[11] Redox Potential of GSH/GSSG Couple: Assay and Biological Significance

By DEAN P. JONES

Glutathione (GSH) is among the most important antioxidants in cells, being used in enzymatic reactions to eliminate peroxides and in nonenzymatic reactions to maintain ascorbate and α-tocopherol in their reduced and functional forms. In these reactions, GSH is converted to its disulfide form, GSSG. An NADPH-dependent reductase is present in cells that functions to reduce GSSG back to GSH. The activity of the reductase increases in response to an increase in concentration of GSSG so that the steady-state balance of GSH and GSSG during oxidative stress is different from that in the absence of oxidative stress (Fig. 1). Synthesis of GSH, export of GSH and GSSG from cells, and formation of protein-bound GSH disulfides (PrSSG) can affect this balance, but under most conditions these rates are slow relative to the rates of GSH oxidation and GSSG reduction. Consequently, the balance of GSH and GSSG provides a dynamic indicator of oxidative stress, i.e., conditions under which prooxidants predominate over antioxidants.

Considerable evidence has accumulated to show that redox-signaling mechanisms function in cell regulation and growth control. Agents altering GSH concentration affect transcription of detoxification enzymes, cell proliferation, and apoptosis.[1] In principle, GSH, GSH S-conjugates, GSSG, or the redox state of the GSH/GSSG couple could provide a mechanistic control or signal for functional changes. Both GSH loss and GSH oxidation have been associated with an increased expression of the rate-limiting enzyme of GSH synthesis, glutamate:cysteine ligase (GLCL), and several other detoxification systems, including glutathione *S*-transferase (GST) and NAD(P)H : quinone reductase (N : QR). A loss or oxidation of GSH also occurs in association with differentiation both *in vitro* and *in vivo* and during apoptosis. In contrast, increases in GSH and/or a reduction of the GSH/GSSG pool is associated with growth stimulation by nutrients and growth factors. Thus, the balance of GSH and GSSG may not only reflect oxidative stress but may reflect changes in redox signaling and control.

The most widely used indicator of the redox state of the GSH pool is the ratio of GSH to GSSG. This ratio provides a simple indicator of redox changes and is

[1] W. G. Kirlin, J. Cai, S. A. Thompson, D. Diaz, T. J. Kavanagh, and D. P. Jones, *Free Radic. Biol. Med.* **27,** 1208 (1999).

0076-6879/02 $35.00

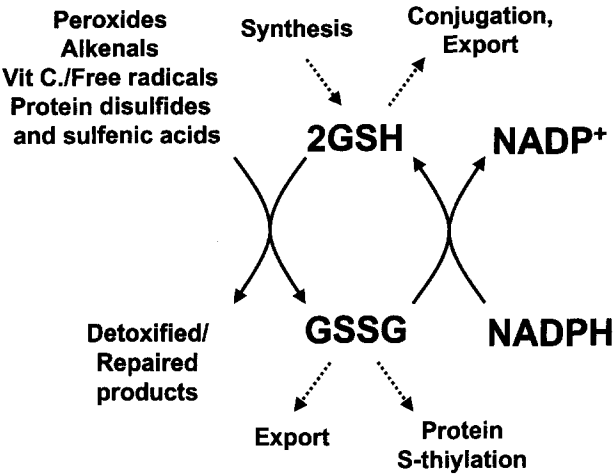

FIG. 1. The cellular balance of GSH and GSSG provides a dynamic indicator of oxidative stress. Although GSH is synthesized continuously and has various fates in cells, these rates are generally slow relative to the turnover by the oxidation–reduction cycle. The activity of the GSSG reductase is dependent on GSSG concentration. During acute oxidative stress, GSH concentration decreases and the associated increase in GSSG concentration results in an increased turnover of the GSH/GSSG cycle.

especially relevant to changes in protein S-thiylation,[2]

$$GSSG + PrSH \rightleftharpoons GSH + PrSSG$$

where the ratio of PrSH/PrSSG is proportional to GSH/GSSG under equilibrium conditions.[3] However, for oxidation–reduction reactions involving GSH and GSSG where the reaction is a $2\,e^-$ transfer, such as those involving proteins with vicinal thiols,

$$GSSG + Pr\text{-}(SH)_2 \rightleftharpoons 2\,GSH + Pr\text{-}(SS)$$

the ratio of donor/acceptor is proportional to $[GSH]^2/[GSSG]$ and not GSH/GSSG. Because $2\,e^-$ transfers appear to be common in redox-sensitive proteins, GSH/GSSG may not be the best measure of redox changes relevant to oxidative stress and redox signaling.

[2] H. F. Gilbert, *Adv. Enzymol. Relat. Areas Mol. Biol.* **63,** 69 (1990).

[3] In redox chemistry, the oxidized component is listed first, i.e., the GSH/GSSG couple is expressed correctly as the GSSG/GSH couple. However, because of the common use of the term "GSH/GSSG" in biochemical and health-related literature, this tradition of expressing donor/acceptor is followed in reference to thiol–disulfide redox couples even though other terms and definitions are those used conventionally for redox chemistry.

A convenient expression for the redox state of the GSH/GSSG pool that incorporates the correct stoichiometry of 2 GSH oxidized per GSSG formed is the redox potential (E_h), calculated according to the Nernst equation:

$$E_h = E_o + (RT/nF)\ln([\text{acceptor}]/[\text{donor}])$$

In this expression, E_h at a defined pH is the electromotive force given in volts (or millivolts) relative to a standard hydrogen electrode (1 atm H_2, 1 M H^+), E_o is the standard potential for the redox couple at the defined pH, R is the gas constant, T is the absolute temperature, F is Faraday's constant, and n is the number of electrons transferred. The E_h value provides a quantitative expression of the tendency of the redox couple to accept or donate electrons. It incorporates both an expression of the inherent affinity of the molecule for electrons (E_o) and the mass action effect of the concentrations of both donor and acceptor forms (logarithmic term). Because E_h values can be estimated for other redox-active biomolecules, the parameter provides a convenient way to compare the tendency of the GSH/GSSG pool to donate or accept electrons from other redox-active biologic components. In such processes, couples with a more negative E_h value are better reductants.

The kinetics of interaction of the GSH/GSSG couple with other redox-active systems, e.g., NADPH/$NADP^+$ and protein thiols, are not sufficient to allow equilibration under usual biologic conditions. In cells and tissues, estimates of E_h for the GSH/GSSG couple range from -260 mV[1] to -150 mV.[4] Earlier studies established that the E_h for NADPH/$NADP^+$, the reductant for the GSH/GSSG couple, is about –400 mV.[5] Although there remains some uncertainty about the absolute accuracy of these values because of the indirect manner in which they are determined, the values for the GSH/GSSG and NADPH/$NADP^+$ couples are so different that it appears very unlikely that they could approach redox equilibrium in cells and tissues. A possible explanation for this lack of equilibration is that the GSSG reductase has a relatively high K_m for GSSG so that its activity limits the reduction of GSSG at normal conditions in cells.[2] If so, this means that the steady-state rate of GSH oxidation is sufficient to maintain the redox balance of the GSH/GSSG couple substantially oxidized relative to the NADPH/$NADP^+$ couple. Accumulating data indicate that the GSH/GSSG redox state is remarkably similar in different cell types but varies according to proliferation rate, differentiation, and apoptosis.[1] Thus, thiol/disulfide redox state, per se, may have a central role in the regulation of cell functions. If so, this implies that specific mechanisms exist to control the GSH/GSSG redox state in cells and tissues.

