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A Fluorometric Method for Determination of Oxidized and Reduced Glutathione in Tissues

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A method for measurement of both oxidized (GSSG) and reduced (GSH) glutathione has been developed, with use of *o*-phthalaldehyde (OPT) as a fluorescent reagent. The method takes advantage of the reaction of GSH with OPT at pH 8 and of GSSG with OPT at pH 12; GSH can be complexed to *N*-ethylmaleimide to prevent interference of GSH with measurement of GSSG. The method gave "recoveries" of 91 to 110% for both GSH and GSSG and was quite specific for glutathione; and none of the manipulations appeared to influence the amount of glutathione present in the tissue. Results for GSH levels agreed well with earlier reports but levels of GSSG estimated here were higher than earlier reported values. The reasons for the apparently higher levels of GSSG are discussed.

Glutathione is widely distributed among living cells and apparently involved in many biological functions (1–4). Glutathione is present in the oxidized (GSSG) form, which is readily converted to the reduced (GSH) form by the enzyme glutathione reductase. It has been reported that glutathione is present mainly in its reduced form in biological tissues, at concentrations as high as 3100 μg/g of tissue (5). However, GSSG was reported to be present in much smaller concentrations, ranging from 0 to 244 μg/g of tissue (8,20,21,24,25).

There are chemical (6) and enzymatic (7–10) methods available for measurement of the relatively high concentration of GSH. However, measurement of GSSG levels has been more difficult. Some methods for measurement of GSSG are based on its estimation from differences in the amount of GSH determined before and after reduction of GSSG by enzymatic (11,12), chemical (14), or electrolytic (13) reactions; these techniques have yielded rather large errors (12,15). Another approach utilized the enzyme glutathione reductase, to convert GSSG to GSH in the presence of the cofactor NADPH (10,17); the disappearance of NADPH was followed spectrophotometrically and was related to the amount of GSSG present. However, any unintentional conversion of endogenous GSH to GSSG, i.e., as a result of oxidation, would cause a larger error in estimating the GSSG present. Some procedures utilize *N*-ethylmaleimide (NEM) to prevent oxidation of GSH to GSSG.

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However, NEM is a potent inhibitor of glutathione reductase and, consequently, methods based on enzymatic reduction of GSSG are dependent on the complete removal of NEM from the incubation mixture. Some methods employ several successive ether extractions to remove NEM (8), while other methods have used column chromatography to remove it (20,21).

Since additional manipulations may lead to lower recovery of GSSG, we undertook studies to develop a reliable procedure that was not influenced by the presence of NEM. Cohn and Lyle (18) used *o*-phthalaldehyde (OPT) as a reagent for a fluorometric assay of GSH. Data are presented here to demonstrate a simple and reliable assay for measurement of GSSG and the method was extended for measurement of both GSH and GSSG on the same tissue preparation.

MATERIALS AND METHODS

Materials. Female Fischer rats were obtained from Charles River Breeding Laboratory, Wilmington, Mass. The animals were individually housed and fed food and water ad libitum.

Reduced and oxidized glutathione (GSH and GSSG) were obtained from Boehringer–Mannheim (New York, N.Y.); *N*-ethylmaleimide (NEM) was obtained from Sigma Chemical Company (St. Louis, Mo.); *o*-phthalaldehyde (Sigma Chemical Co.) was a gift from Dr. Bruce Love, Department of Biochemistry, University of Rochester Medical Center. A Perkin–Elmer fluorescence spectrophotometer (Model MPF-3) was used for all determinations of fluorescence intensity.

GSH and GSSG were prepared daily in 0.1 M sodium phosphate–0.005 M EDTA buffer (pH 8.0) and kept on ice until used. *o*-Phthalaldehyde (OPT) solution was prepared daily in reagent-grade absolute methanol just prior to use. All other reagents employed were reagent-grade quality.

Tissue preparation. Rats were killed by cervical dislocation; livers were removed, blotted, weighed, and used immediately or frozen in liquid nitrogen and stored at −80°C until assayed.

A portion of tissue, usually 250 mg, was homogenized on ice using a Polytron homogenizer. The solution used for homogenization consisted of 3.75 ml of the phosphate–EDTA buffer and 1 ml of 25% HPO_3, which was used as a protein precipitant. The total homogenate was centrifuged at 4°C at 100,000*g* for 30 min to obtain the supernatant for the assay of GSSG and GSH.

