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Assay, Purification, Properties and Mechanism of Action of γ-Glutamylcysteine Synthetase from the Liver of the Rat and *Xenopus laevis*

By J. S. DAVIS,* J. B. BALINSKY, J. S. HARINGTON and J. B. SHEPHERD
Department of Chemistry including Biochemistry, University of the Witwatersrand, Jan Smuts Avenue, Johannesburg, South Africa

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1. An improved radioassay for glutathione synthetase and γ-glutamylcysteine synthetase was developed. 2. *Xenopus laevis* liver γ-glutamylcysteine synthetase was purified 324-fold by saline–bicarbonate extraction, protamine sulphate precipitation, CM-cellulose and DEAE-cellulose column chromatography, and gel filtration. 3. Rat liver γ-glutamylcysteine synthetase was purified 11400-fold by a procedure similar to that employed for the *Xenopus laevis* enzyme. 4. Rat liver γ-glutamylcysteine synthetase activity was inhibited by GSH and activated by glycine. These effects, which were not found in the enzyme from *Xenopus laevis*, may have a regulatory significance. 5. Isotope-exchange experiments revealed fundamental differences in the partial reactions catalysed by the rat and *Xenopus laevis* synthetases. The enzyme from *Xenopus laevis* appears to follow a Bi Bi Uni Uni Ping Pong mechanism, with glutamyl–enzyme as intermediate before the addition of cysteine and the release of γ-glutamylcysteine. The results for the rat liver enzyme are consistent with a Tri Tri sequential mechanism.

The biosynthesis of glutathione was first demonstrated in rat liver slices by Braunstein *et al.* (1948). The first step of this process is catalysed by γ-glutamylcysteine synthetase (EC 6.3.2.2). Partially purified preparations of the enzyme have been obtained from wheat germ (Webster & Varner, 1954), pig liver (Mandeles & Bloch, 1955; Strumeyer & Bloch, 1960), and bovine lens (Rathbun, 1967*a*,*b*). A 40-fold purification of the rat kidney enzyme of high specific activity was reported by Orlowski & Meister (1971*a*). The enzyme has been found in the soluble fraction of bovine lens extracts (Cliffe & Waley, 1961), and in the soluble and mitochondrial fractions of plant cells (Webster, 1953). It catalyses the reaction:

$$\text{Glutamate} + \text{cysteine} + \text{ATP} \rightarrow \gamma\text{-glutamylcysteine} + \text{ADP} + P_i$$

The equilibrium position of the reaction is considered to be on the right-hand side (Webster & Varner, 1954; Mandeles & Bloch, 1955; Strumeyer & Bloch, 1960). The enzyme exhibits an absolute requirement for either Mg^{2+} or Mn^{2+}. In addition the wheat-germ enzyme requires K^+ for activity (Webster & Varner, 1954).

Previous studies on the reaction mechanism indicated some differences in mechanism of the enzyme from plants (Webster & Varner, 1954), pig liver (Strumeyer, 1959; Strumeyer & Bloch, 1960) and rat kidney (Orlowski & Meister, 1971*b*). The present paper compares the mechanism of this enzyme from toad and rat liver. The amount of GSH is much higher in the latter than in the former; its importance in mammals may therefore be greater (Balinsky, 1970). The aim of the investigation was to see whether the difference in amounts is associated with any differences in the biosynthetic mechanism between Amphibia and mammals. A preliminary report of this work has already been published (Davis *et al.*, 1972).

Experimental

Materials

[^{35}S]Cysteine, [U-^{14}C]glutamate and [U-^{14}C]-glycine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Soluene 100, 2,5-diphenyloxazole (PPO) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (DM-POPOP) were purchased from Packard Instruments, Downers Grove, Ill., U.S.A. Nucleotides, phosphoenolpyruvate (tricyclohexylammonium salt), amino acids, GSH and pyruvate kinase were purchased from the Sigma Chemical Co., St. Louis, Mo., U.S.A. A.R.-grade chemicals were used when available, and were usually purchased from BDH Chemicals Ltd., Poole, Dorset, U.K., or E. Merck, Darmstadt, Germany. Whatman CM-11 medium fibrous powder (nominal capacity 0.6mequiv./g) and DE-32 microgranular (mean small ion capacity 1.0mequiv./g) ion-exchange celluloses were obtained from W. and R. Balston, Maidstone, Kent, U.K., and Sephadex G-25 and G-150 from

* Present address: Molecular Enzymology Laboratory, Biochemistry Department, University of Bristol, Bristol BS8 1TD, U.K.

Pharmacia Ltd., Uppsala, Sweden. γ-Glutamylcysteine was prepared by the enzymic method of Strumeyer & Bloch (1962). The γ-glutamylcysteine obtained was shown to be pure by paper electrophoresis at pH3.9 and by performic acid oxidation followed by analysis in a Technicon amino acid analyser.

Xenopus laevis were obtained from the Department of Inland Fisheries, Jonkershoek, Stellenbosch, Cape Province, South Africa. In the laboratory they were kept in water-filled aquaria and fed once a week on chopped liver.

Methods

Preparation of ion-exchange cellulose. CM-cellulose and DEAE-cellulose were prepared as described in the Whatman leaflet 'Advanced ion-exchange celluloses'. The CM-cellulose was adjusted to pH6.8 (for the *Xenopus* preparation) or 6.6 (for the rat preparation), packed into a 2.5cm × 60cm column and equilibrated with 0.01 M-sodium citrate buffer of the same pH. The DEAE-cellulose was adjusted to pH7.8, packed into a 1.5cm × 30cm column and equilibrated with 0.005M-Tris–HCl buffer, pH7.8.

GSH synthesis assay. The standard assay of GSH synthesis from its component amino acids was carried out in stoppered test tubes at 37°C for 60min. The standard incubation medium contained the following reagents (Cliffe & Waley, 1961; Snoke *et al.*, 1953): 10mM-glutamate, 10mM-cysteine, 15mM-glycine, 4mM-ATP, 4mM-phosphoenolpyruvate, 10mM-$MgCl_2$, 15mM-KCN, 100mM-KCl, 2 units of pyruvate kinase/ml, 100mM-Tris–HCl buffer, pH7.8. The radioisotopic assay contained either [U-^{14}C]-glycine or [U-^{14}C]glutamate at the same molar concentrations. The specific radioactivity of the amino acids employed was 0.075mCi/mmol in both cases. The pH of the incubation medium was adjusted to 7.8 with 0.5M-NaOH.