[4] S. Jiang, J. Cai, D. C. Wallace, and D. P. Jones, *J. Biol. Chem.* **274,** 29905 (1999).

[5] H. Sies, *in* "Metabolic Compartmentation" (H. Sies, ed.), p. 205. Academic Press, London, 1981.

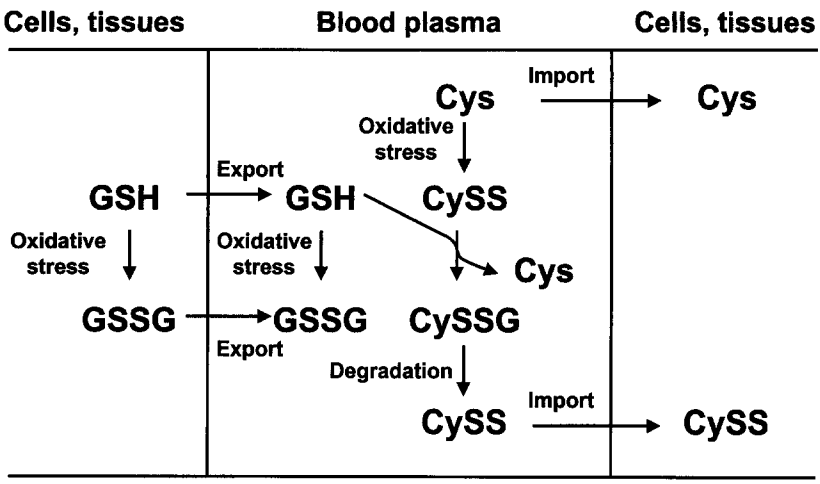

FIG. 2. The redox states of both GSH/GSSG and cysteine/cystine in plasma provide an indicator of oxidative stress *in vivo*. Oxidative stress within the circulatory system can result in enhanced oxidation of cysteine (Cys), the major low molecular weight thiol in plasma, to cystine (CySS), the major low molecular weight disulfide in plasma. Thus, the balance of Cys and CySS can provide an indicator of oxidative stress. An oxidation of the Cys/CySS pool can also result in oxidation of the GSH/GSSG pool because the GSH released from tissues reacts rapidly with cystine to form the disulfide CySSG. During oxidative stress in tissues, decreased GSH is associated with a decreased export of GSH and increased GSSG is associated with an increased export of GSSG. Thus, the GSH/GSSG redox state in plasma can reflect oxidative stress in tissues. However, because of the interaction of the GSH/GSSG and Cys/CySS pools, one cannot readily ascertain whether changes in plasma redox are due to oxidative stress within the circulation or within tissues.

Studies of the GSH/GSSG redox state in human plasma show that this pool is oxidized relative to tissue values.[6] Results indicate that GSH released from tissues reacts with the relatively high concentration of cystine in plasma and thereby helps maintain the redox state of the cysteine/cystine pool (Fig. 2). Oxidative stress in tissues and in the plasma can be expected to alter the redox state of the plasma pool so that measurement of the plasma GSH/GSSG redox state can provide an *in vivo* indicator of oxidative stress.

The following methods describe our approaches to estimate E_h of the thiol/disulfide pools of blood plasma, tissues, and cells from measurements of the thiol and disulfide concentrations.[7] The results obtained to date indicate that the low molecular weight thiol and disulfide pools are in dynamic interaction with ongoing

[6] D. P. Jones, J. L. Carlson, V. C. Mody, Jr., J. Cai, M. J. Lynn, and P. Sternberg, Jr., *Free Radic. Biol. Med.* **28,** 625 (2000).

[7] D. P. Jones, J. C. Kurtz, P. S. Samiec, P. Sternberg, R. L. Reed, and L. A. S. Brown, *Clin. Chem. Acta* **275,** 175 (1998).

oxidation and reduction reactions. This interaction means that application of strategies to prevent oxidation can be expected to have the risk of allowing artifactual reduction, and vice versa. Thus, estimates of E_h obtained by this approach must be considered "operational," i.e., subject to the details of the assay procedures. Nonetheless, accumulating data indicate that extracellular plasma E_h is regulated and that perturbation during aging, disease, and toxicity may contribute to tissue and organ dysfunction.[8,9] In addition, measurements of E_h for the GSH/GSSG couple in cell culture and tissues, along with estimates of E_h for NADPH/NADP$^+$ [5] and thioredoxin,[10] provide evidence that thiol–disulfide redox may be an important characteristic of cell growth and may function in the control of fundamental cell processes, including control of gene expression, cell proliferation, and execution of apoptosis.

Assay of Thiol/Disulfide Redox in Blood Plasma

Background

Technical problems associated with blood collection and sample processing have been described and used as a basis to develop a preservation solution for blood collection.[7] Briefly, one must avoid hemolysis, limit oxidation, and inhibit GSH degradation by γ-glutamyltranspeptidase. The GSH concentration in erythrocytes is about 500 times higher than in plasma. Thus, even 0.1% hemolysis can result in overestimates of plasma GSH, and this level of hemolysis can occur when blood is collected into evaucated tubes (e.g., Vacutainers, Becton-Dickinson, San Diego, CA). γ-Glutamyltranspeptidase activity is measurable in most human plasma samples and elevated in certain diseased states, and this activity must be inhibited to avoid GSH loss during processing. Loss of GSH by oxidation also occurs rapidly in human plasma, and the current protocol was designed to limit this process by chelation of metal ions that catalyze oxidation of thiols by O_2. More recent evidence, however, indicates that thiol–disulfide exchange of GSH with the relatively high concentration of cystine in plasma is probably a more important pathway for GSH oxidation,[6] which is limited by inclusion of an alkylating agent in the preservation solution.

Several different assay methods are available for GSH, but methods based on the thiol group do not allow simultaneous, direct measurement of GSSG. For plasma, this is a significant problem because of the presence of the disulfide of

[8] P. S. Samiec, C. Drews-Botsch, E. W. Flagg, J. C. Kurtz, P. Sternberg, R. L. Reed, and D. P. Jones, *Free Radic. Biol. Med.* **24,** 699 (1998).

