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# Glutathione Turnover in the Kidney; Considerations Relating to the γ-Glutamyl Cycle and the Transport of Amino Acids

**(5-oxoproline/pyrrolidone carboxylate/pyroglutamate/γ-glutamyl amino acids/γ-glutamyl-cysteine)**

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**ABSTRACT** **The overall turnover of glutathione in kidney and liver was determined in studies in which L-[$^{14}$C]glutamate was administered to mice. Turnover was much more rapid (about 5 times greater) in kidney than in liver. Studies were also carried out in which 5-oxo-L-[$^{14}$C]proline was administered; the first order rate constants for glutathione synthesis from 5-oxoproline in liver and kidney were not far from those found for synthesis of glutathione from glutamate in these tissues. The findings are in accord with the fact that the activities of the enzymes of the γ-glutamyl cycle are much higher in kidney than in liver. The findings of high turnover of glutathione in kidney and the rapid utilization of 5-oxoproline by this organ for glutathione synthesis are consistent with the function of the γ-glutamyl cycle *in vivo* and the proposed role of γ-glutamyl derivatives in amino-acid transport.**

Previous studies in this laboratory have provided evidence for the existence of the γ-glutamyl cycle, a series of 6 enzyme-catalyzed reactions which account for the synthesis and degradation of glutathione (1–3). There is evidence that the cycle functions in kidney (1–6), choroid plexus and other regions of the brain (7, 8), ciliary body (9), and in a number of other tissues (1, 2, 4, 10). These and related studies have recently been reviewed (10). The initial step in the breakdown of glutathione is an amino acid-dependent transpeptidation reaction (reaction 1),

$$\gamma\text{-glutamyl-cysteinyl-glycine} + \text{amino acid} \rightleftharpoons \gamma\text{-glutamyl-amino acid} + \text{cysteinyl-glycine}, \quad [1]$$

which is catalyzed by γ-glutamyl transpeptidase, an enzyme localized in the brush borders of the proximal convoluted renal tubules; it is also bound to the membranes of epithelial cells in other tissues. It has been suggested that the γ-glutamyl cycle functions in amino-acid transport (1, 2); a mechanism was postulated in which membrane-bound transpeptidase interacts with extracellular amino acid and with intracellular glutathione to bring the amino acid into the cell as a γ-glutamyl amino acid (1). The amino acid is released from its γ-glutamyl carrier by γ-glutamyl cyclotransferase. The 5-oxoproline formed in this reaction is converted through the action of 5-oxoprolinase to glutamate. Glutamate thus produced, together with cysteine and glycine released by enzymatic hydrolysis of cysteinyl-glycine, is used for the resynthesis of glutathione catalyzed by the actions of γ-glutamyl-cysteine and glutathione synthetases. In addition to glutathione, γ-glutamyl-cysteine and other γ-glutamyl amino acids may also function as γ-glutamyl donors in transpeptidation reactions with amino acids.

The present work, which was begun in an effort to obtain information about the rate of glutathione utilization *in vivo*, deals with the turnover of glutathione in the kidney, an organ which is highly active in amino acid transport. Although a number of studies have been carried out on the turnover of glutathione in other tissues, e.g., liver (11–16), brain (14), erythrocytes (17, 18), lens (19), muscle (15), there appear to be no reports on kidney. Earlier studies in this laboratory have established that the activities of the enzymes of the γ-glutamyl cycle are much higher in kidney than in other tissues. The present studies show that the first order rate constant for glutathione synthesis is much higher in kidney than in liver (or other tissues). The results, which are in accord with the proposed function of γ-glutamyl compounds in amino acid transport, are considered in relation to the function of the γ-glutamyl cycle.