The procedure employed to isolate the [^{14}C]GSH formed from the incubation medium was an extensively modified version of that devised by Johnston & Bloch (1951) and Waelsch & Rittenberg (1941). The reaction was stopped by the addition of a 0.8ml portion of the reaction mixture to an 11cm × 1.5cm centrifuge tube containing 20mg of carrier GSH in 2ml of 12% (w/v) trichloroacetic acid and 1.2ml of deionized water to give a final volume of 4ml. The tubes were shaken to ensure GSH extraction from the precipitated protein, which was removed by centrifugation. The supernatant fraction was decanted into an 11cm × 1.5cm centrifuge tube containing 1ml of 6% (w/v) $CdCl_2,2H_2O$. Sufficient 3M-NaOH was added to cause the formation of a very slight flocculent white precipitate. To achieve an accurate precipitation end-point, the tubes were agitated with a Vortex mixer. Then sufficient 1M-$NaHCO_3$ was added, with agitation, to neutralize the contents of the centrifuge tubes. A heavy white precipitate containing the cadmium salt of GSH resulted. The tubes were cooled to 2°C and centrifuged in the cold. The supernatant fraction, which contained the soluble radioactive material, was discarded and the precipitate was dissolved in 0.5ml of 1M-H_2SO_4, then 7ml of 0.25M-H_2SO_4 was added and the tubes were warmed to 35°C, before adding 1ml of a continuously stirred 1:5 (w/v) Cu_2O aqueous suspension rapidly to each tube. This operation resulted in the transient formation of a white precipitate of the cuprous mercaptide of GSH, which later dissolved in the presence of an excess of Cu_2O. The contents of the tubes were thoroughly stirred with a Vortex mixer before being centrifuged briefly for 2min to sediment the copper oxides. The yellowish-green supernatant fractions were immediately decanted into a series of 15cm × 2cm test tubes and vigorously aerated for 10min. During this process a flocculent white precipitate of the cuprous mercaptide of GSH formed. The precipitate was allowed to settle and the clear blue supernatant discarded. The cuprous mercaptide of GSH was collected by vacuum filtration on a 2.5cm glass-fibre filter disc (Whatman GF/C) by using a Millipore filter unit (Millipore XX 10 025 03) (Davies & Cocking, 1966). The residue was washed thoroughly with deionized water to remove all traces of soluble radioactive material. The discs were then thoroughly dried in a vacuum desiccator over P_2O_5 and placed in Packard scintillation vials. Scintillator (5ml, comprising 0.1g of DM-POPOP and 4.0g of PPO in 1 litre of toluene) was added to each vial. The radioactivity was determined by using a Packard Tri-Carb scintillation spectrometer. Constant quenching pertained in all measurements of radioactivity.

γ-Glutamylcysteine synthetase assay. The incubation conditions were the same as those described for GSH synthesis. The components of the incubation medium included: 10mM-[U-^{14}C]glutamate (specific radioactivity 0.075mCi/mmol), 10mM-cysteine, 4mM-ATP, 4mM-phosphoenolpyruvate, 10mM-$MgCl_2$, 2 units of pyruvate kinase/ml, 100mM-Tris–HCl buffer, pH7.7. A similar procedure to that described for the isolation of [^{14}C]GSH was employed for the isolation of [^{14}C]γ-glutamylcysteine. GSH was used as carrier during the isolation of [^{14}C]γ-glutamylcysteine. The radioactivity present in the isolated γ-glutamylcysteine was determined in a fashion similar to that described for GSH. If pyruvate kinase and phosphoenolpyruvate were included in the reaction mixture, linear progress plots were always obtained over the 1h assay period, and the plots of activity versus enzyme concentration were always linear.

Units. One unit of activity is defined as the amount of enzyme that produces an incorporation of 1c.p.m. into the product in 1h at 37°C under the conditions described. Without an independent chemical method

of assay of the reaction, it was not possible to convert these units into international units of activity. Specific activity is defined as the activity/mg of protein.

Protein concentration. Protein concentration was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard.

Acetone-dried powder extracts. Xenopus laevis and rat liver acetone-dried powders were prepared and extracted by the procedure of Johnston & Bloch (1951).

Cell fractionation. The cell-fractionation procedure employed was devised from several reported procedures (Widnell & Tata, 1964; Campbell *et al.*, 1964). $MgCl_2$ was used in place of $CaCl_2$ in the fractionation medium as Ca^{2+} inhibits γ-glutamylcysteine synthetase.

Purification of Xenopus laevis and rat liver γ-glutamylcysteine synthetases. All operations were carried out at 4°C.

Saline–bicarbonate liver extract. The animals were killed, and their livers were washed in cold 0.14M-NaCl–0.05M-$NaHCO_3$. They were stored frozen at −10°C. After being thawed, a 1:2 (w/v) extract in the same solution was prepared, in an Ultra-Turax blender for 1min. The extract was centrifuged at 15000*g* for 10min and the supernatant fraction obtained was filtered through glass wool.

Protamine sulphate precipitation. The pH of the saline–bicarbonate extract was adjusted to a selected pH (*Xenopus laevis* 6.8; rat 6.6) with 0.25M-H_2SO_4. Salmine sulphate [1vol., 2% (w/v)] solution was added to 5 vol. of extract. The procedure is a modified version of that employed by Mandeles & Bloch (1955). The suspension was stirred for 20min, followed by centrifuging at 23000*g* for 20min. The clear deep-red supernatant fraction was retained.

CM-cellulose column chromatography. The supernatant fraction from the previous step, in portions of less than 150ml, was applied to a Sephadex G-25 (fine grade) column previously equilibrated with 0.01M-sodium citrate buffer at the appropriate pH (*Xenopus laevis* 6.8; rat 6.6). The enzymically active protein was eluted from the column after the void volume. The change in solvent composition delayed the elution of some inactive protein; this material was discarded. The active eluate was applied to the CM-cellulose column and the enzyme was eluted with 0.1M-sodium citrate buffer at the appropriate pH.

