Measurement of Glutathione Transport

The development of methods for accurately measuring the transport of glutathione (GSH) or any metabolite across cellular membranes requires that compartments be efficiently separated. Although some contamination of the space of interest (e.g., intracellular space) is usually unavoidable, quantification of this contamination is critical so that corrections can be made. With GSH as the transport substrate, the redox status of the molecule must be maintained during transport incubations and sample processing. The thiol group of the GSH cysteinyl residue can be readily oxidized in extracellular compartments, either by oxidases or by auto-oxidative processes, particularly in alkaline compartments. In addition, the g-glutamyl residue can be cleaved by g-glutamyltransferase (GGT; EC 2.3.2.2), which is present in large excess in lumenal membranes of several epithelial tissues, such as the renal proximal tubule and small-intestinal epithelium. This oxidation and degradation must be prevented to accurately quantitate the content of GSH in different compartments.

This unit will describe methods for measuring GSH transport into renal proximal tubular cells (see Basic Protocol 1 and Alternate Protocol 1) and into renal cortical mitochondria (see Basic Protocol 2 and Alternate Protocol 2). While these procedures generally apply to measurement of GSH transport in cells or subcellular fractions from other tissues, properties of the kidneys most clearly illustrate the need for appropriate procedures to measure GSH flux accurately. Methods are described for rapidly stopping transport processes at specified times, isolating proximal tubule cells (Support Protocol 1), separating compartments by filtration or centrifugation procedures (Support Protocol 2), quantification of the volume of transport compartments and contaminating space (Support Protocol 4), and ensuring the integrity of the transported substrate by HPLC (Support Protocol 3; also see *UNIT 6.2*). Other methods for incubation and analysis of GSH transport in renal mitochondria have been previously described (Lash, 1995).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety officer (see *APPENDIX 1A*).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

MEASUREMENT OF GSH UPTAKE IN PROXIMAL TUBULAR CELLS FROM RAT KIDNEY

BASIC PROTOCOL 1

This protocol describes a procedure for quantifying GSH uptake by renal proximal tubular (PT) cells. Two critical points in the accurate determination of flux of GSH across renal plasma membranes are that: (1) GSH be neither significantly oxidized nor degraded during transport incubations or sample processing; and (2) contamination of intracellular space with material from the extracellular space be minimized or accounted for so that corrections can be made. The first point is addressed by pretreating cells with acivicin, which is an irreversible inhibitor of GGT (Reed et al., 1980), and by adding an antioxidant during sample processing to prevent auto-oxidation. The second point is addressed by rapid centrifugation of cells through Percoll and monitored by quantifying extracellular volume using a radiolabeled, impermeable solute (i.e., [^{14}C]sucrose; see Support Protocol 4). The following protocol may be used for measuring GSH uptake at incubating concentrations of 0.5 mM or higher using HPLC analysis. A more sensitive method for measuring GSH uptake using radiolabeled substrate is presented in Alternate Protocol 1, which can be used with GSH concentrations as low as 1 μM.

Contributed by Lawrence H. Lash

Current Protocols in Toxicology (1999) 6.3.1-6.3.14

Materials

10× acivicin stock solution (see recipe)
Freshly isolated renal PT cell suspension in 25-ml polypropylene Erlenmeyer flasks (see Support Protocol 1)
10× GSH stock solution (see recipe)
p-aminohippurate (PAH; Sigma)
p-[glycyl-1-,^{14}C]-aminohippuric acid (40 to 60 mCi/mmol; NEN Life Science)
20% (v/v) Percoll (Sigma) in 0.9% (w/v) NaCl
Normal saline: 0.9% (w/v) NaCl (*APPENDIX 2A*)
70% (v/v) perchloric acid
1.5 mM L-γ-glutamyl-L-glutamate (Sigma; prepare fresh in deionized water)
1.5 mM bathophenanthroline disulfonate (Sigma; prepare fresh in deionized water)

25-ml polypropylene Erlenmeyer flasks (e.g., Nalgene)
Dubnoff shaking metabolic incubator (Precision Scientific)
1.5-ml polyethylene microcentrifuge tubes

Treat cells to inhibit GGT activity

1. Add 1 vol of 10× acivicin stock solution to 9 vol renal PT cells (1–5 × 10^6 cells/ml) in a 25-ml polypropylene Erlenmeyer flask.

   Total volume in Erlenmeyer flasks should not exceed 3.0 ml. The final concentration of acivicin is 0.25 mM. Typically, one rat will yield sufficient cells for three to five incubations.

2. Incubate on a Dubnoff metabolic shaking incubator for 15 min at 60 cycles/min, 37°C.

3. Keep cells on ice until needed for transport experiments.

Perform transport incubations

4. In 25-ml Erlenmeyer flasks, gently mix 9 vol of acivicin-pretreated, renal PT cell suspension with 1 vol 10× GSH stock solution.

   Total volume should not exceed 3.0 ml.

   It is important to include a control for measurement of GSH transport in renal proximal tubular cells to demonstrate that the cells are functioning properly. This is accomplished by measuring the transport of a well-characterized substrate, such as p-aminohippurate (PAH). Substitute 1 mM PAH containing 0.1 μCi of p-[glycyl-1-,^{14}C]-aminohippuric acid for the 10× GSH stock solution and process as with the test samples. After step 8, measure radioactivity as described in Alternate Protocol 1, steps 2 to 4, using the ^{14}C channel.