## MATERIALS AND METHODS

Male mice (fed *ad libitum;* strain CF-1) weighing 25–35 g were used. The animals were injected intraperitoneally with a tracer amount (0.68 nmole/g) of L-(*U*-$^{14}$C]glutamate (New England Nuclear Corp.; 269 Ci/mole), and then killed at various intervals by cervical dislocation and decapitation. In some studies the same amount of 5-oxo-L-[*U*-$^{14}$C]proline [prepared from the [$^{14}$C]glutamate by heating at 140° at pH 3 followed by purification on Dowex 50 ($H^+$)] was injected. The tissues were immediately excised and frozen in liquid nitrogen; samples (250–600 mg) were homogenized in 3 ml of 5% trichloroacetic acid. After removal of the precipitated protein by centrifugation (0°; 2000 × *g*), a 2-ml aliquot of the supernatant solution was applied to a column (5 × 0.6-cm) of Dowex 50 ($H^+$), which was then washed with 4 ml of water; elution was then carried out with 4 ml of 3 N $NH_4OH$. The eluates were flash evaporated to dryness and the residues were dissolved in 0.5 ml of 0.25 M (Na-EDTA) sodium ethylenediaminetetraacetate (adjusted to pH 8.65). The solutions were treated with 0.1 ml of a solution containing 15 mg of potassium borohydride (obtained from Alpha Inorganics Inc.) (20); after 15 min at 26°, 0.1 ml of a solution containing 10 mg of iodoacetamide was added and the mixture was allowed to stand at 26° for 15 min. After addition of 0.2 ml of 5 N HCl the precipitate of EDTA was removed by centrifugation. The supernatant solution was analyzed on an automated amino-acid analyzer equipped with a column of Durrum type DC-1A resin. The column was eluted at 37°

Abbreviations: EDTA, ethylenediaminetetracetate.

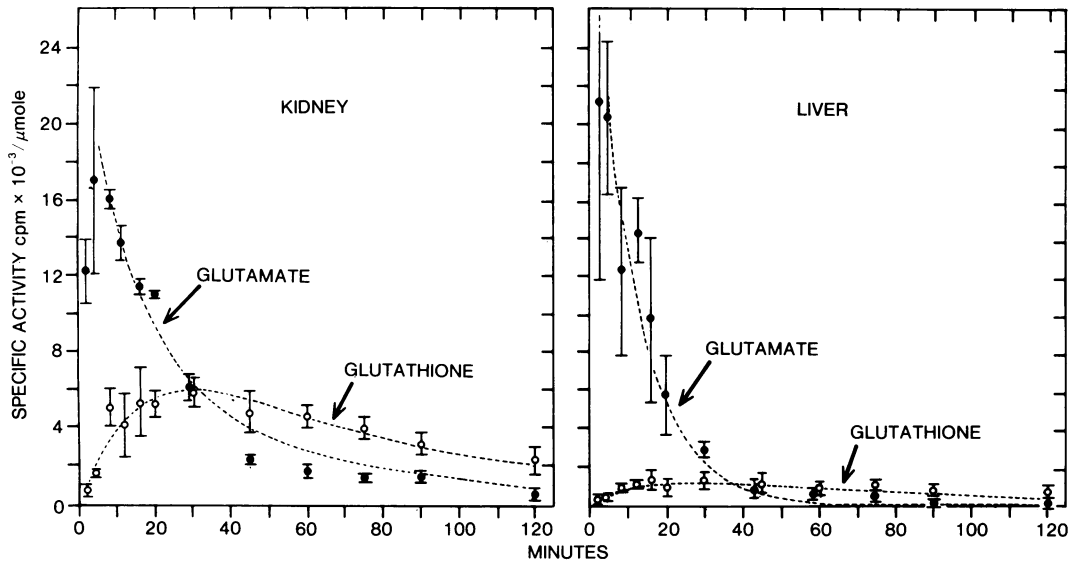


FIG. 1. Values for the specific radioactivity of glutathione and glutamate after administration of L-[$^{14}$C]glutamate to mice. The *bars* represent the standard deviations of the data from the mean; three to four mice were used at each point. The interrupted curves were obtained by calculation; see the *text*.

with 0.16 M lithium citrate (pH 2.90). The column effluent, prior to introduction into the ninhydrin reaction coil, was passed through a Packard scintillation counter equipped with an anthracene flow cell. Other details of the procedure and of the equipment (constructed in this laboratory) will be published in a subsequent communication. Under the conditions employed, *S*-acetamido-glutathione, glutamate, and other amino acids were completely resolved; the specific radioactivities of glutamate and glutathione were obtained from the quantitative ninhydrin values and from the corresponding determinations of $^{14}$C. Satisfactory values for recovery of glutathione added to tissues were obtained by this procedure. Values (μmoles/g) of 6.31 ± 1.30 and 10.7 ± 2.0 were found for glutathione and glutamate in the liver; the corresponding values for kidney were 2.68 ± 0.35 and 3.42 ± 0.37. Similar values have been obtained in this laboratory and by others by other methods for the determination of glutathione. Studies in which the tissues extracts were subjected to acid hydrolysis (6 N HCl, 100°, 6 hr) followed by automated determinations of cysteine and glycine showed no detectable radioactivity in these amino acids.

