme formed when alkaline phosphatase (*Escherichia coli*) and [^{32}P]phosphate esters are rapidly mixed at 25° and pH 8.0 was measured as a function of time from 10 to 200 msec using a rapid mixing and sampling device. There is an initial rapid phosphorylation of more than 30% of the enzyme. The phosphoryl-enzyme level then falls to about 10% in about 50 msec which is somewhat longer than the turnover time. The earliest measurements were made at 10 msec. At this time the level of phosphorylation is on its way down. When P_i was added to the substrate no change was observed, but when P_i was added to the enzyme the initial high level of phosphorylation did not appear; instead there was a slow rise to the steady-state level of 10%. The time required was about 45 msec. Similarly in stopped-flow experiments using *p*-nitrophenyl phosphate as a substrate an extremely rapid burst of *p*-nitrophenol requiring less than 3 msec, the dead time of our instrument, was observed. About 50% of the enzyme was phosphorylated. When P_i was added to the enzyme no burst was found. Addition of P_i to the substrate was without effect. Similar results were obtained with the inhibitor *p*-chloroanilidophosphonate. We interpret these results as indicating that there are two equally stable conformations of the enzyme, E_α and E_β. Only E_β can react with substrates and only E_α with P_i. The slow step in the hydrolysis of substrates is $E_\alpha \rightarrow E_\beta$. The steady-state level of only 10% phosphorylation indicates that dephosphorylation is not the rate-limiting step. Yet a burst is obtained. A complete scheme is presented which explains these observations and also explains why a phosphate acceptor such as Tris increases the rate of utilization of substrate even though dephosphorylation is not rate limiting.

Early studies with alkaline phosphatase from *Escherichia coli* showed that the enzyme catalyzes the hydrolysis of a wide range of phosphate anhydrides and esters at the same rate (Garen and Levinthal, 1960; Heppel *et al.*, 1962). This fact, plus the finding that Tris (and similar compounds) by acting as a phosphate acceptor could accelerate the rate of

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† National Institutes of Health Fellowship, 1968–1970. Present address: Yale University School of Medicine, New Haven, Conn. 06510.

‡ To whom to address correspondence.