Modified Fluorescence Assay for Oxidized and Reduced Glutathione in Tissue

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Abstract □ A previously reported enzymatic-fluorometric assay for reduced glutathione and oxidized glutathione was modified through the use of heat precipitation of sample protein and hydrogen peroxide oxidation of reduced glutathione to oxidized glutathione. These modifications were made to eliminate two critical pH adjustments present in the original assay and the possibility of interference with the assay of oxidized glutathione by the perchloric acid protein precipitant and the cupric-ion oxidant used in the original assay. The reduced glutathione and oxidized glutathione content of Harding–Passey melanoma was determined using the modified assay. The reduced glutathione content was similar to literature values. The precision of the assay was not adversely affected by these modifications.

Keyphrases □ Glutathione, oxidized and reduced—modified fluorometric determination in tissue □ Spectrophotofluorometry—determination of oxidized and reduced glutathione in tissue

Reduced glutathione, a tripeptide (λ-glu-cys-gly), is found widely distributed in tissue (1). It also exists in an oxidized form in which two reduced molecules are joined through a disulfide bridge. To be maximally useful, any assay for these species should measure both in the same sample.

An enzyme, glutathione reductase (EC 1.6.4.2), reduces oxidized glutathione in the presence of reduced nicotine adenine dinucleotide phosphate. One mole of reduced nicotine adenine dinucleotide phosphate is required for conversion of each mole of oxidized glutathione to 2 moles of reduced glutathione (2, 3). This relationship was used by Halprin and Ohkawara (4) to develop an enzymatic assay for reduced glutathione and oxidized glutathione. The amount of oxidized glutathione present in the sample was determined by following the decrease in reduced nicotine adenine dinucleotide phosphate fluorescence. The reduced glutathione content of another aliquot of the sample was determined after its oxidation to oxidized glutathione. This assay as described requires two critical, time-consuming adjustments of the sample pH. The first pH adjustment with potassium hydroxide removes the perchloric acid as potassium perchlorate, and the second optimizes the rate of oxidation of reduced glutathione to oxidized glutathione by cupric ion. When the original assay was modified in this laboratory so that larger volumes of sample could be used, it was found that standards and various volumes of tissue sample did not consistently yield linear fluorescence changes. It was suspected that cupric ion or perchloric acid might be interfering with the oxidized glutathione assay, since cupric ion is known to inhibit glutathione reductase (5) and perchloric acid is a protein precipitant.

The objective of this work was to modify the reduced glutathione–oxidized glutathione assay developed by Halprin and Ohkawara (4) to eliminate the perchloric acid used for protein precipitation and the cupric ion used for reduced glutathione oxidation. By eliminating these two substances, the possibility of their interfering with the assay of oxidized glutathione would be removed and the two critical pH adjustments would be eliminated. Heat precipitation of tissue protein and hydrogen peroxide oxidation of reduced glutathione were investigated as means of accomplishing these objectives. The assay in its modified form was used to determine the oxidized glutathione and reduced glutathione content of Harding–Passey melanoma tissue.

EXPERIMENTAL

Materials—Reduced and oxidized glutathione[1], glutathione reductase[1] (yeast) in 2.8 *M* ammonium sulfate, reduced nicotine adenine dinucleotide phosphate tetrasodium[1], tetrasodium edetate[1], and tromethamine[1] were used. All other chemicals were analytical reagent grade. Stock solutions of reduced nicotine adenine dinucleotide phosphate were prepared in deionized, double-distilled water containing 1% sodium bicarbonate.

Method—Melanoma tissue was rapidly excised from mice and frozen on dry ice. The tissue was prepared for assay by homogenization in five volumes of phosphate buffer (0.05 *M*, pH 6.4) at the temperature of melting ice. The homogenate was placed in a water bath at 100° for 4 min to precipitate protein and was then centrifuged in an ultracentrifuge[2] using a No. 30 rotor (3000×*g*, 5750 rpm for 10 min, 0°). A portion of the supernate was divided into four 1.0-ml samples. The first sample was assayed directly for oxidized glutathione and the second sample was assayed for oxidized glutathione after a known quantity of oxidized glutathione was added as an internal standard. To the third sample were added a known quantity of reduced glutathione and 30% hydrogen peroxide sufficient to make the solution 0.12% (v/v) in hydrogen peroxide. Only hydrogen peroxide sufficient to make 0.12% (v/v) was added to the fourth sample. After 3 hr at room temperature, the third and fourth samples were placed in a water bath (100°, 5 min) to drive off residual hydrogen peroxide; they were then assayed for oxidized glutathione.