DEAE-cellulose column chromatography. The pH of the eluate from the previous step was adjusted to 7.8 with 0.05M-NaOH. A portion containing not more than 2.5g of protein was applied to the column. A linear elution gradient was applied to the column by using a peristaltic pump at a flow rate of 30ml/h. The mixing vessel contained 250ml of 0.005M-Tris–HCl buffer, pH7.8 and the reservoir vessel 250ml of 0.5M-NaCl in 0.005M-Tris–HCl buffer pH7.8; 4.5ml fractions were collected.

Gel filtration. A portion (15ml) of the most active DEAE eluate was concentrated by stirring it with 5g of Sephadex G-25 (coarse grade) and leaving it for 10min. The concentrated interstitial protein was extracted by centrifuging at 1000*g* for 10min in centrifuge tubes fitted with filter adaptors, then 1–3ml of the concentrated protein solution was applied to a 2cm×60cm Sephadex G-150 (fine grade) column previously equilibrated with 0.04M-Tris–HCl buffer, pH7.8. Each column used had been previously calibrated for molecular-weight determination with serum albumin, lactate dehydrogenase, pyruvate kinase and xanthine oxidase as standard proteins (Andrews, 1970). The protein was eluted from the column with the equilibration buffer supplied by a peristaltic pump at a constant flow rate of 10ml/h.

[^{35}S]Cysteine–γ-glutamylcysteine exchange. The isotope-exchange experiments were carried out in stoppered test tubes at 37°C. All solutions were deoxygenated and the incubations performed under N_2. The standard incubation medium contained 10mM-cysteine, specific radioactivity 3mCi/mmol, 10mM-γ-glutamylcysteine, 10mM-dithiothreitol, 10mM-$MgCl_2$ and 100mM-Tris–HCl buffer, pH7.7. Other components of the complete assay mixture were added in specific experiments. These included 10mM-glutamate, 4mM-ATP, 4mM-ADP and 4mM-NaH_2PO_4. All the reagents were adjusted to pH7.7 with 0.5M-NaOH. The reaction was stopped by the addition of 100μl of the incubation mixture to 20μl of acetic acid in a 4cm×0.5cm test tube. The contents of the test tube were frozen immediately at −76°C and stored in a Dewar flask containing solid CO_2 until required. γ-Glutamylcysteine was separated from cysteine by paper electrophoresis. A pyridine–water–acetic acid buffer for electrophoresis was prepared by adjusting 1 litre of 0.6% (v/v) pyridine–water solution at 22°C to pH3.9 with acetic acid. Portions (10μl) of the reaction mixture were applied to three 20cm×2.5cm strips of Whatman no. 1 chromatography paper as described by Shaw (1969). The electrophoretic separation was achieved with a constant voltage of 12.5V/cm for 150min. Cysteine migrated 4.0cm towards the anode and γ-glutamylcysteine 8.6cm. The electrophoretograms were dried at 50°C, and one lane of each triplicate set was stained by dipping in 0.25% (w/v) ninhydrin–acetone solution. The stained strip was used to locate the cysteine and γ-glutamylcysteine spots on the unstained strips. Each of the spots was cut into small pieces and placed flat on the base of a Packard scintillation vial ('5 dram' size). Elution of the ^{35}S-labelled compounds was achieved by the addition of 1ml of Soluene 100 to the vials. During extraction the vials were shaken intermittently over a period of 4h, after which 7ml of toluene scintillator (6g of PPO and 0.3g of DM-POPOP in 1 litre of toluene) were added. The radioactivity was determined with a

Packard Tri-Carb scintillation spectrometer. The results were expressed as a percentage of [^{35}S]cysteine incorporated into γ-glutamylcysteine.

[^{14}C]Glutamate–γ-glutamylcysteine exchange. This isotope-exchange experiment was carried out at 37°C. The standard incubation medium comprised: 10mM-[U-^{14}C]glutamate, specific radioactivity 1.25mCi/mmol, 10mM-γ-glutamylcysteine, 10mM-$MgCl_2$, 100mM-Tris–HCl buffer, pH7.7. Other components of the reaction mixture were added in certain experiments: 10mM-cysteine, 4mM-ATP, 4mM-ADP, 4mM-NaH_2PO_4. The reagents were adjusted to pH7.7 with 0.5M-NaOH. Portions (50μl) of the reaction mixture were withdrawn for assay and their [^{14}C]γ-glutamylcysteine content was determined as described above. The results are expressed as c.p.m. incorporated into γ-glutamylcysteine/50μl of reaction mixture.

Results

GSH synthesis from component amino acids

A linear rate of GSH synthesis was maintained over a period of 60min only in the presence of an ADP phosphorylating system. The rate of overall synthesis of GSH by an acetone-dried powder extract of rat liver was approximately 35 times higher than by a similar extract from *Xenopus laevis* (Table 1).

Table 1. *Comparison of Xenopus laevis and rat liver GSH synthesis from free amino acids in acetone-dried powder extracts*

Xenopus laevis and rat liver acetone-dried powder extracts (5mg) were used. The enzyme was assayed under standard conditions. The incubation period was 30min.

Source	GSH synthesis (c.p.m./mg of protein)
Xenopus laevis	21
Rat	743

Cellular location of γ-glutamylcysteine synthetase

Table 2 shows that γ-glutamylcysteine synthetase appears to be predominantly located in the soluble fraction of the liver cells of both species.

Purification of γ-glutamylcysteine synthetase from Xenopus laevis and rat liver

Typical elution profiles from DEAE-cellulose for *Xenopus laevis* and rat liver γ-glutamylcysteine synthetases are shown in Fig. 1. The *Xenopus laevis* enzyme eluted as one peak at a salt concentration in the range of 0.18 to 0.22M-NaCl and the rat enzyme from 0.21 to 0.27M-NaCl.

Fig. 2 shows typical elution profiles from Sephadex G-150. The enzyme from rat liver moved as a single peak, and that from *Xenopus laevis* showed a similar peak, together with several slower-moving peaks. Comparison with the elution patterns of proteins of known molecular weights suggests that the molecular weight of the main peak in both species is approximately 138000. The slow-moving peaks in the extract from *Xenopus* might represent smaller molecular species, or might be artifacts caused by adsorption of the enzyme on the gel or protein–protein interactions. The results of the purification of γ-glutamylcysteine synthetase from the livers of the two species are summarized in Tables 3 and 4 respectively.