5. Incubate on a Dubnoff shaking metabolic incubator at 60 cycles/min, 37°C.

6. At specified incubation time points (e.g., 1, 2, 3, 5, 10, 15, 20, and 30 min), remove 0.5-ml aliquots and layer on top of 1.0 ml of 20% Percoll in 1.5-ml microcentrifuge tubes at room temperature.

Measure transport

7. Immediately microcentrifuge each sample for 30 sec at 13,000 × *g*, room temperature.

8. Completely remove supernatant with a Pasteur pipet and drain tube dry.

9. Resuspend cell pellets in 0.5 ml normal saline. Add 0.1 ml of 70% (v/v) perchloric acid, 0.05 ml of 1.5 mM L-γ-glutamyl-L-glutamate, and 0.05 ml of 1.5 mM bathophenanthroline disulfonate. Mix vigorously on a vortex mixer.

10. Place 0.5 ml of the acid extract from step 9 into a new 1.5-ml microcentrifuge tube. Store on ice until ready for HPLC analysis of GSH content (see Support Protocol 3).

MEASUREMENT OF GSH TRANSPORT USING RADIOLABELED GSH

ALTERNATE PROTOCOL 1

A more sensitive method of measuring GSH transport uses radiolabeled GSH. This assay can be used with GSH concentrations as low as 1 μM.

Additional Materials *(also see Basic Protocol 1)*

10× GSH stock solution (see recipe) containing 0.01 μCi/ml L-[^{3}H]glycyl-GSH (20 to 50 Ci/mmol; NEN Life Science)
5-ml polyethylene scintillation vials

1. Pretreat PT cells with acivicin and perform time course of incubation with GSH (see Basic Protocol 1, steps 1 to 8), except use 1 vol 10× GSH stock solution containing 0.01 μCi/ml of L-[^{3}H]glycyl-GSH per vol cell suspension, instead of the solution containing cold GSH alone.

   [^{35}S]GSH may be used instead of the ^{3}H-labeled compound.

2. Resuspend each cell pellet in 0.5 ml normal saline. Add 0.1 ml of 70% (v/v) perchloric acid and 0.1 ml of normal saline, then mix vigorously on a vortex mixer.

3. Place 0.5 ml of the acid extract from step 2 in a 5-ml polyethylene scintillation vial.

4. Add 2 ml of scintillation fluid and determine radioactivity by liquid scintillation counting using the ^{3}H channel.

5. Calculate the amount of GSH transported by the following equation:

$$\frac{\text{cpm}}{\text{counting efficiency}} \times \text{dilution factor (ml}^{-1}) \times \text{specific activity (mol/Ci)} \times \frac{1\ \text{Ci}}{2.22\times10^{12}\ \text{cpm}} \times \frac{1\ \text{ml}}{y\times10^{6}\ \text{cells}} = \text{mol GSH transported}/10^{6}\ \text{cells}$$

   where cpm is the result from scintillation counting (see step 4) and *y* (multiplied by 10^6) is the original number of cells in the assay (see step 1).

   The dilution factor is obtained by considering the dilution of the cells during acivicin pretreatment (10/9), the dilution in the incubation (10/9), and the portion sampled for measurement (1/0.5 = 2). Hence, using the dilutions described above, the overall sample dilution = 2.469 ml $^{-1}$ cells.

ISOLATION OF PT CELLS FROM RAT KIDNEY

SUPPORT PROTOCOL 1

This protocol is used to isolate proximal tubule cells from the rat kidney for GSH transport studies. Perfused kidneys are treated with collagenase to release PT cells, which are collected after Percoll gradient centrifugation (Lash and Tokarz, 1989; Lash, 1996).

Materials

Rats (Sprague-Dawley or Fisher 344, 2- to 4-month-old males or 2.5- to 6-month-old females, 175 or 250 g body weight)
50 mg/ml sodium pentobarbital in saline
0.2% (w/v) heparin
Perfusion buffer: Hanks' balanced salt solution (HBBS; *APPENDIX 2A*) without calcium and with 0.12 mM magnesium and 0.5 mM EGTA
Collagenase solution (see recipe)
1× Krebs-Henseleit buffer (see recipe)
45% (v/v) Percoll (Sigma) in HBSS (*APPENDIX 2A*) without calcium and with 0.12 mM magnesium
HBSS (*APPENDIX 2A*) without calcium and with 0.12 mM magnesium

Surgical instruments
4-O silk ligatures
Peristaltic perfusion pump (e.g., MasterFlex), tubing, and cannulas (MasterFlex size 16 to give 0.2 to 20 ml/min; 19-G, cone-shaped)
50-ml round-bottom polycarbonate centrifuge tubes
Sorvall RC-2B or equivalent centrifuge
25-ml polypropylene Erlenmeyer flasks (e.g., Nalgene)
Rubber serum-bottle stoppers (Fisher or VWR)
95% O_2/5% CO_2 gas cylinder with regulator valve