Samples were assayed for oxidized glutathione as in the original assay by following the decrease in fluorescence[3] of reduced nicotine adenine dinucleotide phosphate (340-nm excitation, 450-nm emission, uncorrected). The assay mixture contained 40 nanomoles of reduced nicotine adenine dinucleotide phosphate, 0.05 *M* (pH 7.4) tromethamine buffer (containing tetrasodium edetate, 0.005 *M*, and magnesium chloride, 0.03 *M*), and standards or tissue extracts containing 2–25 nanomoles of oxidized glutathione in a total volume of 3.45 ml. The reaction components were placed in a spectrophotofluorometer cell and the fluorescence was allowed to stabilize. This value was recorded as the initial fluorescence. Glutathione reductase was then added (0.025 ml, 0.3 unit, in 2.8 *M* ammonium sulfate), and the solution was thoroughly mixed. A second fluorescence value was recorded 10 min later. All samples were assayed in triplicate. Blanks contained pH 6.4 phosphate buffer instead of the sample solution and served to correct for

[1] Calbiochem, Los Angeles, Calif.
[2] Beckman Spinco model L or L2-50, Spinco, Beckman Instruments, Palo Alto, Calif.
[3] Aminco-Bowman spectrophotofluorometer or SPF-125.

Table I—Influence of Heat on the Oxidized Glutathione Content of Solutions Containing Reduced or Oxidized Glutathione[a]

Glutathione	Heat Exposure	Oxidized Glutathione Content, nanomoles ± SE[b]	t Value[c]
Oxidized (10 nanomoles)	−	10.00 ± 0.07	
Oxidized (10 nanomoles)	+	9.87 ± 0.15	0.62 (NS)
Reduced (20 nanomoles)	−	0.06 ± 0.06	
Reduced (20 nanomoles)	+	0.05 ± 0.05	0.12 (NS)

[a] Triplicate samples, dissolved in pH 6.4 phosphate buffer, were placed in a water bath (100°) for 4 min (+) before assay for oxidized glutathione or were assayed directly for oxidized glutathione (−). [b] Standard error. [c] (NS) = not significantly different ($p > 0.05$) from unheated samples.

nonenzymatic oxidation of reduced nicotine adenine dinucleotide phosphate. Oxidized glutathione used as an internal standard allowed the conversion of fluorescence units to oxidized glutathione content. Reduced glutathione was used as an internal standard to check on the completeness of oxidation of sample reduced glutathione by hydrogen peroxide.

RESULTS AND DISCUSSION

Heat Stability of Reduced Glutathione and Oxidized Glutathione—The proposed use of heat as a protein precipitant necessitated verification of the heat stability of oxidized glutathione and reduced glutathione. Solutions containing known amounts of either oxidized glutathione or reduced glutathione in pH 6.4 phosphate buffer were assayed for oxidized glutathione content after exposure in a water bath at 100° for 4 min. Reduced glutathione was previously reported to be stable under these conditions (6). Data from this experiment (Table I) indicate that there is no significant destruction of oxidized glutathione or conversion of reduced glutathione to oxidized glutathione by heat exposure of these species at 100° for 4 min.

Evaluation of Reduced Glutathione Oxidation to Oxidized Glutathione by Hydrogen Peroxide—The oxidation of reduced glutathione to oxidized glutathione by hydrogen peroxide was reported previously to be complete (4). Hydrogen peroxide (30%) (to make 0.12% v/v) was added to a solution containing known amounts of reduced glutathione and oxidized glutathione in pH 6.4 phosphate buffer (both reduced glutathione and oxidized glutathione were placed in the same solution to more closely approximate the condition of the tissue homogenate). After 3 hr at room temperature, the solution was heated (100° water bath for 5 min) before assay for oxidized glutathione to drive off excess hydrogen peroxide. If the hydrogen peroxide is not driven off, reduced glutathione values are abnormally high. Data from this experiment (Fig. 1) indicate that the conversion of reduced glutathione to oxidized glutathione is linear over the range of concentrations used (4-12 nanomoles).