Properties of γ-glutamylcysteine synthetase

The enzyme from rat liver lost no activity on storage for 2 weeks in solution at 4°C or in the frozen state at −10°C. The enzyme from toad liver lost 30% and 25% of its activity respectively under the above conditions.

Both enzymes showed maximal activity at pH7.7 under the assay conditions employed.

Table 2. *Cellular distribution of γ-glutamylcysteine synthetase in Xenopus laevis and rat liver*

The standard γ-glutamylcysteine synthetase assay was employed. A 0.4ml portion of cellular fraction derived from a 1:3 (w/v) liver homogenate was used in a final volume of 0.8ml.

	Xenopus laevis		Rat	
Cell fraction	Synthetase activity (c.p.m./h per g wet wt.)	% distribution	Synthetase activity (c.p.m./h per g wet wt.)	% distribution
Nuclear	124	7.5	567	2.4
Mitochondrial	50	3	152	0.6
Microsomal	50	3	168	0.7
Soluble	1480	87	22600	96.2

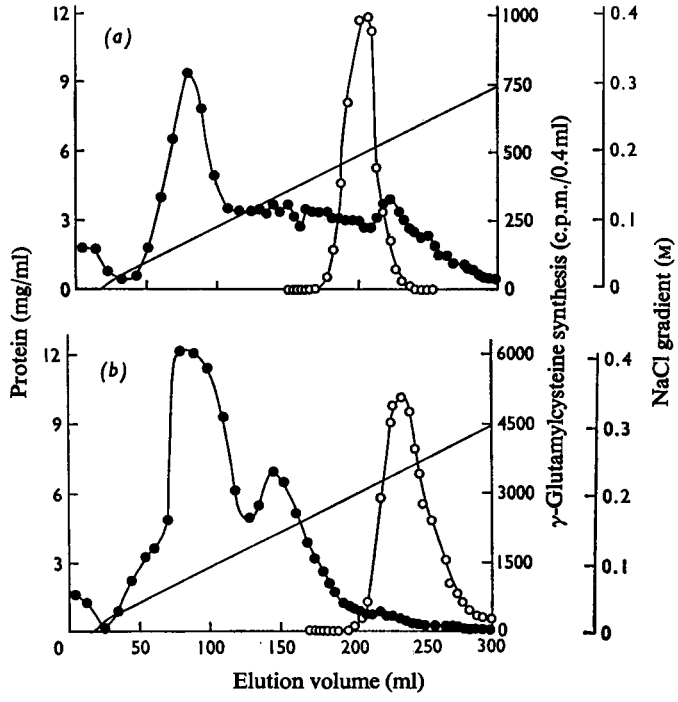


Fig. 1. *DEAE-cellulose column chromatography of γ-glutamylcysteine synthetase (a) from Xenopus laevis liver and (b) from rat liver*

The column, 1.5cm×30cm, was eluted with a linear gradient of 250ml of 0.005M-Tris–HCl buffer, pH7.8 mixing with 250ml of 0.5M-NaCl–0.005M-Tris–HCl buffer, pH7.8. ●, Protein content; ○, γ-glutamylcysteine synthetase activity; ——, NaCl gradient.

The effects of K^+ and Na^+ on *Xenopus laevis* and rat γ-glutamylcysteine synthetases are shown in Fig. 3. K^+ had a strong inhibitory effect on both enzymes. Na^+, on the other hand, had no effect on either enzyme.

Table 5 shows the effects of glycine, glutathione and nucleotides on γ-glutamylcysteine synthetase. Glycine caused a marked stimulation of rat γ-glutamylcysteine synthetase, but had no effect on the toad enzyme. The latter was inhibited by GSSG but not by GSH. This contrasted with the rat enzyme, which was inhibited by both GSH and GSSG. Both enzymes showed some inhibition by $NADP^+$ and NADPH and to a lesser extent by NAD^+ and NADH.

Isotope-exchange experiments

The exchange of isotope between [^{35}S]cysteine and γ-glutamylcysteine in the presence of *Xenopus laevis* γ-glutamylcysteine synthetase is shown in Fig. 4. Isotope incorporation into γ-glutamylcysteine occurred in the absence of ATP and glutamate at a rate equal to that in the presence of these two substrates. The rate of isotope exchange was little affected by addition of various substrates and products of the reaction, either singly or in combination. The incorporation must therefore be due to exchange rather than overall synthesis of γ-glutamylcysteine. In contrast, when the rat enzyme (Fig. 5) was used, a high rate of incorporation of label into γ-glutamylcysteine was only obtained in the presence of the other two substrates, namely ATP and glutamate, i.e. presumably by overall synthesis. Low rates of exchange were observed in the presence of ATP and of ADP, P_i and glutamate; other combinations of substrates and products resulted in no exchange at all.

The incorporation of [^{14}C]glutamate into γ-glutamylcysteine in the presence of the enzyme from *Xenopus laevis* required ATP and cysteine (Fig. 6). A very slight exchange was observed in the presence of ATP alone and of ADP, P_i and cysteine.

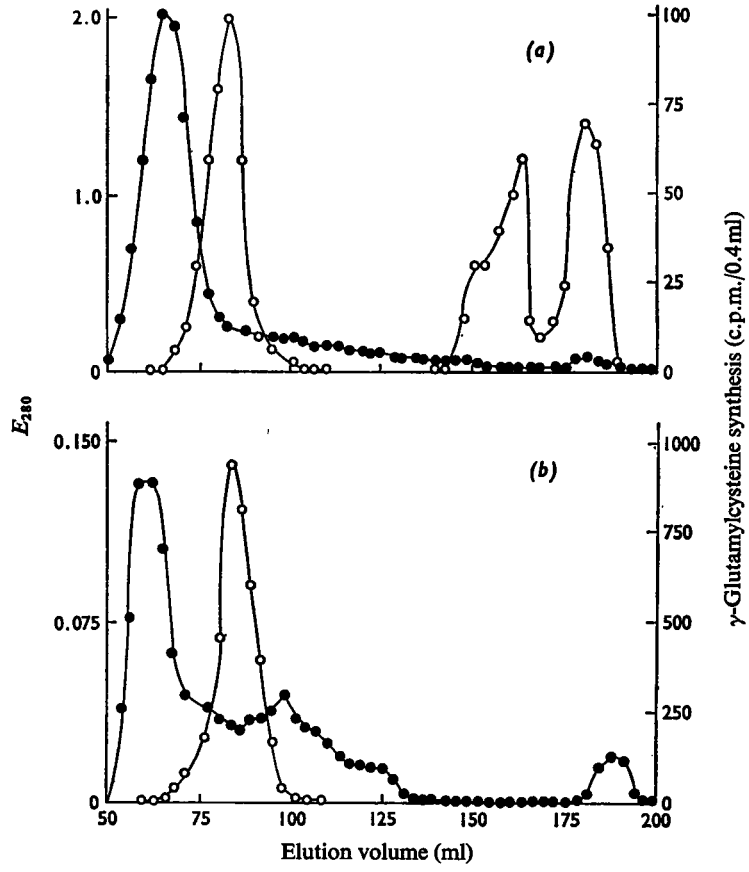


Fig. 2. *Gel filtration on Sephadex G-150 of γ-glutamylcysteine synthetase (a) from Xenopus laevis liver and (b) from rat liver*