1. Anesthetize rats with an intraperitoneal injection of sodium pentobarbital (0.1 ml of a 50 mg/ml solution in saline per 100 g body weight). Prevent blood clotting by injecting 0.2 ml of 0.2% (w/v) heparin into the tail vein just before surgery.
2. Open the peritoneal cavity by making a midventral incision and free the aorta from the surrounding connective tissue and the adjacent inferior vena cava. Place a 4-O silk ligature around the aorta approximately 0.5 inches below the renal artery and a second ligature around the aorta as high up in the abdomen as possible. Ligate the celiac and superior mesenteric arteries to prevent diversion of perfusate.
3. Make an oblique incision through approximately 50% of the diameter of the aorta above the lower ligature, then insert a cone-shaped 19-gauge cannula into the aorta. Secure the cannula with a new ligature.
4. Perfuse kidneys in situ with 37°C perfusion buffer for 10 min at 5 ml/min with recirculation. Remove the kidneys and euthanize the animal by bilateral pneumothorax and exsanguination.
5. Transfer kidneys to a beaker containing 50 ml of 37°C collagenase solution and perfuse with recirculation for 15 to 18 min at 8 ml/min.
6. Remove kidneys from collagenase and release cortical cells into 40 ml fresh 1× Krebs-Henseleit buffer by removing the renal capsule and connective tissue with scissors and gently squeezing with forceps.

   Cortical cells, although primarily of proximal tubular origin, also contain cells from other nephron segments, including the distal convoluted tubules, cortical collecting tubules, and cortical collecting duct. To obtain an enriched preparation of proximal tubular cells, further processing is required (see steps 7 to 9).

7. Layer 5 ml of renal cortical cell suspension on top of 35 ml of 45% Percoll solution in a 50-ml round-bottom, polycarbonate centrifuge tube and centrifuge 30 min at 20,500 × *g*, 0° to 4°C.
8. Harvest the top 8 ml of the gradient (containing the cells), free pool cells from all gradients, and dilute pooled suspension 5-fold with calcium-free and low-magnesium HBSS. Centrifuge in a tabletop centrifuge 5 min at 1000 × *g*, room temperature, and decant the supernatant to remove Percoll.
9. Resuspend cell pellet in Krebs-Henseleit buffer to a concentration of 2–5 × 10^6 cells/ml and place 2- to 3-ml aliquots in 25-ml polypropylene Erlenmeyer flasks. Purge the flasks for 1 min with 95% O_2/5% CO_2, and seal with rubber serum-bottle stoppers. Keep on ice until used for experiments.

   Degree of purity or enrichment of the PT cell preparation can be assessed by measurement of marker enzymes or functional responses (Lash and Tokarz, 1989). Marker enzymes for PT cells include γ-glutamyltransferase and alkaline phosphatase, both of which are present on brush-border membranes and are at high activities in PT cells but very low in other nephron cell types. Hexokinase can be measured as a distal tubular cell marker and should be present at very low activity in PT cells. Cellular respiration in PT cells, which can be measured with a Clark-type oxygen electrode, should be stimulated by addition of succinate

or other citric acid cycle intermediates. In contrast, these metabolites do not affect cellular oxygen consumption in other nephron cell types, because they are not transported across the plasma membranes in those cells.

MEASUREMENT OF GSH UPTAKE IN SUSPENSIONS OF MITOCHONDRIA FROM RAT KIDNEY CORTEX

BASIC PROTOCOL 2

The incubation protocol described below is for measurement of uptake of GSH by mitochondria at an incubation concentration of 0.5 mM or higher using HPLC analysis. For measurement of lower concentrations, see Alternate Protocol 2.

Materials

10× acivicin stock solution (see recipe)
Freshly isolated mitochondrial suspension from rat renal cortical homogenates (1 to 3 mg protein/ml; see Support Protocol 2)
50 mM dithiothreitol (DTT) in mitochondrial isolation buffer (prepare fresh)
Mitochondrial isolation buffer (see recipe)
Metabolite (optional; e.g., malate or 2-oxoglutarate)
10× GSH stock solution (see recipe)
Extraction solution (see recipe)

25-ml polypropylene Erlenmeyer flasks (e.g., Nalgene)
1.5-ml polyethylene microcentrifuge tubes
Dubnoff shaking metabolic incubator (Precision Scientific)
Centrifuge capable of delivering 10,000 × *g*

Pretreat mitochondria to inhibit GGT activity

1. Add 1 vol 10× acivicin stock solution and 1 vol 50 mM DTT to 8 vol renal cortical mitochondrial suspension (1 to 3 mg protein/ml) in 25-ml polypropylene Erlenmeyer flasks.

   Total volume in Erlenmeyer flasks should not exceed 3.0 ml. The final concentration of acivicin is 0.25 mM. Typically, one rat will yield sufficient cells for 3 to 5 incubations.

2. Incubate on a Dubnoff metabolic shaking incubator for 15 min at 60 cycles/min, 25°C.
3. Keep mitochondrial suspensions on ice until needed for transport experiments.

Perform transport incubations

4. *To measure exchange of GSH for intramatrix metabolites other than inorganic phosphate (e.g., malate); optional:* Incubate acivicin/DTT-pretreated mitochondria with 10 mM of the metabolite (e.g., malate or 2-oxoglutarate) for 5 min at 25°C, to preload cells with metabolite. Centrifuge the preloaded mitochondrial suspension for 2 min at 10,000 × *g*, room temperature. Remove supernatant and resuspend at the same protein concentration as before in fresh mitochondrial isolation buffer.