[9] C. R. Jonas, A. B. Puckett, D. P. Jones, D. P. Griffith, E. E. Szeszycki, G. F. Bergman, C. E. Furr, C. Tyre, J. L. Carlson, J. R. Galloway, J. B. Blumberg, and T. R. Ziegler, *Am. J. Clin. Nutr.* **72,** 181 (2000).

[10] W. H. Watson, L. Miller, and D. P. Jones, *Toxicologist* **55**, in press.

Cys and GSH, CySSG, which can result in overestimates of GSSG by methods where GSSG is calculated as the difference between total GSH obtained following reduction and GSH. The "gold standard" for measuring GSH and GSSG is an high-performance liquid chromatography (HPLC) method utilizing iodoacetic acid to modify thiols and 1-fluoro-2,4-dinitrobenzene to add a chromophore to the amino groups,[11] allowing direct measurement of GSH, GSSG, Cys, cystine (CySS), and CySSG in a single run. However, this method does not have the sensitivity to measure GSSG in plasma. We use a modification of this method in which reaction of amine groups with dansyl chloride provides a fluorescent group for quantification.[7] The method is otherwise similar to the method of Reed *et al.*[11] but increases sensitivity by 100-fold. This allows measurement of GSSG in human plasma and in cell culture in individual wells of 96-well plates. In assay development, other approaches were tried and deserve comment. One approach was to modify thiols with monobromobimane followed by dansyl chloride to react with remaining amino groups. In principle, this approach could give identification of thiol compounds with one wavelength pair and disulfides with another wavelength pair. However, this method had problems with quenching and product stability. *O*-Phthalaldehyde derivatization resulted in multiple products with both GSH and cysteine, and derivatives with fluorescamine were found to be unstable. Electrochemical methods were limited by the stability of GSH and GSSG in stored samples and by electrode stability and calibration.

Plasma Sample Collection

This procedure was designed to allow a phlebotomist to collect blood and attend to the subject without need for an assistant to process the plasma sample. Blood is collected by venipuncture to the antecubital vein with a 23-gauge butterfly needle attached to a 5-ml syringe. Approximately 2 ml blood is drawn into the syringe, the butterfly needle is removed while the syringe remains attached to the needle, and the venipuncture site is covered with gauze. The subject is asked to hold the gauze while the collector removes and disposes of the butterfly needle and drips 0.5 ml of blood into a microcentrifuge tube ("A" tube) containing the preservation solution. The composition of this preservation solution is 100 m*M* serine–borate (pH 8.5) containing (per ml) 0.5 mg sodium heparin, 1 mg bathophenanthroline disulfonate (BPDS), and 2 mg iodoacetic acid. This is prepared by mixing 8 ml of a 100 m*M* boric acid stock solution (0.62 g/100 ml), 2 ml of a 100 m*M* sodium tetraborate stock solution (3.81 g/100 ml), 105 mg L-serine, 5 mg sodium heparin, 10 mg BPDS, and 20 mg iodoacetic acid. Once blood is added to tube A, the tube is gently inverted twice for mixing and placed in a rack at room temperature while

[11] D. J. Reed, J. R. Babson, P. W. Beatty, A. E. Brodie, W. W. Ellis, and D. W. Potter, *Anal. Biochem.* **106,** 55 (1980).

the venipuncture site is bandaged. Under these conditions, carboxymethylation of GSH is 80% complete at 2 min.

A tubes are then spun in a microcentrifuge for 30 sec to remove blood cells. This is usually completed within 2 min of collection. If there is any visual evidence of hemolysis, samples are discarded. In more than 500 blood samples in young, healthy individuals, we have had no evidence of hemolysis; however, some samples from aging individuals and subjects with disease or treated with anticancer drugs show evidence of hemolysis even with this collection procedure. Aliquots (200 μl) of supernatant from the A tubes are transferred to microcentrifuge tubes containing 200 μl of a perchloric acid solution[12] (B tubes), and the tubes are inverted to mix. The composition of this solution is 10% (w/v) perchloric acid containing 0.2 *M* boric acid and 10 μM γ-Glu-Glu. This is prepared by adding 6.2 g boric acid and 1.38 mg γ-Glu-Glu to approximately 300 ml water, mixing in 71 ml of 70% perchloric acid, and adjusting to 500 ml total volume. Collected samples are placed at $-80°$ as soon as possible for storage; for multiple collections during a day, samples can be maintained on ice or in a refrigerator. Samples stored at $-80°$ are stable for at least 2 months prior to completion of derivatization and can be shipped overnight on dry ice without deterioration.

Derivatization

Samples are spun for 2 min in a microcentrifuge to pellet protein. An aliquot (300 μl) of each supernatant is transferred to a fresh microcentrifuge tube. An iodoacetic acid solution (60 μl) is added to each tube.[13] The pH is adjusted to 9.0 $\pm$ 0.2 with the KOH/tetraborate solution (approximately 220 μl). After about 3 min to allow complete precipitation of potassium perchlorate, the pH of at least some of the samples should be checked to verify that they are in the correct range. After 20 min, 300 μl of dansyl chloride (20 mg/ml acetone) is added, and the samples are mixed and placed in the dark at room temperature for 16 to 26 hr. Dansylation of GSH is complete by 8 hr, but GSSG has two amino groups that must be modified. The rate of the second dansylation is slower that that for the first with the result that monodansyl derivatives of GSSG (eluting between the *N*-dansyl-, *S*-carboxymethyl-GSH and bisdansyl-GSSG) will be present if dansylation is incomplete. After derivatization, chloroform (500 μl) is added to each

[12] Perchloric acid was used for precipitation of protein because perchlorate is readily precipitated as the potassium salt during adjustment of pH to 9.0. Removal of the anion is useful because derivatives are eluted from the cationic HPLC column by an anion gradient. Thus, variation in injection volume of samples with a high anion concentration can affect retention times.

[13] Carboxymethylation is about 80% complete after 2 min in the original collection of plasma in the preservation solution. In principle, one could allow this reaction to proceed to completion prior to transfer of plasma from tube A to the perchloric acid solution in tube B. This approach was not pursued in the current development because it would require more precise timing for the collection procedure.

tube to extract the unreacted dansyl chloride, and samples are stored at 0–4° in the dark in the presence of both the perchlorate precipitate and the chloroform layer until assay by HPLC. Stability tests show that samples can be stored under these conditions for 12 months with little change in the amounts of GSH and GSSG derivatives.

HPLC Analysis

Samples are centrifuged for 2 min in a microcentrifuge prior to transfer of an aliquot of the upper (aqueous) layer to an autosampler. The typical injection volume is 25 μl. Separation is achieved on 3-aminopropyl columns (5 μm; 4.6 mm × 25 cm; Custom LC, Houston; or Supelcosil LC NH2, Supelco, Bellefonte, PA). Initial solvent conditions are 80% A, 20% B run at 1 ml/min for 10 min. Solvent A is 80% methanol in water and solvent B is 64% methanol, 4 *M* sodium acetate buffer, pH 4.6. A linear gradient to 20% A, 80% B is run over the period from 10 to 30 min. From 30 to 46 min, the conditions are maintained at 20% A, 80% B and returned to 80% A, 20% B from 46 to 48 min. Equilibration time for the next run is 12 min. These conditions have been established to allow simultaneous measurement of CySS and Cys along with GSH and GSSG (Fig. 3); run times can be shortened considerably if resolution of CySS and Cys is not desired.