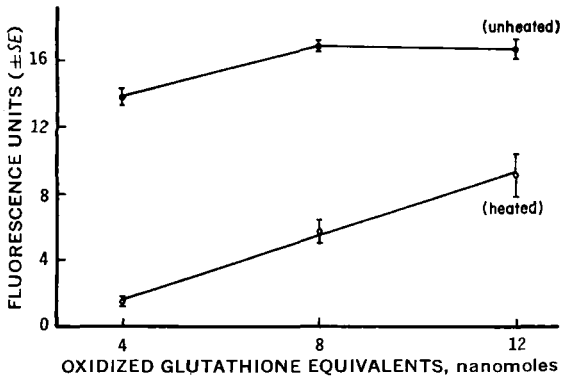

Figure 1—*Evaluation of reduced glutathione oxidation to oxidized glutathione by hydrogen peroxide. An oxidized glutathione–reduced glutathione mixture (1:2) in phosphate buffer was treated with hydrogen peroxide (0.12%, 3 hr). One aliquot was heated (100°, 5 min) to drive off excess hydrogen peroxide and then both aliquots were assayed (triplicate) for oxidized glutathione. Reduced glutathione (nanomoles) is divided by two to obtain oxidized glutathione equivalents (nanomoles). The fluorescence change due to the oxidized glutathione originally added was subtracted from the values shown on the figure.*

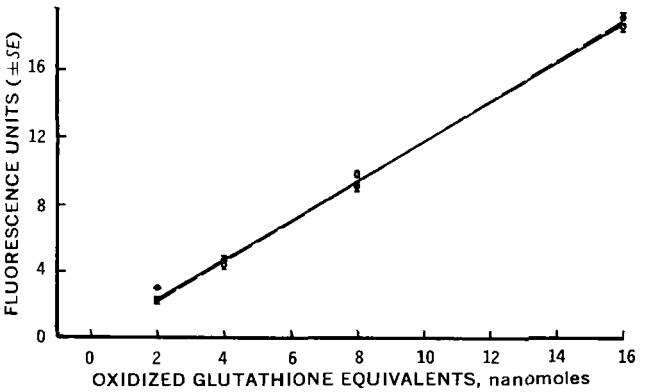

Figure 2—*Linearity and completeness of oxidation of reduced glutathione to oxidized glutathione by hydrogen peroxide. Solutions containing either reduced glutathione* (●) *(4, 8, 16, and 32 nanomoles) or oxidized glutathione* (○) *(2, 4, 8, and 16 nanomoles) were assayed as described under* Method.

Evaluation of Assay Linearity—The decrease in reduced nicotine adenine dinucleotide phosphate fluorescence produced by reduced glutathione (4, 8, 16, and 32 nanomoles) after oxidation by hydrogen peroxide and by oxidized glutathione (2, 4, 8, and 16 nanomoles) was determined. The two curves obtained (Fig. 2) were not significantly different [$F = 0.06$, $df_{1,16}$, by two-way analysis of variance (7)] and were linear. This experiment indicates that the oxidation of reduced glutathione to oxidized glutathione is complete under the conditions employed.

The decrease in reduced nicotine adenine dinucleotide phosphate fluorescence produced by various volumes of tissue homogenate supernate (0.025, 0.05, 0.075, 0.100, and 0.200 ml) from a Har-