   GSH uptake into renal cortical mitochondria is largely accounted for by the activity of two carriers, the oxoglutarate carrier (OGC) and the dicarboxylate carrier (DCC; Chen and Lash, 1998). Preloading mitochondria with either inorganic phosphate or a dicarboxylate substrate (e.g., malate, succinate, malonate) can provide information about the function of one or the other carrier if initial rules are measured. As the DCC exchanges dicarboxylates for inorganic phosphate, it will only be active in the presence of inorganic phosphate. In contrast, the OGC exchanges 2-oxoglutarate for other dicarboxylates and will only be active in the presence of dicarboxylates on both sides of the mitochondrial inner membrane.

5. In 25-ml Erlenmeyer flasks, gently mix 9 vol acivicin- and DTT-treated renal cortical mitochondria (preloaded or not) with 1 vol 10× GSH stock solution. Incubate on a Dubnoff shaking metabolic incubator at 60 cycles/min, 25°C.

   Total volume should not exceed 3.0 ml.

6. At specified incubation time points (e.g., 1, 2, 3, 5, 10, 15, 20, and 30 min), remove 0.5-ml aliquots and place in 1.5-ml polyethylene microcentrifuge tubes.
7. Immediately centrifuge mitochondrial aliquots for 30 sec at 13,000 × *g*, room temperature.
8. Remove supernatant, resuspend pellets in 30 ml ice-cold mitochondrial isolation buffer, and centrifuge again for 30 sec at 13,000 × *g*, room temperature.

Measure transport

9. Resuspend pellets in 0.7 ml extraction solution and mix vigorously on a vortex mixer.
10. Place 0.5 ml of the acid extract from step 9 into a new 1.5-ml microcentrifuge tube. Store on ice until ready for HPLC analysis of GSH content (see Support Protocol 3).

ALTERNATE PROTOCOL 2

MEASUREMENT OF MITOCHONDRIAL TRANSPORT USING RADIOLABELED GSH

Basic Protocol 2 is limited by the sensitivity of the assay method. As with cellular GSH transport (see Basic Protocol 1 and Alternate Protocol 1), a more sensitive measurement of mitochondrial GSH transport can be performed using radiolabeled GSH. The protocol below is applicable to measuring uptake of GSH at any incubating concentration of GSH; it can be used with any incubating concentration of GSH, but is applicable to lower concentrations, down to 1 μM GSH.

Additional Materials *(also see Basic Protocol 2)*

10× GSH stock solution (see recipe) containing 0.01 μCi/ml L-[3H-glycyl] GSH (20 to 50 Ci/mmol; NEN Life Science)
10% (v/v) perchloric acid
5-ml polyethylene scintillation vials

1. Pretreat and (optionally) preload mitochondria, and perform time course of incubation with GSH (see Basic Protocol 2, steps 1 to 8), using 10× GSH stock solution containing 0.01 μCi/ml of L-[^{3}H-glycyl]-GSH instead of the solution containing cold GSH alone.

   [^{35}S]-GSH may be used instead of the ^{3}H-labeled compound.

2. Resuspend pellets in 0.5 ml of 10% (v/v) perchloric acid and mix vigorously on a vortex mixer.
3. Place 0.5 ml of the acid extract into a 5-ml polyethylene scintillation vial. Add 2 ml scintillation fluid and determine radioactivity by scintillation spectroscopy using the ^{3}H-channel.
4. Calculate initial rate of GSH uptake.

   The first-order rate constant of GSH uptake, k, is calculated by performing linear curve-fitting on the plot of $\ln[P_{total}/(P_{total} - P_t)]$ versus time. P_{total} represents the total uptake of GSH at equilibrium, which is estimated by performing an exponential decay curve-fitting on the time course data of GSH uptake. P_t represents the GSH uptake at time t. Thus, the initial rate of GSH uptake is determined from the first-order rate equation $v = k(P_{total})$.

SUPPORT PROTOCOL 2

ISOLATION OF MITOCHONDRIA FROM RAT RENAL CORTEX

The method for preparation of isolated mitochondria from rat renal cortex has been described elsewhere and will not be presented here in detail. Refer to Lash and Sall (1993) for a more thorough description.

Materials

Rats (Sprague-Dawley or Fisher 344, 2- to 4-month-old males or 2.5- to 6-month-old females, 175 to 250 g body weight)
50 mg/ml sodium pentobarbital in saline
0.2% (w/v) heparin
Mitochondrial isolation buffer (see recipe), without EGTA

Hand-held Dounce homogenizer (40-ml capacity)
50-ml round-bottom, polycarbonate centrifuge tubes
Sorvall RC-2B or equivalent centrifuge

1. Anesthetize rats with an intraperitoneal injection of sodium pentobarbital (0.1 ml of a 50 mg/ml solution in saline per 100 g body weight). Prevent blood clotting by injecting 0.2 ml of 0.2% (w/v) heparin into the tail vein just before surgery.
2. Open the peritoneal cavity by making a midventral incision. Euthanize the animal by bilateral pneumothorax and exsanguination, then remove the kidneys and place in 2 to 3 ml of ice-cold mitochondrial isolation buffer in a petri dish.
3. Decapsulate kidneys by making a small incision with scissors on one end and peeling the capsule around the kidney. Slice cortices into small pieces, transfer to homogenizer, add 30 ml of ice-cold mitochondrial isolation buffer, then homogenize with 10 to 15 strokes of the hand-held Dounce homogenizer.
4. Isolate mitochondria by differential centrifugation in the following three steps.
   a. Centrifuge homogenate 10 min at 650 × *g*, 0° to 4°C. Transfer supernatant into another tube.
   b. Centrifuge supernatant 5 min at 15,000 × *g*, 0° to 4°C.
   c. Remove supernatant and resuspend pellet in 30 ml isolation buffer. Centrifuge again for 5 min at 15,000 × *g*.
5. Remove supernatant and resuspend pellet in mitochondrial isolation buffer (without EGTA) at a protein concentration of 1 to 5 mg protein/ml. Keep mitochondria on ice until used in transport experiments.