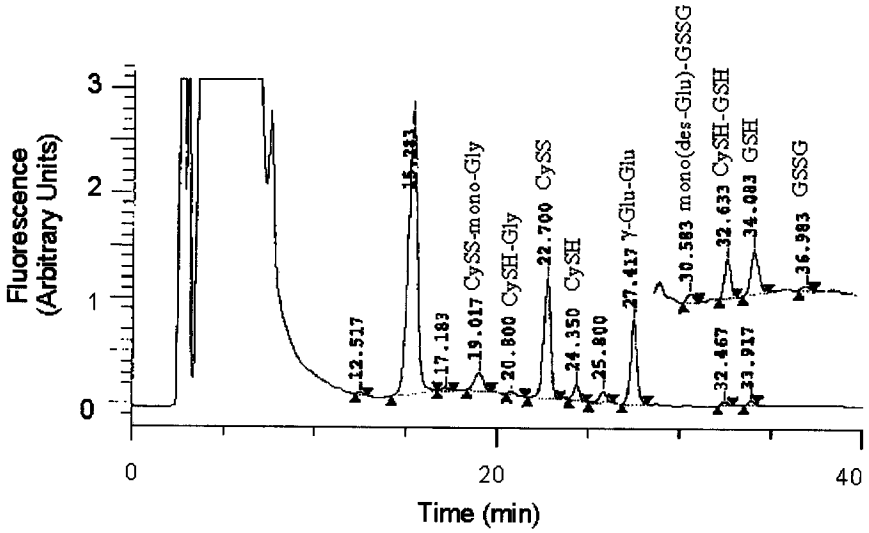

FIG. 3. Typical HPLC trace of human plasma with identification of *S*-carboxymethyl-, *N*-dansyl derivatives, or *N,N'*-bisdansyl derivatives of cysteine (Cys), cystine (CySS), disulfide of cysteine and cysteinylglycine (CySS-mono-Gly), cysteinylglycine (CySH-Gly), mono(des-Glu)-GSSG, CySSG, GSH, and GSSG.

Detection is obtained by fluorescence monitoring with bandpass filters, 305–395 nm excitation and 510–650 nm emission (Gilson Medical Electronics, Middleton, WI). To facilitate simultaneous measurement of CySS (typically >40 μM) and GSSG (typically <200 nM), two detectors with different sensitivity settings are used in series. Fluorometric detectors with monochromators set at 335 nm for excitation and 515 nm for emission can also be used with equivalent results, but sensitivity is substantially less because the narrower bandwidths limit the intensity of both excitation and emission light. Quantification is obtained by integration relative to the internal standard with a correction factor of 2.67 for the dilution of the plasma upon collection of the blood into the preservation solution. This correction factor assumes a hematocrit of 40% and should be adjusted for different hematocrit values. For quality control and to allow calculation relative to external standards, a mixture of external standards (10 μM each of Cys, CySS, CySSG, GSH and GSSG, with 20 μM internal standard) are run daily.

The fluorescence of dansyl derivatives of GSH and Cys is identical to that for an equimolar amount of γ-glutamyl glutamate. However, disulfides have two amine groups, which complicates quantification because the fluorescence of the bisdansyl derivative is between 1.2 and 1.4 times that of the internal standard. For precise measurement of disulfides, calculation relative to external standards is needed.

Calculation of E_h

For GSH/GSSG, the Nernst equation simplifies to E_h (in mV) $= E_o +$ 30 log([GSSG]/[GSH]^2), where GSH and GSSG are molar concentrations and E_o is taken as -264 mV for pH 7.4. This latter value is based on a value of -240 mV for pH 7.0[14] with an adjustment of -5.9 mV/0.1 increase in pH.[14] For calculation of E_h of the Cys/CySS couple, an E_o value of -250 mV is used.[6]

Assay of Thiol/Disulfide Redox in Tissue Samples and Cell Culture

Background

Oxidation of thiols occurs rapidly on cell and tissue disruption so freeze clamping or addition of samples directly into ice-cold acid, followed by disruption, is preferable to tissue disruption prior to acidification. Because of this, studies of subcellular compartments require special techniques, such as nonaqueous fractionation, and little information is currently available on thiol–disulfide redox in subcellular compartments. This issue is important because these compartments could contain relatively high concentrations of disulfides, which could have effects

[14] J. Rost and S. Rapoport, *Nature* **201,** 185 (1964).

on the calculated cellular redox values (see Comments). Consequently, one must recognize that estimates provided by tissues and other whole cell measurements largely represent the aqueous cell volume but also include all other aqueous compartments. The quantitative contributions of these other compartments are largely unknown and this contribution could result in misinterpretation.

Sample Collection

Precautions needed for sample collection vary according to experimental conditions. For tissue culture, aspiration of the medium followed by the addition of ice-cold 5% (w/v) perchloric acid containing 0.2 *M* boric acid and 10 μM γ-glutamyl glutamate (γ-Glu-Glu) is usually adequate for accurate measurements of GSH and GSSG. If oxidants or compounds that are reactive with thiols under alkaline conditions are present, it is necessary to wash cells prior to the addition of acid. Otherwise, on addition of KOH/tetraborate, oxidation and alkylation reactions will compete with carboxymethylation, and thiols will be underestimated.

For tissues, freeze clamping in liquid nitrogen is usually preferred, but rapid excision of samples less than 10 mg followed by direct addition to ice-cold 5% (w/v) perchloric acid containing 0.2 *M* boric acid and 10 μM γ-glutamyl glutamate provides comparable results.

Derivatization and Analysis

GSH concentrations in cells and tissues are at least 100-fold greater than plasma so that samples need to be diluted when using the procedures described earlier for plasma. This is done most readily during the extraction procedure by selection of a suitable volume of acid for addition to samples. Cellular GSH most often is in the range of 2 to 40 nmol/mg protein, and the range of 2 to 40 μM GSH works well for the assay procedure. Thus, extraction is conveniently performed with 5% (w/v) perchloric acid containing 0.2 *M* boric acid and 10 μM γ-Glu-Glu with a volume of 1 ml added per milligram protein. Medium should normally be aspirated prior to the addition of acid because medium contains a relatively high concentration of cystine, which can react with GSH during the derivatization procedure. GSSG is 50- to 200-fold lower than GSH and normally can be quantified under these conditions of extraction. Occasionally, however, a smaller volume of acid must be used per milligram protein.

