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Maintenance of Glutathione Levels in Renal Cortex Slices of the Rat[1] (39671)

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Glutathione (GSH) is purported to play a central role in the transport of amino acids and perhaps other substances in mammalian kidney. For each amino acid molecule that is transported into a renal tubular cell, a molecule of GSH is broken down and later resynthesized. The synthetic steps require metabolic energy in the form of ATP. The flux of amino acids through kidney is high; accordingly, turnover of GSH is rapid. Maximal rates of degradative and synthetic enzymes of rat kidney are sufficient to catalyze the breakdown and resynthesis of several grams of GSH per day (1).

Kidney slices are frequently used in transport studies (2) but relatively little attention has been paid to GSH levels in the slices. Amino acids released from GSH degradation would not all be expected to be utilized for resynthesis of the tripeptide. Furthermore, considerable leakage of free amino acids from kidney slices is known to occur (2). Thus, GSH levels would be expected to fall off with time in kidney slices being incubated in a medium unfortified with the component amino acids of GSH.

This work was carried out in order to determine how well GSH levels were maintained in kidney cortex slices under standard incubation conditions, and to study the influence of metabolism and added amino acids or protein on GSH levels. In addition, since maleic acid has been shown to depress renal GSH levels *in vivo* (3) and to inhibit renal amino acid transport *in vitro* (4) and *in vivo* (5), the effect of this compound on GSH levels in the kidney slices was studied.

Methods. Animals. Male Holtzman rats of weight range 175-380 g were used in all experiments as the source of kidney tissue. These animals were maintained in an air-conditioned room with an automatic 12 h light/darkness cycle and fed Purina Rat Chow (Ralston Purina Co., St. Louis, Missouri) and water *ad libitum*.

Chemicals. All buffers and solutions were prepared in deionized distilled water and analytical reagent grade chemicals were used except as specified below.

The following chemicals were obtained as the highest purity available: glutathione and L-cysteine (Calbiochem, Los Angeles, California); glycine, L-glutamic acid, and 5,5'-dithiobis-(2-nitrobenzoic acid), (DTNB), (Fisher Scientific, Fair Lawn, New Jersey); bovine serum albumin (Miles Laboratories, Kankakee, Illinois); maleic acid and L-methionine (Eastman Kodak, Rochester, New York); and L-glutamine and dithiothreitol (Sigma Chemical, St. Louis, Missouri). 2,4-dinitrophenol was synthesized in the Department of Nutrition, Harvard School of Public Health.

Solutions added to tissue slice preparations were corrected to pH 7.4 with NaOH or HCl. Cysteine solutions were always prepared immediately before use to avoid oxidation of the sulfhydryl group.

Tissue slices. Renal cortex slices were prepared from rat kidney as described by Rosenberg *et al.* (2). The average thickness obtained with the Stadie-Riggs tissue slicer was 0.50 mm. Each kidney yielded four cortical slices. Prior to incubation, slices were removed from storage in ice-cold

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Krebs-Ringer buffer, gently blotted on buffer-moistened filter paper, and weighed by suspending three slices at a time from a steel wire hung inside a Mettler analytical balance. The average wet-weight of tissue per flask (three slices) was 110 mg. After weighing, slices were placed in 25 ml conical flasks containing Krebs-Ringer buffer and kept on ice until the start of the preincubation interval.

Incubations. Slices were incubated at pH 7.4 either in Krebs-Ringer bicarbonate (KRB) or Krebs-Ringer phosphate (KRP) buffer containing 10 m*M* glucose (6). These buffers were freshly prepared on the day of the experiment. Incubations were carried out in a Dubnoff incubator agitating at about 85 oscillations/min. Flasks were covered by an air-tight hood and gassed with 95% O_2/5% CO_2 when KRB was used, or 100% O_2 when KRP was used. In anaerobic experiments 95% N_2/5% CO_2 (for KRB) was used. Flow rate was adjusted to 4 ft^3/hr, sufficient to produce bubbling from under the hood. Temperature was maintained at 37 ± 2° except in reduced temperature experiments when 25 ± 1° or ice-bath temperature was maintained. Unless otherwise specified, slices were preincubated at the temperature to be maintained in the experiment for 15 min. Incubations were then continued for 10–120 min in time-course experiments or 60 min in single time-point experiments. At the end of the incubation interval, flasks were removed from the incubator to an ice bath and the slices quickly removed with forceps, dipped briefly into fresh, cold buffer, blotted with buffer-moistened filter paper and placed into micro ground-glass hand-driven homogenizers containing 0.90 ml of cold 5% trichloroacetic acid (TCA) containing 1 m*M* disodium ethylenediaminetetraacetic acid (EDTA). After thorough homogenization, aliquots of 0.8 ml were removed and placed into 10 × 70 mm test tubes and centrifuged at 1000*g* for 15 min to sediment the TCA-precipitated proteins. Aliquots of the clear supernatant (0.40 ml) were transferred to 13 × 100 mm test tubes for assay of GSH.