The column, 2cm×60cm, was equilibrated and eluted with 0.04M-Tris–HCl buffer, pH7.8. ●, Protein content; ○, γ-glutamylcysteine synthetase activity.

Table 3. *Purification of Xenopus laevis liver γ-glutamylcysteine synthetase*

The standard γ-glutamylcysteine synthetase assay was employed. An 0.4ml sample of each enzyme fraction, previously adjusted to pH7.7 with 0.5M-NaOH, was used. The incubation period was 60min.

Stage	Volume (ml)	Total protein	10^{-3}×Total activity (c.p.m./h)	Specific activity (c.p.m/mg)	Purification	Yield (%)
Saline–bicarbonate extract	95	5.23g	54.9	10.5	1	100
Protamine sulphate precipitation	100	3.50g	152	43.4	4.1	277
CM-cellulose chromatography	110	2.16g	162	75	7.1	295
DEAE-cellulose chromatography	45	0.189g	128	677	64.5	233
Gel filtration (Sephadex G-150)	15	2.7mg	9.19	3400	324	17

Table 4. *Purification of rat liver γ-glutamylcysteine synthetase*

The standard γ-glutamylcysteine synthetase assay was employed. A 0.4ml sample of each enzyme fraction, previously adjusted to pH7.7 with 0.05M-NaOH, was used. The incubation period was 60min.

Stage	Volume (ml)	Total protein	10^{-3}×Total activity (c.p.m./h)	Specific activity (c.p.m./mg)	Purification	Yield (%)
Saline–bicarbonate extract	111	6.11g	339	55	1	100
Protamine sulphate precipitation	118	3.68g	745	202	3.7	220
CM-cellulose chromatography	148	1.39g	703	506	9.2	207
DEAE-cellulose chromatography	37.8	8.5mg	552	64900	1170	163
Gel filtration (Sephadex G-150)	8	84μg	52.5	625000	11400	15

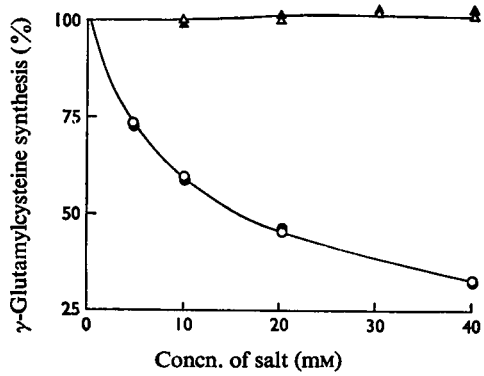


Fig. 3. *Effects of K^+ and Na^+ on Xenopus laevis and rat liver γ-glutamylcysteine synthetase activity*

Various concentrations of NaCl and KCl were added to the standard assay medium. *Xenopus laevis*: ○, KCl; △, NaCl. Rat: ●, KCl; ▲, NaCl.

Table 5. *Effect of various metabolites on Xenopus laevis and rat liver γ-glutamylcysteine synthesis*

The standard γ-glutamylcysteine synthetase assay was employed; the DEAE-cellulose eluate was used as a source of the enzyme. Additions to the system were made at the concentrations listed in the Table. Experiments were done at pH7.7 and 37°C.

Additions to standard assay	γ-Glutamylcysteine synthetase activity (% of control)	
	Xenopus laevis	Rat
None	100	100
10mM-Glycine	98	222
2mM-GSH	102	52
2mM-GSSG	76	54
2mM-NAD^+	94	79
2mM-NADH	90	67
2mM-$NADP^+$	83	50
2mM-NADPH	65	52

Surprisingly, when the rat enzyme was used, rapid incorporation was observed in the presence of ATP whether or not cysteine was present (Fig. 7). No other combinations of substrates and products were able to promote this exchange.

Discussion

The rate of synthesis of GSH in rat liver is comparable with that encountered in other mammals (Yanari *et al.*, 1953). The rate of synthesis in *Xenopus laevis* liver was 35 times lower. It seems that the low amount of GSH in Amphibia is associated with a much lower rate of synthesis.

The low γ-glutamylcysteine synthetase activity encountered in *Xenopus laevis* liver necessitated development of a suitable assay. Various methods for the estimation of the products of γ-glutamylcysteine and GSH synthetases were investigated. Of these, only the radioisotopic GSH assay of Johnston & Bloch (1951) proved satisfactory. This assay was improved by increasing its sensitivity and decreasing its duration. Mandeles & Bloch (1955) determined γ-glutamylcysteine synthetase activity by the addition of [^{14}C]glycine, K^+ and GSH synthetase to the system to convert the γ-glutamylcysteine formed into labelled GSH. This assay could not be used in the present study as K^+ inhibits both enzymes (Fig. 3) and GSH is also an inhibitor of rat γ-glutamylcysteine synthetase