   Protein concentration in suspensions of isolated mitochondria are determined with the Coommassie Blue G dye from Bio-Rad. The Lowry method cannot be used, as the sucrose in the isolation buffer interferes with the assay.

HPLC ANALYSIS OF GSH AND RELATED COMPOUNDS

SUPPORT PROTOCOL 3

This HPLC method is based on that described by Fariss and Reed (1987) and involves derivatization of thiols (e.g., GSH) with iodoacetic acid and of amino groups with 1-fluoro-2,4-dinitrobenzene. Separation of derivatives is achieved by reversed-phase, ion-exchange chromatography. Methanol is used in the mobile phase to rapidly elute the excess 2,4-dinitrophenol and the dinitrophenyl derivatives of basic and neutral amino acids. The low pH of the acetate in the mobile phase (pH = 2.5) maintains the bonded-phase amino groups in the protonated form. Selective elution of the acidic dinitrophenyl derivatives is accomplished by increasing the sodium acetate concentration of the mobile phase, and the eluted derivatives are measured by their absorbance at 365 nm. An internal standard (L-γ-glutamyl-L-glutamate) corrects for errors in dilution and alterations in derivatization efficiency, derivatization stability, and chromatographic conditions.

Materials

Acid extract of PT cells (see Basic Protocol 1, step 10) or mitochondria (see Basic Protocol 2, step 10)

100 mM iodoacetic acid (free acid; made fresh in deionized water)
Neutralization solution (see recipe)
1% (v/v) 1-fluoro-2,4-dinitrobenzene in ethanol (made fresh)
HPLC mobile phases (see recipe)

Tabletop centrifuge (e.g., Clay Adams Dynac)
HPLC column: μBondapak amine 10 μm cartridge (8 mm × 10 cm; Waters)

Derivatize sample

1. Add 50 μl of 100 mM iodoacetic acid to 0.5 ml of acid extract.

   The acid extract contains the internal standard (L-γ-glutamyl-L-glutamate) and antioxidant (bathophenanthroline disulfonate).

2. Bring the acidic solution to pH 8 to 9 by adding 0.48 ml of neutralization solution and incubate for 10 min in the dark at room temperature.

3. Add 1 ml of 1% (v/v) 1-fluoro-2,4-dinitrobenzene. Cap the reaction mixture and mix with a vortex mixer. Store overnight at 4°C.

4. Centrifuge the *S*-carboxymethyl-*N*-dinitrophenyl derivatives for 15 min at 1000 × *g* in a tabletop clinical centrifuge to remove insoluble material. Store supernatant at 4°C in the dark until analysis.

   Samples are stable for up to 2 weeks.

Separate by HPLC

5. Perform HPLC analysis by monitoring absorbance at 365 nm using the following elution conditions.

   Maintain mobile phase at 75% A/25% B for 5 min at a flow rate of 1.5 ml/min;
   Run 30-min linear gradient to 1% A/99% B;
   Hold mobile phase at 1% A/99% B until last compound (usually GSSG) has eluted (10 to 15 min);
   Run 1 min linear gradient to 80% A/20% B;
   Hold at 80% A/20% B for 5 min to reequilibrate to initial conditions.
   These separation conditions will vary depending on the efficiency of the column, which decreases with the age of the column. As resolution decreases, the acetate concentration may need to be lowered and/or the gradient made more shallow.

SUPPORT PROTOCOL 4

MEASUREMENT OF INTRACELLULAR OR MITOCHONDRIAL MATRIX VOLUME

The contamination of intracellular (intramitochondrial) space with extracellular (extramitochondrial) material must be accounted for when measuring GSH uptake so that corrections can be made. This can be accomplished by quantifying the extracellular volume using a radiolabeled, impermeable solute and comparing this with total volume determined using radiolabled water.

Materials

Isolated cells (see Support Protocol 1) or mitochondria (see Support Protocol 2)
Substrate of interest (e.g., GSH or malate)
[U-^{14}C]Sucrose (1.7 mCi/mg; Amersham)
[^{3}H]$_2$O (1 mCi/ml; Amersham)

Additional materials and equipment for GSH transport determination and sample processing (see Basic Protocol 1 or Basic Protocol 2).

1. Incubate isolated cells or mitochondria with the substrate of interest in the appropriate buffer containing 0.1 μCi/ml of [U-^{14}C]sucrose and 1 μCi/ml of [^{3}H]$_2$O.
2. At various time points, remove 0.5-ml aliquots of the isolated cell or mitochondrial suspensions and process by centrifugation as in Basic Protocol 1 or Basic Protocol 2, steps 6 and 7.
3. Determine the radioactivity of pellet and supernatant fractions separately using the ^{14}C and ^{3}H channels.
4. Calculate intracellular or mitochondrial matrix volume according to the following equation:

$$V_{cell} \text{ or } V_{matrix} = V_{H_2O} - V_{sucrose}$$
$$= ([\text{Net (H)}_{pellet}/A^{3}H] - [\text{Net (C)}_{pellet}/A^{14}C]) \times 1/\text{mg protein}$$

where V_{cell} or V_{matrix} are the volume of ^{3}[H]$_2$O in the pellet (V_{H_2O}) minus the volume of [U-^{14}C]sucrose in the pellet (Vsucrose), V_{H_2O} is the total pellet volume calculated from net ^{3}H counts divided by specific ^{3}H counts ($A^{3}H$), and $V_{sucrose}$ is the extracellular or extramitochondrial medium calculated from the net ^{14}C counts divided by specific ^{14}C counts ($A^{14}C$); $A^{3}H$ and $A^{14}C$ are determined by standard samples containing only a known amount of either [^{3}H]$_2$O or [U-^{14}C]-sucrose.