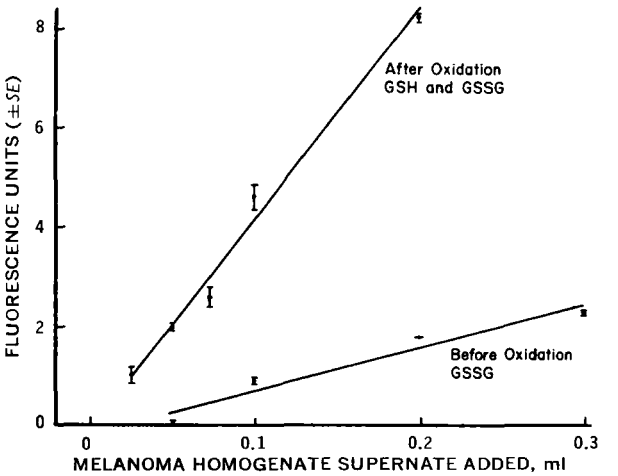

Figure 3—*Linearity of the assay for tissue oxidized glutathione (GSSG) before and after oxidation of tissue reduced glutathione (GSH) to oxidized glutathione by hydrogen peroxide. Melanoma homogenate supernate, prepared as described under* Method, *was divided into two aliquots. One aliquot was assayed directly for oxidized glutathione. The other aliquot was treated with hydrogen peroxide.*

Table II—Reduced and Oxidized Glutathione Content of Harding–Passey Melanoma Tissue[a]

Tissue Oxidized Glutathione, nanomole/mg ± *SE*[b]	Tissue Reduced Glutathione, nanomoles/mg ± *SE*	Ratio Reduced Glutathione to Oxidized Glutathione
0.141 ± 0.029	1.473 ± 0.054	12.5

[a] Melanomas were excised from four mice and triplicate samples were assayed for oxidized glutathione and reduced glutathione following tissue preparation as described under *Method*. [b] Standard error.

ding–Passey melanoma homogenate, prepared as described under *Method*, was determined both before and after addition of hydrogen peroxide. As shown in Fig. 3, the fluorescence change produced is linear with respect to the volume of supernate employed both before and after the oxidation of reduced glutathione to oxidized glutathione.

Determination of Oxidized Glutathione and Reduced Glutathione Values for Harding–Passey Melanoma—The Halprin and Ohkawara assay (4) as modified through the use of heat precipitation of sample protein and hydrogen peroxide oxidation of reduced glutathione was performed on a Harding–Passey melanoma homogenate supernate prepared as described under *Method*. The value for reduced glutathione (Table II) is similar to the value of 1.4 nanomoles/mg (sulfosalicylic acid-soluble sulfhydryl) reported (6) for this tissue. More than 90% of the glutathione in the melanoma is in the reduced form, which is similar to the percentage reported for other tissues (4, 9). This is indicated by the experimentally determined ratio, reduced glutathione–oxidized glutathione of 12.5, obtained in the present study.

The enzymatic–fluorometric assay for reduced glutathione and oxidized glutathione previously described (4) was modified to eliminate the use of a strong acid protein precipitant and of cupric ion for the oxidation of reduced glutathione. These procedures were replaced by the use of heat as a protein precipitant and of hydrogen peroxide as an oxidant. These modifications obviate the necessity of a critical pH adjustment and remove the possibility of copper interference with glutathione reductase activity.

It was determined that heat treatment of reduced glutathione- and oxidized glutathione-containing solutions does not interfere with the precision of oxidized glutathione determination and does not convert reduced glutathione to oxidized glutathione. It appears that heat precipitation of protein is an acceptable substitute for perchloric acid precipitation of protein and is free of the mentioned disadvantages.

Substitution of hydrogen peroxide for cupric ion in the oxidation of reduced glutathione to oxidized glutathione eliminates the need for making a critical pH adjustment and the possibility of cupric-ion interference with the reduced glutathione assay. Cupric ion (10^{-5} *M*) was reported to inhibit glutathione reductase by approximately 50% (5). Hydrogen peroxide appears to be an acceptable substitute for cupric ion in that the oxidation of reduced glutathione standards is complete and the oxidation of reduced glutathione to oxidized glutathione in tissue samples is linear.

The reduced glutathione content determined with the modified assay for Harding–Passey melanoma was nearly identical to the 1.4-nanomoles/mg (sulfosalicylic acid-soluble sulfhydryl) value reported (8) for this tissue. No literature value for oxidized glutathione in the Harding–Passey melanoma is available; but human epidermis, which has a similar amount of reduced glutathione to Harding–Passey melanoma, was reported (4) to have 0.078–0.111 nanomole/mg of oxidized glutathione, values similar to those determined experimentally for the melanoma with the modified assay.

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