GSH assay. Determination of GSH was performed by a modification of the method of Cohn and Lyle (18). To 0.5 ml of the 100,000*g* supernatant, 4.5 ml of the phosphate–EDTA buffer, pH 8.0, was added. The final assay mixture (2.0 ml) contained 100 μl of the diluted tissue

supernatant, 1.8 ml of phosphate–EDTA buffer, and 100 μl of the OPT solution, containing 100 μg of OPT. After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette. Fluorescence at 420 nm was determined with the activation at 350 nm.

GSSG assay. A 0.5-ml portion of the original 100,000*g* supernatant was incubated at room temperature with 200 μl of 0.04 M NEM for 30 min to interact with GSH present in the tissue. To this mixture, 4.3 ml of 0.1 N NaOH was added. A 100-μl portion of this mixture was taken for measurement of GSSG, using the procedure outlined above for GSH assay, except that 0.1 N NaOH was employed as diluent rather than phosphate–EDTA buffer.

Recovery studies. The recovery of GSH was estimated in the following way. Equal amounts of tissue were homogenized in three different tubes. In the first tube, prior to homogenization, a known amount of GSH, usually 100 μg, was added. To the second tube, after homogenization, 100 μg of GSH was added. The third tube acted as the control and no additions of GSH were made prior to or after homogenization. The three homogenates were subsequently handled identically for GSH measurement. Estimation of GSSG recovery was performed in a similar manner.

In addition, the stability of GSH under these experimental conditions was also examined. Prior to tissue homogenization, 100 μg of GSH was added to the tissue–diluent mixture, followed by homogenization. After centrifugation, the supernatant was incubated with NEM and a portion was obtained for measurement of GSSG. These results were then compared with the data on levels of GSSG obtained in a similar tissue supernatant preparation to which no GSH had been added.

RESULTS

Measurement of GSH

GSH was shown to react specifically with OPT at pH 8.0, yielding a highly fluorescent product that could be activated at 350 nm with an emission peak at 420 nm (18). The reaction was dependent on the final pH, as the fluorescence intensity decreased below pH 8.0. These observations as originally reported by Cohn and Lyle (18) were confirmed in our laboratory. Therefore, dilution of the original supernatant with buffer was required to maintain the mixture at a final pH of about 8.0. On the other hand, an increase above pH 8.0 caused the conversion of GSH to GSSG. The fluorescence intensity for the OPT-GSH reaction was directly related to GSH concentration and was linear over the concentration range of 10 ng to 2 μg (Fig. 1).

Specificity of the reaction of OPT with GSH was investigated by

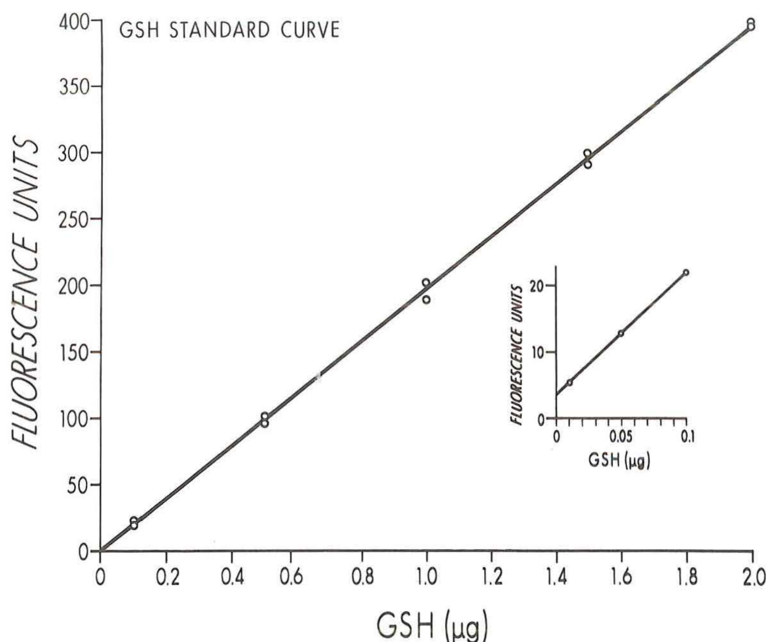

FIG. 1. GSH was dissolved in phosphate–EDTA buffer, pH 8.0. Known amounts of this solution were mixed with 100 μl of the OPT solution. The final volume of this mixture was adjusted to 2 ml with buffer. The solution was mixed, incubated at room temperature for exactly 15 min, and transferred to a quartz cuvette. Fluorescence intensity was measured at 420 nm (excitation was at 350 nm). Inset: Enlargement of data obtained from 0.01 to 0.1 μg of GSH.

examining the reaction of OPT with other amino acids. Data in Table 1 demonstrate that the fluorescence intensity of the compounds exposed to OPT under similar conditions (pH 8.0) was negligible compared to that for GSH. These data agree with those of Cohn and Lyle, who examined a wider spectrum of compounds (18).