REAGENTS AND SOLUTIONS

*Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Acivicin stock solution, 10×

Prepare a 2.5 mM (10×) stock solution of L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin; Sigma) in Krebs-Henseleit buffer (see recipe). Store at –20°C for up to 1 month.

Collagenase solution

Prepare a 0.15% (w/v) solution of collagenase, type I (Sigma) in Hanks' buffered salt solution (HBSS; *APPENDIX 2A*) with 1.2 mM magnesium and 2 mM calcium chloride. Prepare fresh daily.

Extraction solution

Stock solutions:
70% (v/v) perchloric acid
1.5 mM L-glutamyl-L-glutamate (Sigma)
1.5 mM bathophenanthroline disulfonate (BPDS; Sigma)
0.9% (w/v) NaCl (normal saline; *APPENDIX 2A*)

Add 1 vol 1.5 mM L-γ-glutamyl-L-glutamate, 1 vol BPDS, and 2 vol 70% perchloric acid to 10 vol 0.9% NaCl. Prepare fresh.

GSH stock solution, 10×

Prepare a 1 to 100 mM (choose concentration as needed) solution of reduced glutathione (GSH) in Krebs-Henseleit buffer (see recipe) for isolated cells or mitochondrial isolation buffer (see recipe) for isolated mitrochondria. Prepare fresh daily and keep on ice.

HPLC mobile phases

Mobile phase A: Prepare a solution of 80% (v/v) HPLC-grade methanol in water.

Mobile phase B: Prepare 2.6 M sodium acetate stock solution by adding 272 g sodium acetate and 122 ml of water to 378 ml of glacial acetic acid. Stir with low heat until sodium acetate is in solution. Cool and filter with a 4.0- to 5.5-µm polypropylene filter. Add 200 ml of the sodium acetate stock solution to 800 ml of 80% (v/v) methanol to yield 0.5 M sodium acetate in 64% (v/v) methanol.

Krebs-Henseleit buffer

Stock solution: Prepare a 10× stock solution as follows:

1.18 M NaCl
48 mM KCl
12 mM $MgSO_4 \cdot 7H_2O$
9.6 mM KH_2PO_4
250 mM $NaHCO_3$
25 mM $CaCl_2 \cdot 2H_2O$
250 mM HEPES

Prepare the 10× stock solution in advance and store in a 1-liter polycarbonate bottle at 0° to 4°C for up to 1 month. Add all of the ingredients except $CaCl_2$ and allow to dissolve with stirring. Purge the 10× stock with 95% O_2/5% CO_2 for 30 min, then slowly add the $CaCl_2$ in small aliquots with slow stirring to prevent precipitation of calcium phosphate.

CAUTION: *If the $CaCl_2$ is added too quickly, a cloudy precipitate will form and the solution must be discarded and prepared again.*

Working solution: Prepare the working Krebs-Henseleit buffer (1×) just prior to use by mixing 1 vol 10× stock with 9 vol deionized water. After bubbling with 95% O_2/5% CO_2 for 30 min, adjust the pH of the buffer to 7.4 with 1 M HCl or 1 M NaOH as needed.

Mitochondrial isolation buffer

20 mM triethanolamine·HCl, pH 7.4
225 mM sucrose
3 mM potassium phosphate, pH 7.4
5 mM $MgCl_2$
20 mM KCl
0.1 mM phenylmethylsulfonyl fluoride (PMSF, 99% purity; Sigma)
2 mM EGTA (added only in preparatory steps except final resuspension and incubation of mitochondria)
Adjust pH to 7.4 with 1 M HCl or 1 M KOH as needed
Prepare buffer in advance and store in a 1-liter polycarbonate bottle at 0° to 4°C for up to 1 month

Phenylmethylsulfonyl fluoride is added to inhibit proteolysis.

Neutralization solution

Make a working solution by adding 1 vol of 10 M KOH to 4 vol of 3 M $KHCO_3$, for a final concentration of 2 M KOH/2.4 M $KHCO_3$. Store up to 2 months at 0° to 4°C.

COMMENTARY

Background Information

Until the late 1970s the dogma was that many tissues, in particular the liver, had transport systems for efflux of GSH into the extracellular space but that GSH was not transported into cells as the intact tripeptide. Observations with isolated, perfused kidneys in the late 1970s through the mid 1980s (Fonteles et al., 1976; Griffith and Meister, 1979; Häberle et al., 1979; Anderson et al., 1980; Ormstad et al., 1982; Rankin and

Curthoys, 1982; Rankin et al., 1985) provided evidence that either endogenous or administered GSH was extracted from plasma by both a basolateral and a lumenal mechanism. While the latter was ascribed to glomerular filtration, degradation by brush-border enzymes (i.e., GGT and dipeptidase), and uptake of the constituent amino acids by renal PT cells (Griffith and Meister, 1979; Lash et al., 1988), there was controversy over whether a basolateral transport mechanism for the intact tripeptide of GSH actually existed or if the apparent basolateral extraction of plasma GSH was due to degradation by extralumenal GGT followed by renal cellular uptake of the constituent amino acids and intracellular resynthesis of GSH (Anderson et al., 1980; Abbott et al., 1984; Inoue et al., 1986).