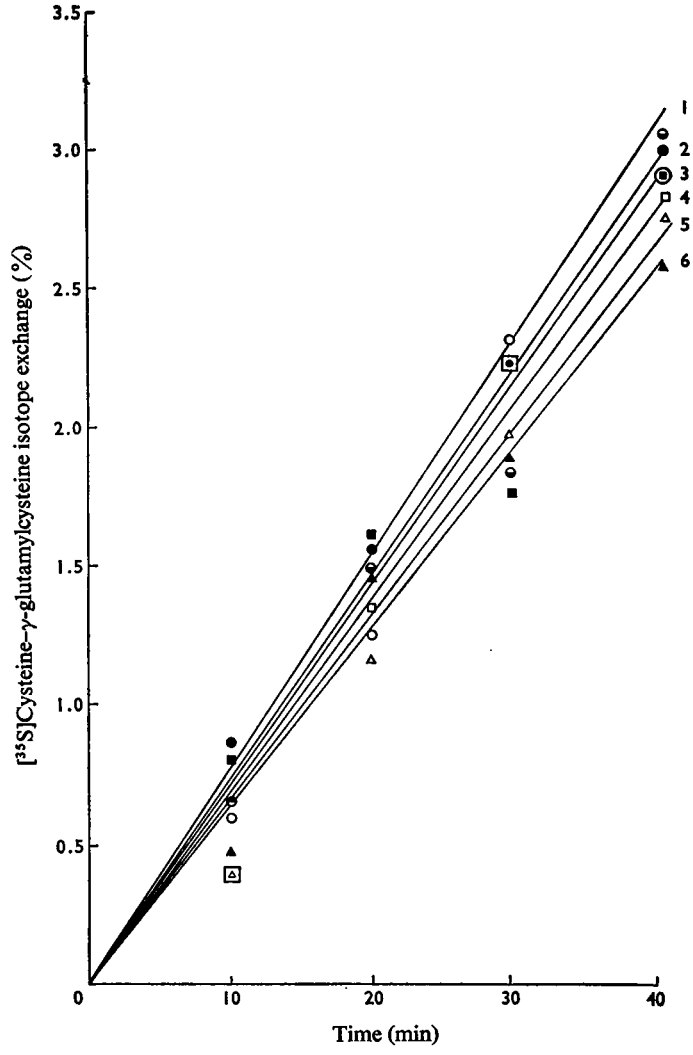


Fig. 4. *Effects of various reaction components on [^{35}S]cysteine–γ-glutamylcysteine isotope exchange catalysed by γ-glutamylcysteine synthetase from Xenopus laevis liver*

The standard cysteine–γ-glutamylcysteine exchange assay was employed. A 0.4ml portion of the DEAE-cellulose eluate containing 1.9mg of protein/ml was used in a final volume of 0.8ml. The following additions were made to the incubation medium: ● (line 1), ATP and glutamate; ■ (line 2), ATP; ○, ◒ (line 3), no addition, P_i; □ (line 4), ADP; △ (line 5), ADP and P_i; ▲ (line 6), ADP, P_i and glutamate.

(Table 5). It was, however, found that γ-glutamylcysteine synthetase could be conveniently assayed by following the incorporation of [^{14}C]glutamate into γ-glutamylcysteine. The [^{14}C]γ-glutamylcysteine formed was subsequently isolated as its cuprous mercaptide derivative by using GSH as carrier. The linearity of the kinetic studies lends further support to the validity of the assay procedure. Unfortunately, samples of GSH and γ-glutamylcysteine of known specific radioactivity that could be used to determine the degree of recovery were not available. No suitable alternative assay method was available to calibrate the radioassay.

The inhibition by K^+ is very similar in both γ-glutamylcysteine synthetases. In contrast, it has been shown that K^+ is essential for activity of the plant

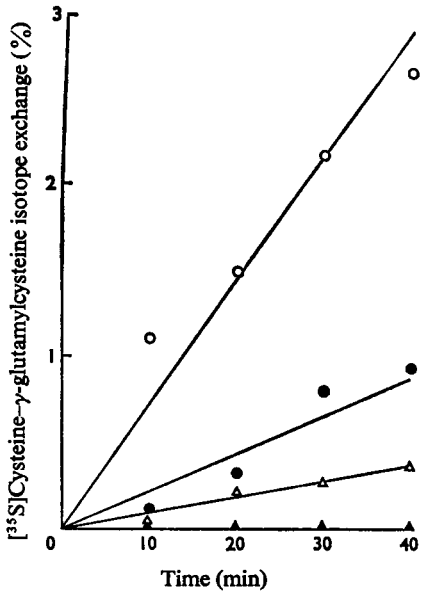


Fig. 5. *Effects of various reaction components on [35S]cysteine–γ-glutamylcysteine isotope exchange catalysed by rat liver γ-glutamylcysteine synthetase*

The standard cysteine–γ-glutamylcysteine exchange assay was employed. A 0.4ml portion of DEAE-cellulose eluate containing 0.21 mg of protein/ml was used in a final volume of 0.8ml. The following additions were made to the incubation medium: ○, ATP and glutamate; ●, ATP; △, ADP, P_i and glutamate; ▲ (a single line representing similar results from the following experiments), no addition, P_i only, ADP only, and ADP and P_i.

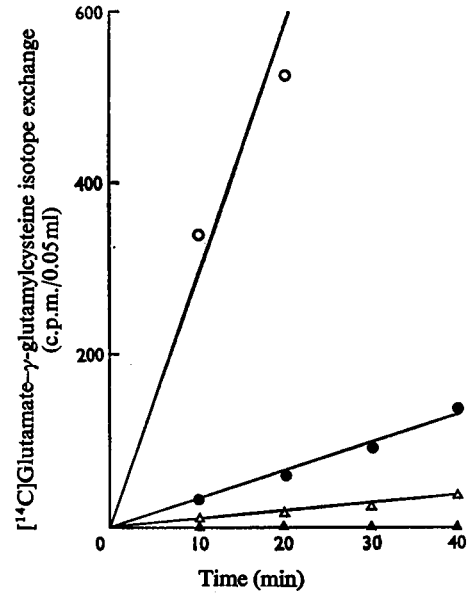


Fig. 6. *Effects of various reaction components on [14C]glutamate–γ-glutamylcysteine isotope exchange catalysed by γ-glutamylcysteine synthetase from Xenopus laevis liver*

The standard glutamate–γ-glutamylcysteine exchange assay was employed. A 0.4ml portion of the DEAE-cellulose eluate containing 2.3mg of protein/ml was used in a final volume of 0.8ml. The following additions were made to the incubation medium: ○, ATP and cysteine; ●, ATP; △, ADP, P_i and cysteine; ▲ (a single line representing similar results from the following experiments), no addition, P_i, ADP and P_i, and ADP and cysteine.

enzyme (Webster & Varner, 1954) and has no appreciable effect on the enzyme from pig liver (Mandeles & Bloch, 1955).