Oxidation of thiols in anoxic tissues does not appear to occur, but intentional preparation of samples under anoxic conditions, such as preperfusion of tissues with anoxic solutions, could result in artifactual reduction of disulfides. Thus, it appears most reasonable to follow procedures in which tissue samples are frozen or placed in ice-cold acid as rapidly as possible following excision rather than to introduce steps to enhance reduction.

Determination of Cytoplasmic Volume

Accurate expression of cellular GSH and GSSG concentrations requires a means to convert measured amounts per milligram protein or cell number to concentrations (mol/liter). Estimates based on cytoplasmic volume per milligram of cell protein are usually of sufficient accuracy, but measurements of cell volume can be done conveniently with a dual-label radiotracer method with [^{3}H]H_2O and [^{14}C]polyethylene glycol.[15] With this method, added [^{3}H]H_2O equilibrates rapidly with all aqueous compartments, wheares [^{14}C]polyethylene glycol remains in the extracellular compartment. An aliquot of the extracellular medium allows one to determine the counts per minute per microliter (cpm/μl) in the extracellular space. Following removal of as much of the extracellular water as possible, the remaining cells plus adherent water are treated with a tissue solubilizer and counted by scintillation counting. From the [^{3}H]H_2O counts associated with the cells and the measured cpm/μl for [^{3}H]H_2O, one can calculate the volume of water (intracellular plus extracellular). From the [^{14}C]polyethylene glycol associated with the cells and the measured cpm/ml for the [^{14}C]polyethylene glycol in the medium, one can calculate the amount of extracellular water associated with the cells. The difference between total water and extracellular water provides an estimate of cell volume.[15] Volume measurements with this approach are frequently within 20% accuracy. A twofold error in volume introduces 9 mV error in E_h for the GSH/GSSG pool. Thus, with volume measurements, one can virtually eliminate this potential source of error.

pH Measurements

pH has a relatively large effect on E_h (5.9 mV/0.1 pH unit) through an effect on E_o.[16] Thus, accurate estimates of E_h require that the pH is known. For extracellular media, this is accomplished readily with a pH electrode, but intracellular estimates are done more conveniently with radiotracer methods involving the distribution of the weak acid, [^{14}C]dimethadione (DMO).[17] The uncharged form of [^{14}C]dimethadione (HDMO) distributes freely across the cell membrane, whereas the ionized form (DMO^-) does not. Because the pK of 6.13, the total concentration of HDMO + DMO^- in the cellular compartment and extracellular compartment differs according to the difference in pH. By measurement of the cpm/ml in the cells and medium and using correction for the adherent water as determined earlier, one can calculate the cytoplasmic pH from the Henderson–Hasselbalch equation.

[15] T. Y. Aw and D. P. Jones, *FASEB J.* **3,** 52 (1989).

[16] W. M. Clark, "Oxidation-Reduction Potentials of Organic Systems." Williams & Wilkins, Baltimore, 1960.

[17] W. J. Waddell and T. C. Butler, *J. Clin. Invest.* **38,** 720 (1959).

Because cytoplasmic pH can be measured to within 0.1 pH unit, one can typically minimize error due to pH to less than 6 mV.

Comments and Perspectives

Accuracy of E_h Measurements

Direct potentiometric analysis of the redox states of specific thiol–disulfide couples in biologic systems is complicated by the limited specificity and stability of the electrodes in the presence of thiol compounds. Potentiometric measurements of tissue culture media show that cell density is maximal with E_h values about −60 mV,[18] i.e., only 20 mV more oxidized than the E_h value of the cysteine couple in human plasma.[10] Calculated E_h values for cysteine and cystine in conditioned medium are similar to the values for human plasma.

The E_h for the GSH/GSSG couple in human plasma is 50 mV more reduced than for the Cys/CySS couple, indicating that GSH release and Cys uptake by tissues *in vivo* create a steady state in which the redox states of the GSH/GSSG and Cys/CySS couples are not equilibrated.[10] The liver is a major source of GSH in the plasma, and previous studies have shown that the GSH concentration differs in blood samples taken from different blood access sites.[19] Thus, differences in GSH redox are also likely to occur in plasma according to the sampling site, especially those distal to the liver. Based on this, one can extrapolate that variations in sampling and processing are likely to affect the measured E_h value. Attempts to detect such variation with the current method by timed collection did not reveal such error,[7] but the design of the experiments and the extent of variation are such that one cannot absolutely exclude a systematic error in E_h that is specifically due to the method. In any case, interstitial space and unstirred layers adjacent to cells are likely to be more reduced than venous samples and are probably intermediate between the estimated plasma values (−137 for GSH/GSSG and −80 mV for Cys/CySS) and the more reduced tissue values (approximately −220 mV).

Accuracy of E_h Estimates for Tissues and Cells

Potentiometric data are not available for E_h in tissues and cell because of the technical problems described earlier. Calculations based on measurements of the concentrations of specific thiols and disulfides provide a more practical way to assess thiol–disulfide redox. As indicated earlier, errors due to the estimation of cytoplasmic volume and pH can be minimized, and with reasonable precautions, neither should contribute to over 10 mV error.

[18] C. Hwang and A. J. Sinskey, *in* "Production of Biologicals from Animal Cells in Culture" (R. E. Spier, J. B. Griffiths, and B. Meignier, eds.), p. 548. Halley Court, Oxford, 1991.

[19] B. H. Lauterburg, J. D. Adams, and J. R. Mitchell, *Hepatology* **4,** 586 (1984).

Sequestration of a relatively high concentration of GSSG into an intracellular compartment could result in an error in E_h values, but this is not likely to result in large errors, e.g., of a magnitude that could account for the >100-mV difference between the NADPH/$NADP^+$ and GSH/GSSG couples. For example, if one assumed that the cisternae of the endoplasmic reticulum constituted 1% of the cell volume and had a 100-fold higher concentration of GSSG than present in the cytoplasm, then the calculated E_h estimate would only be 9 mV more oxidized than the correct cytoplasmic value. Experiments with digitonin to selectively permeabilize the plasma membrane without disruption of the mitochondria or the secretory pathway showed a selective retention of cystine by permeabilized HT29 cells, but GSSG was not selectively retained.[1] Together with data showing that erythrocytes, which have no internal organelles, have a E_h value for the GSH/GSSG couple that is similar to other differentiated cells,[1,8] these data indicate that calculations based on concentrations of cellular GSH and GSSG are reasonable estimates of the cytoplasmic pool.