This controversy was resolved by the biochemical description of a Na^+-dependent transport process for uptake of GSH in basolateral membrane vesicles (Lash and Jones, 1983, 1984) and isolated kidney cells (Hagen et al., 1988) and the further demonstration that this system also mediated the uptake of GSH *S*-conjugates (Lash and Jones, 1985). Basolateral uptake of GSH was subsequently shown to be a general property of many epithelial cells, including those found in the small-intestinal jejunum (Lash et al., 1986; Hagen and Jones, 1987), type II pulmonary alveolar cells (Hagen et al., 1986; Bai et al., 1994), and retinal pigment epithelial cells (Davidson et al., 1994; Zlokovic et al., 1994; Kannan et al., 1995; Lu et al., 1995; Mackic et al., 1996).

Within renal PT cells, as well as isolated hepatocytes, GSH is known to be compartmentalized into distinct cytosolic and mitochondrial pools. There are two possible sources of mitochondrial GSH—de novo synthesis from precursor amino acids and transport of GSH from the cytosol. As the enzymes for synthesis of GSH are localized predominantly, if not exclusively, in the cytosol (Griffith and Meister, 1985), transport must supply the mitochondria with their pool of GSH. Because the GSH molecule has a net charge at physiological pH and there exists a pH gradient and membrane potential across the mitochondrial inner membrane, GSH transport into mitochondria cannot occur passively but must occur by a specific, carrier-mediated process. Several investigators subsequently demonstrated the presence of specific, carrier-mediated uptake of GSH in hepatic (Kurosawa et al., 1990; Martensson et al., 1990; Garcia-Ruiz et al., 1995) and renal (McKernan et al., 1991; Schnellmann, 1991) mitochondria. GSH is taken up into renal cortical mitochondria in electroneutral exchange for dicarboxylates such as malate and succinate (McKernan et al., 1991; Chen and Lash, 1997), suggesting that transport is mediated, at least in part, by the dicarboxylate and oxoglutarate carriers that are present in the mitochondrial inner membrane of mitochondria in kidney and several other tissues, including the liver (Klingenberg, 1979; Palmieri et al., 1996).

Critical Parameters

Measurement of transport

This unit examines GSH uptake by isolated renal PT cells and renal cortical mitochondria. Although the kidney has several unique properties with regard to the disposition and metabolism of GSH, the principles should apply to analysis of GSH uptake in isolated cells or mitochondria from most mammalian tissues.

Methods for accurate measurement of GSH transport must take into account not only concerns that exist with measurement of transport of any substrate, but concerns that are specific to the study of GSH transport. General concerns about the accuracy of transport measurements include efficient separation of compartments, prevention of changes in compartment volume during transport measurements and sample processing, and loss of transported substrate during sample processing. Compartment volumes can be readily quantified by use of radiolabeled water, which gives total space, and a radiolabeled impermeant molecule, such as sucrose (see Support Protocol 4). This is a critical parameter to monitor in many cases. With isolated mitochondria, matrix volume can often change due to metabolic state. Antimycin A (2 μM) can be added to incubations to inhibit substrate metabolism and thus substrate-induced changes in matrix volume. Loss of transported substrate during sample processing can be eliminated by addition of a specific, irreversible inhibitor of transport in the incubation stop solution, if one is available. Alternatively, efflux of transported substrate can be measured under the same conditions of sample processing (usually 0° to 4°C, <5 min) and can be shown to be negligible (Chen and Lash, 1998).

Concerns specific to the study of GSH transport include prevention of GSH oxidation and degradation. Oxidation of GSH during sample processing is usually prevented by stopping transport under acidic conditions and including an antioxidant, such as bathophenanthroline disulfonate (BPDS). BPDS is used in preference to EDTA, because the latter contains free amino groups and interferes with detection of GSH by the HPLC method described in Support Protocol

3. Degradation of GSH is not a major concern in tissues such as skeletal muscle or the liver, which have negligible amounts of GGT activity. In the kidney, however, extremely high activity of GGT is present on the brush-border membrane (Lash et al., 1988). Consequently, the presence of even a small amount of contaminating brush-border membranes in a preparation of basolateral membranes (Lash and Jones, 1984) or isolated mitochondria (McKernan et al., 1991) can result in a significant amount of degradation of GSH. This concern is minimized by pretreatment of the preparation with an irreversible inhibitor of GGT, such as acivicin. One must be cautious not to use too high a concentration of acivicin. Although acivicin is a specific glutamine antagonist at relatively low concentrations (usually <1 mM), it becomes a nonspecific alkylating agent at higher concentrations and can inhibit GSH transport (Lash and Jones, 1984). In suspensions of isolated renal cells, which possess both brush-border and basolateral membranes, it is essential to inhibit GGT activity. Buthionine sulfoximine (BSO) can also be added to inhibit GSH synthesis, thereby excluding the possibility that detection of intact GSH arose due to extracellular degradation of GSH, uptake of the constituent amino acids, and intracellular resynthesis of GSH.