Apart from minor differences, the *Xenopus laevis* and rat γ-glutamylcysteine synthetases behaved in a similar manner during purification. Slight structural differences are indicated by the fact that the toad enzyme eluted at a slightly lower salt concentration than the rat enzyme. On gel filtration, both enzymes gave peaks of similar molecular weight. It would seem very probable, therefore, that the enzymes from toad and rat are homologous. Yet there appear to be a number of important differences between the two.

From Table 5 it can be seen that the enzyme from rat liver is inhibited by GSH and activated by glycine. The *Xenopus laevis* enzyme is unaffected by similar concentrations of these compounds. The effect of glycine is unlikely to be due to possible contamination by glutathione synthetase (EC 6.3.2.3), for which glycine functions as substrate. This is because a linear initial velocity is measured by the assay, and this is unlikely to be affected by coupling to the next step in the metabolic sequence. Inhibition by GSH and activation by glycine are more likely to be allosteric regulatory effects. Such inhibition of the first step in the pathway by the end-product of the biosynthetic pathway, and the activation of this step by glycine, a substrate for the second reaction, are both plausible regulatory effects. The effect of this regulatory mechanism would be to accelerate the first step in the synthesis when glycine is available and to inhibit it when glutathione concentration is high. It is thus tempting to suppose that a higher amount of GSH in mammals is associated with the evolution of regulatory mechanisms for the first enzyme in the pathway of its biosynthesis. The enzymes from both sources are inhibited by GSSG but this may well be a non-specific effect involving the oxidation of enzyme and/

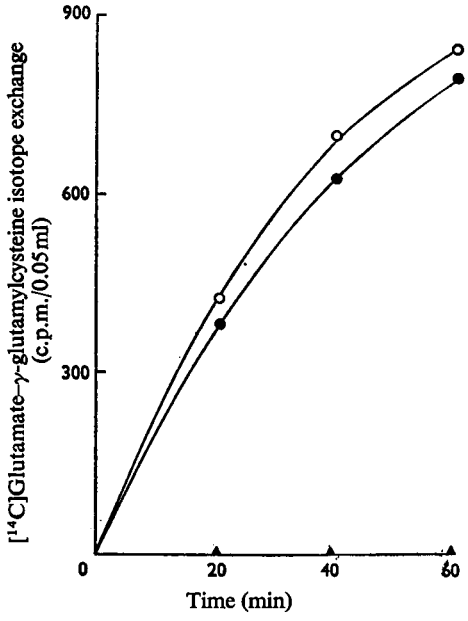


Fig. 7. *Effect of various reaction components on [^{14}C]glutamate–γ-glutamylcysteine isotope exchange catalysed by rat liver γ-glutamylcysteine synthetase*

The standard glutamate–γ-glutamylcysteine exchange assay was employed. A 0.4ml portion of DEAE-cellulose eluate containing 0.18mg of protein/ml was used in a final volume of 0.8ml. The following additions were made to the incubation medium: ○, ATP and cysteine; ●, ATP; ▲ (a single line representing similar results from the following experiments), no addition, P_i, ADP and P_i, ADP and cysteine, and ADP, P_i and cysteine.

or substrate thiol groups. Both enzymes show inhibition by oxidized and reduced forms of nicotinamide–adenine nucleotides; this is in agreement with the findings of Jackson (1969) with the human erythrocyte enzyme.

Because of the virtual irreversibility of the reaction catalysed by γ-glutamylcysteine synthetase (Mandeles & Bloch, 1955), the equilibrium position of the reaction is unknown. As a result, no attempt was made to carry out the exchange experiments at chemical equilibrium. In fact the cysteine– and glutamate–γ-glutamylcysteine exchanges took place, albeit at a slow rate, in the presence of all the products, namely γ-glutamylcysteine, ADP and P_i together with the one substrate selected for exchange (Figs. 4–7). This result is similar to the findings of Orlowski & Meister (1971*b*) for rat kidney γ-glutamylcysteine synthetase with the cysteine analogue α-amino-*n*-butyrate as substrate. These exchanges could only occur if one assumes that the complete reversal of the reaction, involving as it does the synthesis of ATP from ADP and P_i, is too slow to compete with the partial reactions mediating the exchange under these conditions.

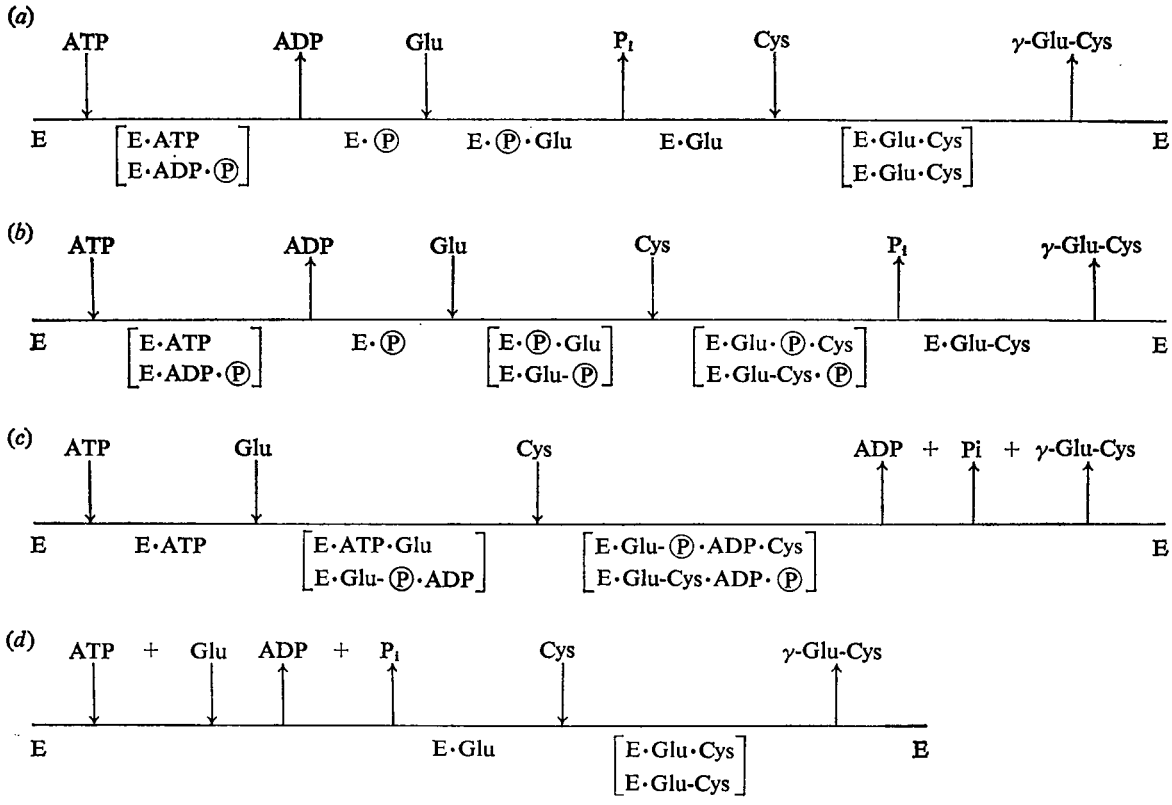
Previous experiments on isotope-exchange reactions catalysed by this enzyme indicated some differences between the enzyme from plants (Webster & Varner, 1954), pig liver (Strumeyer, 1959; Strumeyer & Bloch, 1960), and rat kidney (Orlowski & Meister, 1971*b*). Wheat germ and pig liver γ-glutamylcysteine synthetases both catalyse an ATP–ADP exchange reaction with enzyme alone (Webster & Varner, 1954; Strumeyer, 1959; Strumeyer & Bloch, 1960). Phosphorylation of the enzyme by ATP thus appears to be the first step in the reaction sequence of these two enzymes. However, the facility with which rat kidney enzyme preparations catalyse this exchange was found to diminish with purification (Orlowski & Meister, 1971*b*). These workers demonstrated the firm binding of ATP to the enzyme but were unable to decide conclusively whether enzyme phosphate was formed.