Measurement of GSSG

Cohn and Lyle (18) reported that *o*-phthalaldehyde reacted with GSSG, yielding readily measureable fluorescent intensity at pH 12. We confirmed that finding by analyzing the OPT-GSSG reaction; as seen in Fig. 2, the fluorescence intensity was observed to be linear over the concentration range of 5 ng to 2 μg of GSSG (levels above 2 μg of GSSG were not investigated). Unlike the measurement of GSH, measurement of GSSG was not affected by small variations in pH (lower recoveries of GSSG were obtained when the pH was below 10). At the high pH of the medium (~pH 12), the conversion of GSH to GSSG was

TABLE 1

SPECIFICITY OF OPT FOR GSH AT pH 8[a]

Substance	Final concentration (μg)	Relative fluorescent intensity (%)
GSH	5	100
GSSG	5	0.1
Cystine	5	0.4
Cysteine	5	1.0
Leucine	5	0.6
Glutamic acid	5	0.3
Glycine	5	0.4

[a] Substances (100 μl of a solution containing 5 μg) were added to 1.8 ml of buffer and mixed. OPT (100 μl containing 100 μg) was then added to this mixture. The mixture was incubated for 15 min and assayed at pH 8.0 as indicated in methods section.

negligible. The absorbance and emission spectra for the OPT-GSSG reaction were similar to that for GSH, namely 350 and 420 nm, respectively. Thus, we obtained results in complete accordance with the data of Cohn and Lyle (18). Furthermore, their suggestion (18) that the OPT-GSSG reaction at pH 12 might be suitable for an assay of GSSG was borne out by our results.

The specificity of the OPT-GSSG reaction at pH 12 was investigated. The fluorescence intensity of other substances treated with OPT was negligible compared to that of GSSG (Table 2).

Recovery Studies

Estimation of recovery of added GSH and GSSG was ascertained by adding a known amount of these substances to the homogenization solution (tissue plus diluent) prior to homogenization. Three homogenate mixtures were prepared: tissue alone (no added GSH or GSSG), tissue plus 100 μg of GSH, and tissue plus 100 μg of GSSG. All were treated identically and assayed according to the procedure outlined in the methods section. Results from several representative experiments are presented in Tables 3 and 4, showing that the recovery of either GSH or GSSG was approximately 100%, ranging from 91 to 110%. Although most of the recovery studies were performed with 250 mg of tissue, similar results were obtained with use of 100 mg of tissue. A summary of recovery experiments, employing 50 to 1000 μg of GSH and/or GSSG, is given in Table 5. The data show that comparable recovery was obtained over this range of added standards. For convenience, we usually employed 100 μg of GSH or GSSG for routine assays. As seen in Table 5, recovery of 100 μg of GSH (46 samples)

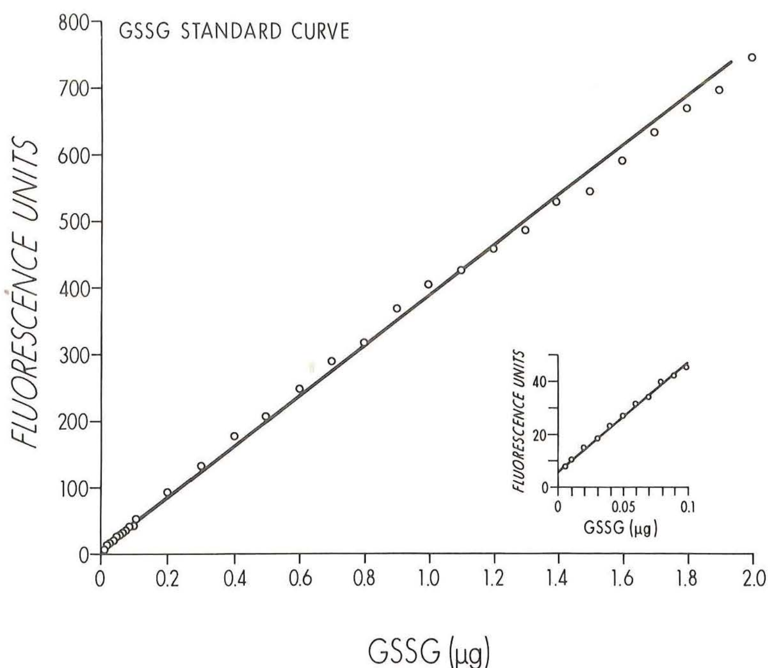

FIG. 2. GSSG was dissolved in 0.1 N NaOH. Known amounts of this solution were mixed with 100 μl of the OPT solution. The final volume of this mixture was adjusted to 2 ml with 0.1 N NaOH. The solution was mixed, incubated at room temperature for exactly 15 min, and transferred to a quartz cuvette. Fluorescence intensity was measured at 420 nm (excitation was at 350 nm). Inset: Enlargement of data obtained from 0.005 to 0.1 μg of GSSG.