Other factors that could contribute to error include noncovalent binding of GSSG to proteins, chelation of GSSG in a redox-inactive complex with a metal ion, or equilibration of the GSH/GSSG pool with of a sizable concentration of glutathione sulfenic acid. Equilibrium dialysis of rat plasma with radiolabeled GSSG under anaerobic conditions provided no evidence for noncovalent binding of GSSG, but this possibility has not been formally excluded for cellular proteins. From the Nernst equation and measured GSSG values, if 50% were bound, the E_h would be off by 9 mV; and if 99% were bound, it would be off by 30 mV; and if 99% were bound, it would be off by 60 mV. For noncovalent GSSG binding to account for the difference between NADPH/$NADP^+$ and GSH/GSSG couples, >99.99% of the GSSG could have to be noncovalently bound. Similarly, removal of a large fraction of GSSG from the redox pool by binding to metal ions has not been formally excluded. Again, >99.99% would have to be bound, and candidate metal ions with concentrations in the range of 20 μM are not known. The possibility that the measured GSH/GSSG pool contains a sizable amount of GSH–sulfenic acid that is converted to GSSG during extraction and assay seems unlikely because sulfenic acids are not stable in aqueous media.[20]

Significance of Thiol–Disulfide Redox in Plasma

Data are now available on thiol–disulfide redox in plasma,[6] intestinal lumen,[21] cultured cells,[1] and culture medium.[22] Studies on plasma redox show that the E_h values of the low molecular weight thiol/disulfide pools are correlated with each

[20] A. Claiborne, J. I. Yeh, T. C. Mallett, J. Luba, E. J. Crane III, V. Charrier, and D. Parsonage, *Biochemistry* **38,** 15407 (1999).

[21] L. J. Dahm and D. P. Jones, *J. Nutr.* **130,** 2739 (2000).

[22] L. T. Miller and D. P. Jones, unpublished results.

other when compared among different individuals, but the GSH/GSSG couple is 50 mV more reduced than the Cys/CySS pool.[6] Older individuals and diabetics have more oxidized values,[8] and values are oxidized following high-dose chemotherapy for bone marrow transplantation.[9] These observations indicate that E_h in plasma varies according to physiology, disease, and toxicity. Because there is little variation among young healthy individuals, measures of plasma redox may provide a useful means for clinical assessment of the balance of oxidative stress and opposing defense mechanisms.

The consequences, if any, of having a more oxidized plasma redox are not yet clear. Hwang and Sinskey[18] showed in cell culture that cell density varies according to E_h, of the culture medium. While their study does not distinguish between effects on cell proliferation and apoptosis, we have found that cell proliferation increases with a more reduced E_h for Cys/CySS over the range measured *in vivo*.[23] Apoptosis is inhibited by thiols,[24] but whether this effect occurs over the physiologic range of E_h is not known.

The quantitatively important mechanisms for maintaining E_h in the plasma have not been established experimentally. Values for the GSH/GSSG pool are considerably more reduced than for the Cys/CySS pool,[6] indicating that GSH release and Cys uptake contribute to maintenance of different E_h values for the pools. However, uptake of CySS also occurs in many cell types, and the small intestine regulates extracellular thiol–disulfide redox by a mechanism in which enhanced CySS uptake is associated with stimulated Cys release.[21] This cysteine–cystine shuttle functions in both the lumen and the vascular perfusate[25] to regulate extracellular redox in response to added GSSG. In addition, the basolateral membranes of small intestinal enterocytes and renal proximal tubules also have a thiol oxidase that oxidizes low molecular weight thiols.[26] Whether this system functions along with the transport systems to maintain plasma redox is not known.

In individuals who have a more oxidized plasma redox, it may be possible to nutritionally or therapeutically shift the redox to the range of young, healthy individuals. Studies to assess this are not available, but calculations using data by Stofer-Vogel *et al.*[27] indicate that such an effect can be achieved by mercaptoethane sulfonate (mesna). Possible benefits from such changes in redox have not been investigated.

Significance of Thiol–Disulfide Redox in Cells and Tissues

E_h values for GSH/GSSG in cells and tissues are considerably more reduced than those for extracellular fluids. Essentially all available cellular and tissue values

[23] L. T. Miller, J. Cai, W. H. Watson, P. Sternberg, and D. P. Jones, *Toxicologist* **54,** 211 (2000).
[24] D. P. Jones, E. Maellaro, S. Jiang, A. F. G. Slater, and S. Orrenius, *Immunol. Lett.* **45,** 205 (1995).
[25] L. J. Dahm and D. P. Jones, *Toxicol. Appl. Pharmacol.* **129,** 272 (1994).
[26] L. H. Lash and D. P. Jones, *Arch. Biochem. Biophys.* **247,** 120 (1986).
[27] B. Stofer-Vogel, T. Cerny, A. Kupfer, E. Junker, and B. H. Lauterburg, *Br. J. Cancer* **68,** 590 (1993).

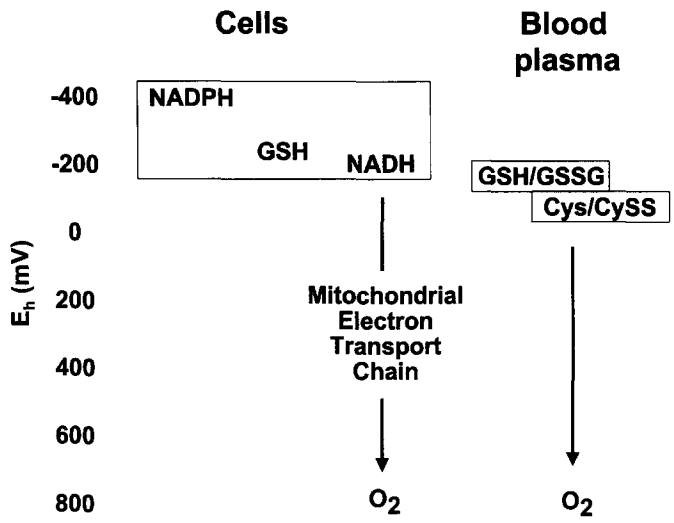

FIG. 4. Steady-state redox potentials (E_h) for cellular GSH/GSSG are between the values for the NADPH/$NADP^+$ pool and the NADH/NAD^+ pool, whereas that for the plasma GSH/GSSG pool is between that for the cellular pool and the plasma cysteine/cystine (Cys/CySS) pool.

are in the range of −260 to −150 mV, with the more oxidized values only present in cells undergoing apoptosis. The range of values is relatively small considering that the NADPH/$NADP^+$ values is about −400 mV and that of O_2 as the terminal oxidant is at least 600 mV (Fig. 4). The apparent displacement from equilibrium and the relatively small range of values suggest that the redox state of the GSH/GSSG pool is regulated. Change in cellular redox due to varying extracellular redox typically has little effect unless high concentrations of thiol are added.[24]

A provocative aspect of the cellular E_h values is that the cellular E_h tends to vary according to cell growth conditions. Rapidly proliferating cells and tissues have 30 to 60 mV more reduced values than differentiated and growth arrested cells (Fig. 5). Cells undergoing apoptosis are further oxidized by 30 to 60 mV relative to differentiated and growth-arrested cells. These observations indicate that thiol–disulfide redox could give a context for cell control, essentially providing an optimal redox for the function of enzymes, transcriptions factors, and other proteins. This could function in much the same way as pH optimum determines the activity of acid phosphatases and alkaline phosphatases in different cellular compartments. An important difference, however, is that redox changes occur in the cytoplasm so that if specific systems for proliferation, differentiation, or apoptosis have different redox optima, then changes in steady-state cytoplasmic redox could provide a means to optimize conditions for proliferation, differentiation, or apoptosis.