Glutathione (GSH) assay. The method of Sedlak and Lindsay (7) was used. The yellow color produced by release of 5-thio-2-nitrobenzoate by thiol cleavage of 5,5′-dithiobis(2-nitrobenzoic acid), (DTNB), at pH 8.0 was read at 412 nm in a final volume of 5.00 ml.

Statistical treatment. Glutathione concentrations are expressed as μg GSH/g wet weight of tissue. Data are presented as mean ± SE based on values from three incubation flasks. Significance of differences between means was determined using Student's *t* test, with $P < 0.05$ being regarded as significant.

Results. Time-course of GSH maintenance and effect of added protein. Kidney cortex slices were incubated in KRB buffer at 37° under 95% O_2/5% CO_5 for 30, 60, 90, and 120 min. Glutathione levels in the slices fell during the incubation interval as shown in Fig. 1. Addition of bovine serum albumin (BSA) to the incubation medium at a concentration of 4% (w/v) had no effect on the disappearance of GSH.

Effect of maleate on time-course. Following a 10-min preincubation at 25°, slices were transferred to flasks containing KRB buffer or buffer containing 3 m*M* disodium maleate and incubated at 37° for 10, 20, 30, 60, 90, and 120 min (Fig. 2). Maleate had no effect on the rate of disappearance of GSH between 10 and 60 min of incubation, but GSH levels in maleate-treated slices dropped to lower levels than control slices at 90 and 120 min.

Effect of amino acids and dithiothreitol. Slices were preincubated in KRB buffer at 37° for 10 min before addition of amino acids or dithiothreitol (DTT). Incubation was then continued for 60 min (Fig. 3). Addition of glycine or glutamic acid singly

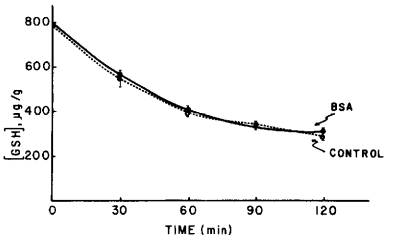

FIG. 1. Time-course of disappearance of glutathione (GSH) from renal cortex slices. Slices were incubated for 30, 60, 90, and 120 min in KRB (control) or KRB containing 4% (w/v) bovine serum albumin (BSA).

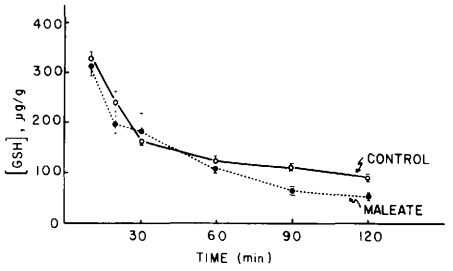

FIG. 2. Time-course of disappearance of glutathione (GSH) from renal cortex slices. Slices were stored at 0° and then preincubated in the same flasks for 10 min at 25°. At zero time slices were transferred to fresh KRB buffer (control) or KRB containing disodium maleate (3 m*M*) and incubated at 37° for 10, 20, 30, 60, 90, and 120 min.

in 0.1 m*M* concentraton did not affect the GSH level. However, adding these amino acids in combination raised the GSH level somewhat above control. The same combination at 3 m*M* concentration produced a marked effect, even though no source of sulfur had been added. Methionine was added at 3 m*M* concentration as a sulfur source, but the result was a depression of the GSH level below the control value. When this amino acid was added to the glutamic acid and glycine combination, the resulting GSH level was slightly lower than with only glycine and glutamate present. Cysteine alone was effective in significantly raising the GSH level and was nearly twice as effective if DTT was included. Dithiothreitol on its own had little or no effect on the GSH level, indicating that DTT acts by preserving the free sulfhydryl groups of cysteine rather than by furnishing sulfur itself. This effect was seen again with the inclusion of all three component amino acids of GSH: the combination was markedly effective, but inclusion of DTT with glycine, glutamate, and cysteine produced the most effective combination tested. Glutamine at 1 m*M* concentration was somewhat more effective than the mixture of 0.1 m*M* glycine and glutamic acid.

Metabolic influences. Table I summarizes the results of three separate experiments testing different aspects of the dependence of GSH maintenance on metabolism. Replacement of oxygen by nitrogen in the incubator atmosphere produced over a twofold drop in GSH concentration. Incubation with dinitrophenol, an uncoupler of oxidative phosphorylation, also resulted in lowering the GSH levels below that of controls. Reducing the temperature to 25° or 0° resulted in successively increasing the GSH level in the slices.

Discussion. Albumin is sometimes added to incubation media as a nonspecific stabilizer of cell membranes (8) but it had no effect on the parameter being measured here. Glutathione disappears rapidly from solutions containing albumin due to the formation of mixed disulfides with the protein (9). If free GSH were simply diffusing out of the slices, a faster rate of disappearance of GSH might have been expected with BSA acting as a trapping agent in the medium. Such was not the case, however.