## RESULTS

As shown in Fig. 1, liver glutamate reached maximal specific activity within 2 min after intraperitoneal injection of L-[$^{14}$C]glutamate, and the specific activity of liver glutamate fell rapidly, reaching 50% of its highest value at about 12 min after injection. Kidney glutamate reached, after 4 min, almost the same maximum specific activity that was found in liver; it then declined, more slowly than in liver, reaching 50% of its highest value at about 22 min. The increase in specific activity of kidney glutathione was much more rapid than that found in liver, and the maximum specific activity attained by glutathione in the kidney was about four times higher than found in liver. In both kidney and liver, the glutathione specific activity curves were maximal at the points at which they intersected the curves for the specific activities of glutamate, a finding consistent with a precursor-product relationship between glutamate and glutathione (21).

In previous studies, glutathione turnover in animal tissues has been estimated (*a*) from the amount of labeled amino-acid precursor incorporated into glutathione in a given period of time (11–13), (*b*) from a semi-logarithmic plot of the decay of specific activity of glutathione with time (14, 17, 18), and (*c*) by application of procedures in which the relationship between the specific activities of the pools of precursor and product are considered (15, 16, 19). In the present work, the first order rate constants for glutathione synthesis were calculated from the observed specific activities of glutathione and glutamate (Fig. 1). Equations similar to those developed by Reiner (22, 23) and Koch (24) for a 2-compartment system, i.e., glutamate, glutathione, were used. The first order rate constants for glutathione synthesis were obtained by a curve fitting procedure. A nonlinear minimization method was used in which the Davidon algorithm (25, 26, see*) was employed to iteratively modify an initial approximation of the solution; constants were selected (by the program) such that the root mean square deviations of the experimental data (for the specific activities of glutamate and glutathione) from the generated values were at a minimum. The experimental and theoretical curves for the specific activity of glutathione agree within the experimental error, but there is some deviation in the later portions of the curves for the specific activity of glutamate. The first order rate constants for glutathione synthesis were calculated to be 0.0240 ± 0.0057 and 0.00477 ± 0.00054 $min^{-1}$ for kidney and liver, respectively.

Similar studies were carried out in which 5-oxo-L-[$^{14}$C]-proline was administered; first order rate constants for glutathione synthesis of 0.0168 and 0.00384 $min^{-1}$ were obtained, for kidney and liver, respectively.

## DISCUSSION

The data given in Table 1 summarize previously published estimates of glutathione turnover in various animal tissues.

* The programs used for these estimations were written in APL/360. The Davidon program was made available by the SECOS APL/360 system, Poughkeepsie, N.Y.

TABLE 1. *Data reported on turnover of glutathione*

| Tissue (species) | Compound given | Half-time* minutes | Glutathione synthesis first-order rate constant ($min^{-1}$) × $10^3$ | Reference, date |
|---|---|---|---|---|
| Liver (rat) | [$^{15}$N]glycine | < 480 | < 1.4 | (11), 1941 |
| Liver (rat) | DL-[$^{15}$N]glutamate | 120–240 | 2.9–5.8 | (12), 1942 |
| Liver (rabbit) | DL-[$^{15}$N]glutamate | 120–240 | 2.9–5.8 | (12), 1942 |
| Liver (rat) | DL-[$^{35}$S]cysteine | < 180† | | (13), 1951 |
| Erythrocyte (man) | [$^{15}$N]glycine | 5760 | 0.12 | (17), 1955 |
| Liver (rabbit)§ | [$^{14}$C]glycine | 616‡ | 1.1 | (15), 1955 |
| Muscle (rabbit) | [$^{14}$C]glycine | 6200‡ | 0.11 | (15), 1955 |
| Erythrocyte (rat) | [$^{14}$C]glycine | 3900 | 0.18 | (18), 1956 |
| Liver (rat) | [$^{14}$C]glycine | 240 | 2.9 | (14), 1956 |
| Brain (rat) | [$^{14}$C]glycine | 4200 | 0.17 | (14), 1956 |
| Liver (rat) | [$^{14}$C]glycine | 77‡ | 9.0 | (16), 1957 |
| Lens (rat) | [$^{14}$C]glycine | 4000‡ | 0.17 | (19), 1959 |
| Liver (mouse) | L-[$^{14}$C]glutamate | 145 | 4.8 | Present work, 1974 |
| Kidney (mouse) | L-[$^{14}$C]glutamate | 29 | 24 | Present work, 1974 |