and 100 μg of GSSG (35 samples) was ±10% (standard deviation); the standard error of the mean for these experiments represented ±2 to 3%.

The effect of homogenization on the levels of GSH was investigated by experiments in which 100 μg of GSH was added, in one case, before and, in the other case, after homogenization of the tissue. Table 6 presents data showing that the recovery of GSH was identical in both instances, indicating that the mechanical procedures used did not influence the amount of GSH estimated by the methods employed.

The instability of GSH, due to its oxidation to GSSG upon exposure to air, has been implied. As mentioned in the methods for assay of GSSG, the supernatant was incubated with NEM to prevent oxidation of GSH to GSSG. Cohn and Lyle (18) had demonstrated that incubation of GSH and NEM completely prevented fluorophore formation upon addition of OPT. Preliminary results indicated that a level of 10^{-4} M NEM, as reported by Cohn and Lyle (18), was insufficient to inhibit the GSH-OPT reaction completely when using 1000 μg of GSH;

TABLE 2

SPECIFICITY OF OPT FOR GSSG AT pH 12[a]

Substance	Final concentration	Relative fluorescent intensity (%)
GSSG	5	100
Glutamic acid	5	0.2
Cysteine	5	0.1
Cystine	5	0.7
Leucine	5	0.6
Glycine	5	0.3

[a] Substances (100 μl of a solution containing 5 μg) were added to 1.8 ml of 0.1 N NaOH and mixed. OPT (100 μl containing 100 μg) was then added to this mixture. The mixture was incubated for 15 min and assayed at pH 12 as indicated in methods section.

at 4×10^{-2} M NEM, complete inhibition of fluorescence was observed when 1000 μg of GSH was added to tissue preparations. Therefore, it was decided that 4×10^{-2} M NEM was adequate to react with the anticipated levels of GSH present in the tissues to be analyzed. We also examined NEM to determine if (a) any significant conversion of GSH to

TABLE 3

RECOVERY OF GSH

Tissue	Amount of tissue used (mg)	Amount of GSH added[a] (μg)	Amount of GSH measured (μg)	Percent recovery
Liver	250	—	405 ± 5	
	250	100	496 ± 4	91
Liver	250	—	455 ± 5	
	250	100	553 ± 3	98
Liver	250	—	392 ± 8	
	250	100	497 ± 2	105
Liver	250	—	452 ± 8	
	250	100	550 ± 10	97
Liver	100	—	195 ± 5	
	100	100	293 ± 3	98
Lactating	250	—	85 ± 5	
mammary gland	250	100	182 ± 1	97
Lactating	250	—	89 ± 1	
mammary gland	250	100	186 ± 2	97

[a] GSH was added prior to homogenization of tissue. Representative experiments indicating recoveries of 100 μg of GSH. Calculated levels of GSH presented as means ± SEM; each sample assayed in duplicate.

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TABLE 4

RECOVERY OF GSSG

Tissue	Amount of tissue used (mg)	Amount of GSSG added[a] (μg)	Amount of GSSG measured (μg)	Percent recovery
Liver	250	—	85 ± 1	
	250	100	184 ± 1	99
Liver	250	—	75 ± 1	
	250	100	180 ± 5	105
Liver	250	—	83 ± 3	
	250	100	188 ± 3	105
Liver	100	—	39 ± 2	
	100	100	134 ± 1	95
Liver	100	—	40 ± 1	
	100	100	141 ± 1	101
Lactating	250	—	29 ± 2	
mammary gland	250	100	137 ± 1	107
Lactating	250	—	24 ± 1	
mammary gland	250	100	134 ± 2	110

[a] GSSG was added prior to homogenization of tissue. Representative experiments indicating recoveries of 100 μg of GSSG. Calculated levels of GSSG presented as means ± SEM; each sample assayed in duplicate.