The effect of maleate on the slice preparations was in marked contrast to the effect on renal GSH *in vivo* (3) when maximal effects on GSH depletion were produced in 30 min. In the *in vivo* situation, however, GSH levels in kidneys of control animals are maintained constant rather than decreasing as in the slice preparation. Glutathione depletion *in vitro* may be difficult to achieve unless the conjugation product is constantly removed from the medium (it is excreted in the urine *in vivo*) since some GSH-conjugating en-

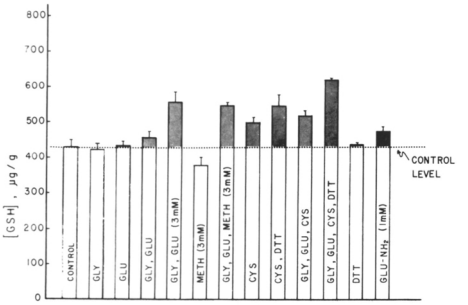

FIG. 3. Effect of amino acids and dithiothreitol on glutathione (GSH) levels in kidney cortex slices. Substances were added to KRB medium at zero time following a 15 min preincubation interval and incubation continued for 60 min. Control = buffer only; GLY = glycine; GLU = glutamic acid; METH = methionine; CYS = cysteine; DTT = dithiothreitol; GLU-NH_2 = glutamine. Except when indicated inside the bars, final concentration of each added component was 0.10 m*M*. Shaded portion of bar indicates net increase of GSH concentration over mean control level.

TABLE I. METABOLIC INFLUENCES ON GLUTATHIONE (GSH) LEVELS IN KIDNEY SLICES.[a]

	Oxygen[b]		Temperature,[c] °C			Dinitrophenol[d]	
	Present	Absent	37	25	0	Absent	Present
GSH, μg/g	460 ± 14	211[e] ± 7	310 ± 20	650[e] ± 40	770[e] ± 70	370 ± 5	280[e] ± 10

[a] Results of three separate experiments. Renal cortex slices prepared as outlined in Methods and incubated for 60 min in the appropriate buffer system following a 15-min preincubation.

[b] KRB buffer, 37°, under 95% O_2/5% CO_2 or 95% N_2/5% CO_2.

[c] Modified KRP buffer containing 0.11 *M* $CaCl_2$, under 100% O_2.

[d] Standard KRP buffer, under 100% O_2 at 37°. Dinitrophenol (final concentration 1.0×10^{-4} *M*) added at beginning of preincubation interval.

[e] Significantly different from each respective control ($P < 0.01$).

zymes are inhibited by their products (10).

The results obtained with the amino acids indicated that the GSH lost by degradation could be at least partially replaced by supplying amino acids for resynthesis. Glutamate and glycine are utilized in a number of pathways other than GSH synthesis. It was of interest that GSH levels could be boosted by including only these amino acids in the medium, even though cysteine was not supplied. These results have considerable significance in studies of amino acid transport. Increasing glutathione levels can increase the effectiveness of amino acid transport by the γ-glutamyl cycle (1). Thus, increasing the concentration of one of the component amino acids of GSH could increase its rate of transport through its effect on the level of GSH maintained in the slice.

The metabolism experiments verify first of all that the enzymic degradation of GSH can occur anaerobically whereas synthesis requires oxidative metabolism. These experiments also discount the possibility that GSH disappears from the slices due simply to oxidation of the SH group. Finally, cooling of the slices would be expected to depress degradative and synthetic processes equally, thus preserving initial levels of GSH, as was observed.

While this work was in progress, Hewitt *et al.* (11) reported the same steady decline in GSH levels in kidney slices as reported here. They were able to drastically accelerate the rate of the disappearance by treating the slices with high concentrations of diamide, a selective agent for the intracellular oxidation of GSH. Amino acid transport was concomitantly inhibited in the treated slices.

Thus, even though GSH levels may simply reflect the overall metabolic state of a slice preparation, the existence of relatively specific inhibitors of GSH metabolism and the possible direct interaction between amino acid transport and GSH suggest that GSH levels should be regarded as an index of viability in kidney slices with respect to transport.

Summary. The level of reduced glutathione (GSH) in renal cortex slices was shown to be a function of overall metabolism and availability of the component amino acids of GSH. Incubation of slices in oxygenated Krebs–Ringer media at 37° resulted in a steady loss of GSH. Nearly 50% of the initial GSH was lost in 1 hr. This loss was offset when the incubation was carried out a reduced temperature. Anaerobic conditions or incubation with dinitrophenol enhanced the disappearance of GSH. Fortification of the medium with glycine and glutamic acid at 3 m*M* concentration each or glycine, glutamic acid and cysteine each at 0.1 m*M* resulted in a marked increase of GSH levels after 60 min incubation compared to unfortified slices. Dithiothreitol enhanced the effect of cysteine, but had no significant effect on its own. The presence of 4% (w/v) bovine serum albumin in the medium had no effect on loss of GSH. Disodium maleate had no effect for up to 1 hr of incubation and produced only slight additional loss of GSH after 1.5 and 2.0 hr of incubation.

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