* Half-Time = 0.693 (first order rate constant)$^{-1}$.
† Time within which maximum labeling of glutathione was observed.
‡ Calculated by multiplying reported renewal times by 0.693.
§ A half-time of less than 1080 min was also reported (11).

The first of these was reported by Waelsch and Rittenberg (11, 12), who administered [$^{15}$N]glycine, [$^{15}$N]glutamate, or $^{15}NH_4^+$ to animals and determined the isotope content of the glutathione isolated from liver at various intervals. It is interesting to note that the value for liver glutathione turnover obtained in the present work is not very far from those found by Waelsch and Rittenberg and by other investigators over the period 1941–1959.

The present findings show that the turnover of glutathione in the kidney is substantially higher than in the liver and in the other tissues that have previously been studied. Such a high turnover of glutathione is in accord with the presence in kidney of high activities of the enzymes of the γ-glutamyl cycle. However, the rate of utilization (and synthesis) of glutathione by the kidney found here is much lower on a molar basis than the estimated renal reabsorption of amino acids. Thus, the overall rate of turnover by the kidney may be estimated from the glutathione pool size and the rate constant, to be 64.3 ± 15.4 nmoles/min per g, while the amount of amino acid filtered by the mouse kidneys may be estimated to be about 2.4 μmoles/min per g (assuming a glomerular filtration rate of 0.8 ml/min per g and a blood plasma amino-acid concentration of 3 mM).

It should be emphasized, however, that certain of the assumptions made in obtaining the estimate of glutathione turnover rate are open to serious question, especially the assumptions of homogeneous pools of glutamate and glutathione. Thus, it is probable that the distribution of glutathione in kidney, as well as that of the enzymes that synthesize and degrade it, is not homogeneous. Boyne and Ellman (27) have reported evidence consistent with a heterogeneous distribution of glutathione in the kidney. Furthermore, it would be expected that if the γ-glutamyl cycle functions in reabsorption of amino acids, the glutathione involved in the cycle, as well as the other substrates and enzymes, would be localized at the sites of reabsorption; at these sites, the localized glutathione would be expected to turnover very rapidly, perhaps much more rapidly than glutathione elsewhere in the kidney. Indeed, histochemical studies have shown that the transpeptidase is found mainly in the brush borders of the proximal convoluted tubules. These considerations suggest the need for studies on the turnover of glutathione in the proximal convoluted tubular cells as well as in other regions of the kidney. One may also consider the apparently analogous situation in regard to the overall turnover of glutathione in brain, which is very slow (ref. 14 and Table 1). Thus, the very high activities of the γ-glutamyl cycle enzymes found in the choroid plexuses (7) suggest that, at least in these regions of the brain (which represent a very small fraction, perhaps as little as 0.1%, of the total brain mass), the turnover of glutathione may be very much higher than indicated by the overall turnover rate of the brain.

It seems notable that the theoretical curves for the specific activity of glutamate deviate significantly in the later portions from the experimentally determined points (Fig. 1); this also suggests that the system may be more complex than assumed here for purposes of calculation. It thus appears that the results can be interpreted in several ways. For example, the presence of a separate small and very rapidly turning over pool of glutathione would be obscured in the present approach by the existence of a larger pool that turns over at a slower rate. Furthermore, if glutathione were synthesized from a closed glutamate pool, the observed turnover rates might reflect the rate at which glutamate entered the pool rather than the actual rate of glutathione turnover within the pool. Further information might be derived from data on the rates of formation and utilization of other γ-glutamyl cycle intermediates, e.g., γ-glutamyl amino acids, 5-oxoproline. Although exact data are not yet available, there is reason to think that the capacity to utilize 5-oxoproline is quite large. The rate of 5-oxoproline degradation by patients with 5-oxoprolinuria is evidently great, i.e., as much as 60 g/day (28). In the present work the first order rate constants for glutathione synthesis from 5-oxoproline were found to be not far from those for glutathione synthesis from glutamate; this is consistent with substantial utilization of 5-oxoproline via the γ-glutamyl cycle.