In this unit, two methods are described for analysis of transported GSH: analysis by HPLC and use of radiolabeled GSH. The HPLC method has the advantage of demonstrating recovery of intact tripeptide. With the radiolabel method, which must be used when incubating concentrations of GSH are lower than the HPLC limit of detection (<0.5 mM), samples can be spiked with unlabeled GSH and fractions from the HPLC eluate can be collected to demonstrate that the radiolabeled molecule coincides with the derivative of intact GSH.

Sensitivity of method

Sensitivity of the HPLC method is based, in part, on the number of stationary amine groups available with which the chromophore can interact, as well as the sensitivity of the detector. With variable wavelength detectors, disulfides are measured at concentrations as low as 0.5 nmol/ml or 25 pmol/injection, and thiols are measured at concentrations as low as 1.0 nmol/ml or 50 pmol/injection. Approximately 5- to 10-fold greater sensitivities can be achieved with some fixed-wavelength, filter-type detectors. In addition to GSH and GSSG, other relevant compounds that are measurable by this method include glutamate, cystine, cysteine, aspartate, γ-glutamyl-L-cysteine, and the mixed disulfide of GSH and cysteine (also see *UNIT 6.2*).

As the time period during which both isolated renal cells and isolated mitochondria are viable is somewhat limited (2 to 3 hr for isolated cells and 30 min for isolated mitochondria after removal from storage on ice), it is critical that experiments be conducted as soon as possible after preparation of the cell or mitochondrial suspension.

Troubleshooting

Potential problems with measurement of GSH uptake can arise from three areas: (1) quality of the biological preparation, (2) alterations in GSH status during incubation or sample processing, or (3) artifacts during sample processing. There are numerous methods to assess the quality of the biological preparation being used, and these have been detailed elsewhere (e.g., Lash, 1989, 1993, 1996; Lash and Sall, 1993). Problems with the viability of isolated cells or mitochondria will often lead to changes in membrane permeability and hence, changes in apparent transport. If analysis of intracellular or intramitochondrial contents after incubation with GSH reveals significant oxidation or degradation of GSH, incubation conditions should be carefully checked to see if the pH is correct and if acivicin pretreatment is effective. If the incubating pH is too alkaline, auto-oxidation of GSH is more likely to occur. Although previous studies have shown than a 15-min preincubation of renal tissue with 0.25 mM acivicin inhibits ≥98% of GGT activity, this should be assessed if degradation is a problem. As mentioned above, concentrations of acivicin of >1 mM can lead to nonspecific alkylation of proteins. Potential artifacts during sample processing can be assessed by measurement of compartment volumes and determination of sample recovery.

Methods for the accurate measurement of GSH uptake by renal cortical mitochondria from the rat may be compromised by the following factors.

1. The presence of even small amounts of contaminating brush-border membranes, which contain GGT, can lead to significant degradation of GSH, thus either causing underestimation of uptake or making measurement of uptake impossible.

2. Efflux of transported GSH can occur during sample processing, which can lead to underestimation of uptake.

3. Contamination of the mitochondrial pellet with extramitochondrial medium can take place, thus leading to overestimation of uptake.

4. Changes in mitochondrial matrix volume during transport measurements with or without other added substrates can lead to altered transport kinetics.

5. Induction of the membrane permeability transition during transport measurements with or without other added substrates can markedly alter membrane permeability to GSH.

Anticipated Results

The choice of either the HPLC or radiolabel method for quantifying GSH uptake in either cells or mitochondria is wholly dependent on the incubating concentration of GSH and the sensitivity of the HPLC detector. Typically, a limit of detection of 25 to 50 pmol GSH is seen with the HPLC method. This is usually adequate with incubating concentrations of ≥0.5 mM. Using radiolabeled GSH and scintillation counting, incubating concentrations of GSH as low as 1 μM have been successfully used. This lower range is critical because physiological concentrations of GSH in renal plasma are 5 to 20 μM, depending on species. In contrast, renal mitochondria are typically exposed to 1 to 5 mM GSH in the cytosol.

Using two kidneys from 175- to 250-g male rats as starting material, the typical yield of isolated renal PT cells is 30×10^6 cells and that of isolated renal cortical mitochondria is 15 to 25 mg of mitochondrial protein. In both cases, this is usually enough material for 10 to 15 individual samples.

Time Considerations

The amount of time required to measure GSH uptake in isolated renal cells or mitochondria can be divided into three phases: (1) biological preparation, (2) transport incubations and sample preparation, and (3) analysis of GSH. The first and third phases require significantly more time than the second phase. The isolated cell preparation method requires ~3.5 hr to complete. If GSH transport is assessed in cells from other tissues, this time will of course vary depending on the method used to prepare the cells. The mitochondrial isolation method typically requires 45 min to 1 hr to complete. The HPLC method requires ~1 hr per sample while the radiolabel method requires ~5 to 10 min per sample, depending on the number of counts per sample. Longer counting times should be used at low incubating concentrations of GSH to decrease counting error. In contrast to the times required for the first and third phases, the actual transport incubations are done in real time and typically take at most 60 min for experiments with isolated cells and at most 30 min for experiments with isolated mitochondria. In both cases, sample preparation then requires 2 to 5 min per sample.

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Key References

Lash, 1995. See above.

Describes other methods for uptake of mitochondrial GSH.

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