The wheat-germ enzyme in addition catalysed a glutamate-facilitated ATP–P_i exchange and an ADP-facilitated cysteine–γ-glutamylcysteine exchange (Webster & Varner, 1954). These data for the wheat-germ enzyme were used to propose mechanism (*a*) in Scheme 1 (Webster & Varner, 1954; Cleland, 1963). According to this mechanism, ATP reacts with enzyme to form enzyme–phosphate, which in turn reacts with glutamate to form glutamyl–enzyme.

The pig liver enzyme did not catalyse an ATP–P_i exchange under any circumstances (Strumeyer, 1959). Strumeyer & Bloch (1960) showed that when [γ-*carboxyl*-^{18}O]glutamate was incubated with pig liver enzyme, Mg^{2+}, ATP and cysteine, the P_i formed contained one ^{18}O atom per molecule. On the strength of these experiments, they proposed mechanism (*b*) in Scheme 1, which involves enzyme-bound glutamyl phosphate as a reaction intermediate.

The rat kidney enzyme does not catalyse ATP–ADP or ATP–P_i exchanges, and incorporation of label from glutamate or α-amino-*n*-butyrate into γ-glutamyl-α-amino-*n*-butyrate requires both ADP and P_i (Orlowsky & Meister, 1971*b*). A fully sequential mechanism (*c*, Scheme 1) was proposed by these writers in which ATP, glutamate and cysteine all add on to the enzyme before any product is formed.

The isotope exchanges described in the present paper suggest a difference in mechanism between the toad and rat liver enzymes. The enzyme from *Xenopus laevis* appears to be able to catalyse cysteine–γ-glutamylcysteine exchange in the absence of glutamate, ATP, ADP and P_i, whereas the rat liver enzyme does not catalyse this exchange. The exchange could conceivably be due to an impurity present in the toad enzyme, for example a γ-glutamylcysteine di-



Scheme 1. *Mechanisms of γ-glutamylcysteine synthetase*

For details see the Discussion section.

peptidase. But if this were the case, the preparation could hardly be expected to catalyse a linear rate of synthesis of glutamylcysteine. It seems much more likely, therefore, that the exchange is catalysed by toad liver γ-glutamylcysteine synthetase. This suggests that the enzyme from toad liver catalyses a Ping Pong mechanism somewhat similar to that formulated by Cleland (1963) for the wheat-germ enzyme (Webster & Varner, 1954), though there is no evidence from the present results of the existence of a phosphoryl enzyme as intermediate. A possible mechanism is indicated in Scheme 1 (*d*). It is implicit in this mechanism that a glutamyl–enzyme complex occurs as an intermediate during the reaction. Only the formation of such a complex with the release of cysteine on the reaction of γ-glutamylcysteine and enzyme can explain the observed exchange in the absence of the other components of the reaction. Apart from the existence of this complex and its reaction with cysteine no other conclusions can be drawn about the order of addition and release of substrates and products to and from the enzyme, or whether the reaction order is compulsory or random.

The results obtained with the rat liver enzyme, on the other hand, are consistent with the sequential mechanism proposed by Orlowski & Meister (1971*b*) for the rat kidney γ-glutamylcysteine synthetase, though a random order of addition cannot be ruled out by the present data.

A rather surprising result is the stimulation by ATP of all the exchanges save that between cysteine and γ-glutamylcysteine as catalysed by the *Xenopus laevis* enzyme. In particular, the rat liver enzyme catalyses an ATP-facilitated glutamate–γ-glutamylcysteine exchange at a velocity comparable with that of the forward reaction. This stimulation by ATP could be mediated by a number of possible mechanisms. One is that the reaction of ATP with the enzyme and γ-glutamylcysteine leads to a partial reversal of the reaction with the release of one of the amino acid substrates followed by its replacement with a labelled molecule. Another possibility is that the incorporation of labelled amino acid occurs via a modification of the forward reaction in which γ-glutamylcysteine acts as a substitute for the amino acid not present in the reaction mixture. For example, during the in-

corporation of [^{14}C]glutamate into γ-glutamylcysteine, γ-glutamylcysteine would replace cysteine in the normal forward reaction with enzyme, ATP and glutamate. The net result is the release of unlabelled glutamate and the incorporation of [^{14}C]glutamate into γ-glutamylcysteine. Both the above mechanisms result in the net hydrolysis of ATP, and it may be significant that adenosine triphosphatase activity has indeed been shown to be associated with the enzyme from rat kidney (Orlowski & Meister, 1971*b*).

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