At present, such a possibility is only hypothetical in that no specific examples of such control have been established. Modeling of redox sensitivities of proteins

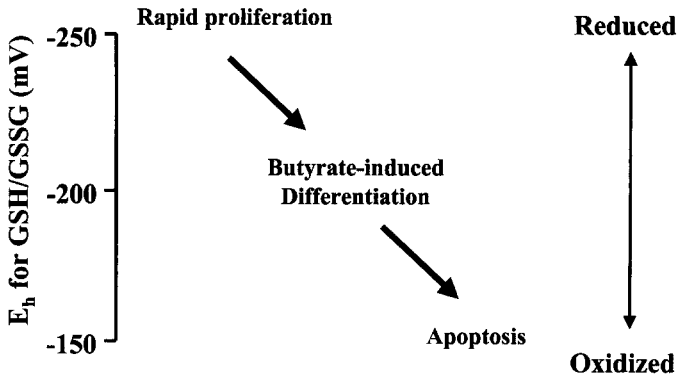

FIG. 5. The steady-state redox potential (E_h) for the cellular GSH/GSSG pool in HT29 cells differs among proliferating, differentiated, and apoptotic cells. Cells with a doubling time of 33 hr are about 60 mV more reduced than cells differentiated by treatment with 5 m*M* sodium butyrate. These latter cells had a slow doubling time (> 100 hr). On treatment with agents that induced apoptosis, E_h became even more oxidized.

with E_o values for vicinal dithiols in the range of −200 to −260 mV shows that gains or losses in function of 55-fold could occur with a change of 60 mV (Fig. 6). The magnitude of functional change could be amplified by coupling multiple thiol–disulfide couples together or by having redox-sensitive groups in chaperones, docking/assembly proteins, or nuclear transport systems.

Two key conditions would be required for the redox-dependent regulation of proteins by GSH/GSSG: enzymes would be required to control protein thiol/disulfide redox in association with changes in GSH/GSSG redox and metabolic systems would be needed to control GSH/GSSG redox at appropriate values. Enzymes are needed to catalyze exchange between the GSH/GSSG pool and protein dithiol–disulfide motifs because the nonenzymatic exchange rates are too slow to achieve effective regulation under biologic conditions. A protein family, consisting of glutaredoxin and related proteins, catalyzes such exchange reactions.[28] These enzymes are widely distributed and well characterized. However, specific examples of their function in cellular regulation are not well established; this leaves open the possibility that the GSH/GSSG system serves largely in detoxification and has little role in regulation. If so, the GSH/GSSG redox changes associated with proliferation, differentiation, and apoptosis may be a secondary indicator of redox control by other thiol–disulfide systems, such as those dependent on members of the thioredoxin family of proteins.

[28] W. W. Wells, Y. Yang, T. L. Deits, and Z. R. Gan, *Adv. Enzymol. Relat. Areas Mol. Biol.* **66**, 149 (1993).

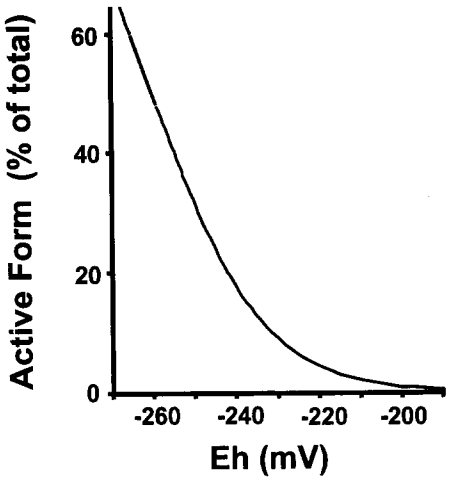

FIG. 6. Modeling of activity of a hypothetical protein with vicinal dithiols responsive to the GSH/GSSG redox state. Assuming that vicinal dithiols have an E_o value of -260 mV and that only the dithiol form is active, the percentage of the total protein in the active form would be 55-fold higher at -260 mV than at -200 mV. If such a protein were present in the replicative machinery, it could provide a means to prevent replication unless the cell maintained the more reduced redox potential.

As detailed elsewhere in this volume, considerable evidence is available to indicate that thioredoxins are required for enzymatic and transcription factor function. In addition, Trx has chaperone-like activity and controls apoptosis signal-regulating kinase-1 (ASK-1)[29] and tumor necrosis factor α-receptor activity via redox-dependent associations.[30] Preliminary data[9] indicate that E_h for thioredoxin is in the range of -330 mV in proliferating cells and becomes oxidized during growth arrest and apoptosis. Data suggest that the redox of the thioredoxin system responds to changes in growth and apoptosis in much the same way as the GSH/GSSG redox system. However, the magnitudes of change are less, indicating that the redox of the Trx system is maintained independently. Thioredoxin functions are distinct from those of GSH/GSSG, and this difference may provide specificity in redox control and signaling.

In the absence of constitutively active enzymes to catalyze rapid equilibration of redox-sensitive thiol motifs in proteins with the redox of either the GSH/GSSG or the thioredoxin system, regulation could involve transient activation followed by a slower inactivation. Thiols in close proximity to a cationic amino acid are relatively reactive. Under basal conditions, these thiols could autoxidize to a stable

[29] M. Saitoh, H. Nishitoh, M. Fujii, K. Takeda, K. Tobiume, Y. Sawada, M. Kawabata, K. Miyazono, and H. Ichijo, *EMBO J.* **17,** 2596 (1998).

[30] H. Liu, H. Nishitoh, H. Ichijo, and J. M. Kyriakis, *Mol. Cell. Biol.* **20,** 2198 (2000).

sulfenic acid or internal disulfide to achieve an OFF state. These proteins could be activated by reduction involving either a thioredoxin or a GSH/GSSG system. This could occur following assembly of a complex that allows reduction (ON state). Such a system could have the gain of function (i.e., 55-fold for 60 mV) similar to phosphorylation mechanisms and have the recovery characteristics of many phosphatase-dependent mechanisms (decay to a resting state following activation). Indeed, such a mechanism could control phosphatase activities as some are thiol dependent and inhibited by oxidation.