In addition to the possibilities discussed above, we must consider other phenomena that are consistent with the findings. For example, information derived from earlier enzyme

studies suggests that glutathione does not function as the sole donor of the γ-glutamyl moiety in transpeptidation, and thus that other γ-glutamyl derivatives may also function in amino-acid transport. γ-Glutamyl-cysteine has been shown to be a good substrate for transpeptidase (29, 30), and it was previously suggested that γ-glutamyl-cysteine might function in transpeptidation in 5-oxoprolinuria in which there is a deficiency of glutathione synthetase (31).

In addition, glutathione might serve as a reservoir for the γ-glutamyl moiety, and γ-glutamyl amino acids formed initially by transpeptidation with glutathione might function significantly as γ-glutamyl donors. γ-Glutamyl-glutamine is very active in transpeptidation (29, 30), and this compound is also one of the best substrates of γ-glutamyl cyclotransferase. In previous work, it was found that the most active substrates of γ-glutamyl cyclotransferase are the γ-glutamyl derivatives of glutamine, methionine, cysteine, glycine, and alanine and that the γ-glutamyl derivatives of other amino acids are much less active. However, the γ-glutamyl amino acids which are poor substrates for γ-glutamyl cyclotransferase (γ-GCT) may be degraded by coupled reactions involving γ-glutamyl transpeptidase (γ-GTP):

$$\gamma\text{-glutamyl-valine} + \text{glutamine} \xrightarrow{\gamma\text{-GTP}} \gamma\text{-glutamyl-glutamine} + \text{valine} \quad [2]$$

$$\gamma\text{-glutamyl-glutamine} \xrightarrow{\gamma\text{-GCT}} \text{5-oxoproline} + \text{glutamine} \quad [3]$$

Such transpeptidation reactions could facilitate amino acid transport. The concentration of glutamine in blood plasma and in many tissues is much higher than the concentrations of methionine, cysteine, glycine, and alanine; thus, it is likely that glutamine would be more readily available for reactions of the type depicted in Eqs. 2 and 3. Glutamine might therefore perform a special function in the utilization of amino acids via the γ-glutamyl cycle.

It is possible that a number of successive transpeptidation reactions occur *in vivo*; indeed, many such reactions have been demonstrated *in vitro*. A multiple transpeptidation model could conceivably function as a facilitated diffusion carrier system. The transpeptidation reaction of glutathione with an amino acid might theoretically be driven in the direction of γ-glutamyl amino-acid formation by removal of the dipeptide product by enzymatic cleavage; however, the products of cleavage (cysteine and glycine) are themselves substrates of the transpeptidase. Transpeptidation reactions between a γ-glutamyl amino acid and an amino acid would be expected to be associated with relatively little change in free energy, and therefore it would appear that such reactions could not mediate active transport unless they were coupled to reactions associated with greater changes in free energy. The possibility exists that there are other reactions (in addition to those catalyzed by γ-glutamyl cyclotransferase, 5-oxoprolinase, and the enzymes that catalyze γ-glutamyl-cysteine and glutathione synthesis) which can drive a γ-glutamyl transpeptidase-mediated carrier system without removal of the γ-glutamyl moiety by hydrolysis or cyclization. The existence of such mechanisms (which might conceivably be associated with energy-dependent conformational changes in membrane-bound transpeptidase, or the effects of $Na^+$) would be consistent with a molar turnover of glutathione which is lower than that of amino acid transport. Further studies on the interrelationships between the reactions of the γ-glutamyl cycle and other metabolic and membrane phenomena are needed in order to evaluate these possibilities. The data now available do not exclude the possibility that glutathione turnover may reflect several cellular functions. Although a number of experimental findings support the existence of the γ-glutamyl cycle in various cell types, the proposed function of the cycle in amino-acid transport remains to be proven, and other possible functions cannot be ruled out, including for example, a role in peptide transport or metabolism, or another metabolic activity. The finding of a relatively high turnover of glutathione in kidney suggests that this tripeptide is of special significance in the function of this organ.

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