The second key condition for the function of such a redox control mechanism is the requirement for a means to control GSH/GSSG (and/or thioredoxin) at appropriate redox values. For GSH/GSSG, this control could be regulated simply by the activity of GSSG reductase. Principal control could be exerted by a change in the concentration of the substrate GSSG, and secondary control could involve a change in enzyme expression. However, given the apparent importance of regulating thiol/disulfide redox, it would appear that additional mechanisms would exist. During apoptosis, the redox shift is dependent on increased generation of reactive oxygen species by mitochondria,[31] and mitochondria could also control thiol/disulfide redox during cell proliferation and differentation by a variation in the rate of generation of reactive oxygen species. This control mechanism could involve the known induction of GSH synthesis in response to reactive oxygen species. An increase in pool size would allow cells to maintain a more reduced E_h due to increased GSH and due to an enhanced reduction of GSSG (at a higher steady-state GSSG concentration). Enhanced generation of reactive oxygen species by NADPH oxidases that have been associated with cell proliferation could similarly result in a reduction of the thiol–disulfide redox state by increasing the GSH pool size. Alternatively, recruitment of GSSG reductase into a complex could determine its activity in specific pathways.

Other Hypotheses

Although missing experimental evidence, two other possibilities need to be considered. The first of these could provide a function for thiol (sulfhydryl) oxidases. A variety of these enzymes have been characterized and some have the paradoxical property that they produce H_2O_2. This is unusual in that GSH, the major cellular thiol, is used as a reductant in the elimination of H_2O_2 by the GSH peroxidase reaction in cells. Together, thiol oxidases and GSH peroxidase would appear to create a futile cycle, one in which reducing equivalents are used to both form and eliminate H_2O_2. However, the activity of these enzymes together with GSSG reductase could provide an autoregulatory mechanism to control thiol–disulfide redox (Fig. 6). H_2O_2 production would be stimulated by increased thiol concentration, and the increased steady-state H_2O_2 would stimulate GSH oxidation by

[31] J. Cai and D. P. Jones, *J. Biol. Chem.* **273,** 11401 (1998).

GSH peroxidase. H_2O_2 would accumulate until the rate of GSH oxidation matched the rate of GSSG reduction by GSSG reductase. If NADPH and O_2 are constant, the GSH/GSSG redox would be inherently stable by this mechanism, and redox could be varied by a change in expression of the thiol oxidase or GSSG reductase (Fig. 6).

A second possibility is that the principal, direct oxidant of thiols is neither O_2 nor H_2O_2 but rather NAD^+ (see Fig. 7). While this may seem heretical, cytoplasmic NADH/NAD^+ varies in association with the availability of dehydrogenase substrates over a range (-150 to -250 mV)[5] where it could function as an oxidant in control of the thiol–disulfide redox. Under conditions of substrate deficiency,

A. Reactions of GSH oxidation:

Thiol oxidases $\quad 2\,GSH + O_2 \longrightarrow H_2O_2 + GSSG$

GSH peroxidases $\quad H_2O_2 + 2\,GSH \longrightarrow ROH + GSSG$

If O_2 and thiol oxidases (TO) are constant, the rate of formation of GSSG by TO can be approximated as $v_{TO}=k_{TO}[GSH]^2$. At steady state, oxidation of GSH by GSH peroxidase equals the rate of oxidation by TO, so the total rate of GSSG formation approximates $2*v_{TO}= 2*k_{TO}\,[GSH]^2$.

B. Reaction of GSSG reduction:

GSSG reductase $\quad GSSG + NADPH \longrightarrow 2\,GSH + NADP^+$

If NADPH is saturating, rate of GSSG elimination by GSSG reductase (GR) is approximated by $v_{GR}=k_{GR}[GSSG]$. At steady state, $2*v_{TO}= v_{GR}$, so $2*k_{TO}\,[GSH]^2 = k_{GR}[GSSG]$. Thus,

$$\frac{[GSH]^2}{[GSSG]} = \frac{k_{GR}}{2*k_{TO}} = \text{Constant}$$

FIG. 7. Hypothetical mechanism for steady-state control of GSH/GSSG redox state via thiol or sulfhydryl oxidases. Redox of the GSH/GSSG couple could be maintained by a combination of GSH peroxidases and GSSG reductase along with H_2O_2-producing thiol oxidases. (A) If one assumes that O_2 is constant and the K_m for GSH is high for a thiol oxidase-catalyzed oxidation of 2 GSH by O_2 to produce H_2O_2 plus GSSG, then the rate of the reaction will increase as the square of GSH concentration. Under steady-state conditions, the H_2O_2 will be metabolized by GSH peroxidase at the same rate. (B) At high NADPH conditions, the rate of reduction of GSSG back to GSH is pseudo-first order in GSSG and equal to twice the rate of the thiol oxidase reaction. Rearrangement of the equations shows that $[GSH]^2/[GSSG]$ is constant under these conditions and is defined by the ratio of rate constants for the thiol oxidase and GSSG reductase reactions. Thus, the GSH/GSSG redox state can be controlled by the expression of thiol oxidases and/or GSSG reductase. In principle, H_2O_2 production by any sulfhydryl oxidation, i.e., enzymatic or nonenzymatic and low molecular weight thiol or protein sulfhydryl, could function in this regulation so long as glutaredoxin or a similar catalyst were present to equilibrate the thiol–disulfide pools.

the NADH/NAD^+ pool becomes oxidized. If thiol–disulfide pools were linked to this, it could explain the oxidation of GSH/GSSG that occurs in association with nutrient deprivation.[32] Such a mechanism would provide an inherently simple way to control thiol–disulfide redox. It would be energetically efficient in that electron flow from NADPH could flow through NADH to the mitochondrial electron transport chain. Under conditions of nutrient depletion, GSH/GSSG oxidation would follow NADH/NAD^+ oxidation. This could arrest cell division and enhance apoptosis, paralleling the natural redox changes associated with terminal differentiation.[1] However, such a mechanism could also create a futile cycle in that there is an energetic cost in the reduction of $NADP^+$ by NADH catalyzed by the energy-dependent transhydrogenase.

In conclusion, the redox of thiol–disulfide components in biological systems is conveniently and simply expressed in terms of an E_h value calculated from concentrations of the reduced and oxidized forms. Quantification of the major cellular (GSH/GSSG) and extracellular (Cys/CySS) aminothiol compounds can be obtained readily by derivatization and HPLC separation with fluorescence detection. E_h for cellular GSH/GSSG is remarkably constant among different cell types under the same conditions of growth. The value differs considerably among proliferation, differentiation, and apoptosis, following a progression from most reduced to most oxidized values. Under all conditions, values are considerably more oxidized than for the donor NADPH/$NADP^+$ pool, indicating that specific mechanisms exist to maintain steady-state GSH/GSSG redox. Blood plasma redox also varies little among young healthy individuals. However, the plasma values become oxidized in association with aging, toxicity, and certain diseases, indicating that blood plasma redox measurements may be useful to clinically detect oxidative stress and assess potential interventional strategies.

[32] C. R. Jonas, C. F. Estivariz, D. P. Jones, L. H. Gu, T. M. Wallace, E. E. Diaz, R. R. Pascal, G. A. Cotsonis, and T. R. Ziegler, *J. Nutr.* **127,** 1278 (1999).