GSSG occurred prior to addition of NEM, and (b) the amount of NEM would react with all of the GSH present in the preparation prior to addition of NaOH. Experiments were performed such that 100 μg of GSH was added to one aliquot of the homogenate, 100 μg of GSSG was added to a second aliquot, and neither GSSG nor GSH was added to a third aliquot. The data in Table 7 show that the amount of GSH converted to GSSG under these experimental conditions was within experimental error (±10%) and that the amount of NEM added was effective in preventing any conversion of GSH to GSSG.

DISCUSSION

Data presented above indicate that a satisfactory method for measurement of both GSH and GSSG was developed, utilizing the fluorescent agent OPT. The reaction was shown to be specific for GSH and GSSG, since the fluorescence observed with a variety of other substances appeared to be minimal compared to that seen for GSH and GSSG. These results agree with the report of Cohn and Lyle (18). In addition, the sensitivity of this reaction allows one to use small samples and

TABLE 5

RECOVERY OF GSH AND GSSG[a]

Amount added (μg)	GSH recovered (%)				GSSG recovered (%)			
	Number of samples	Mean	SD	SEM	Number of samples	Mean	SD	SEM
50	2	100	10	6	2	102	2	1
100	46	91	11	2	35	102	10	2
200	3	96	13	8	9	100	15	5
250	—	—	—	—	7	93	9	3
500	3	101	16	9	—	—	—	—
1000	10	96	11	4	3	94	10	6

[a] Summary of experiments measuring recovery of various amounts of GSH and/or GSSG added to liver samples. Data presented as means with the standard deviation (SD) and standard error of the mean (SEM).

thereby minimize the presence of interfering substances in the tissue homogenate and incubation mixture. Poor recovery was obtained when the original tissue supernatant (100 μl) was used directly for the assay. This problem was resolved by incorporating a second dilution step prior to reaction with OPT, which we attribute to dilution of the HPO_3 present in the original homogenization mixture.

The recovery studies (Tables 3–5) indicate the reliability of this method for the measurement of both GSH and GSSG from the same tissue preparation. These studies also show that the homogenization treatment did not affect the levels of GSH, since the recovery of GSH added before or after homogenization was identical. The data shown in Table 7 indicate that GSH was stable under the experimental

TABLE 6

THE EFFECT OF THE HOMOGENIZATION PROCEDURE UPON THE RECOVERY OF GSH

Tissue	Amount of tissue used (mg)	Amount of GSH added (μg)	Amount of GSH measured (μg)
Liver	250	–	405
	250	100[a]	496.5
	250	100[b]	498
Liver	250	—	455
	250	100[a]	553
	250	100[b]	549

[a] 100 μg of GSH added before homogenization of tissue.
[b] 100 μg of GSH added after homogenization of tissue.

TABLE 7

EFFECT OF GSH ON THE MEASUREMENT OF GSSG

Tissue	Amount of GSSG added (μg)	Amount of GSH added (μg)	Amount of GSSG measured (μg)	Percent recovery
Liver (250 mg)[a]	—	—	100	
	100	—	190	90
Liver (250 mg)[b]	—	—	105	
	—	100	115	
	100	—	210	105

[a] NEM was added before tissue homogenization and centrifugation.
[b] NEM was added to the tissue supernatant.

conditions, presumably due to the acidity of the supernatant. Therefore, the observed GSSG values correspond to the amount present in the tissue and are not due to conversion of GSH to GSSG.

The GSH values reported here with this method agree with those reported by others (5,7,8,13,22,23); GSH levels in liver ranged from 1500 to 2100 μg/g of tissue. However, the levels for GSSG reported here

TABLE 8

REPORTED VALUES FOR REDUCED AND OXIDIZED GLUTATHIONE IN TISSUE

	GSH (μg/g)	GSSG (μg/g)	Reference
Rat kidney	875	0	(5)
	764	38	(8)
Perfused rat heart	1074	25	(20)
Freeze-clamped liver	1570	244	(24)
Rat liver	2180	0	(5)
	1695	0	(13)
	1930	78	(8)
	—	21	(21)
	1753 ± 53	288 ± 22	(a)
Harding–Passey melanoma	452	86	(19)
Mouse lactating mammary gland	326 ± 14	157 ± 15	(b)
Mouse mammary carcinoma	583 ± 28	151 ± 21	(b)
Human mammary gland	83 ± 6	32 ± 8	(b)
Human breast carcinoma	466 ± 78	135 ± 15	(b)

[a] Values reported here. Levels of GSH and GSSG are presented as means ± SEM for 15 samples, each sample having been assayed in duplicate.
[b] R. Hilf, R. Ickowicz, S. Abraham, and J. C. Bartley, in preparation; means ± SEM.

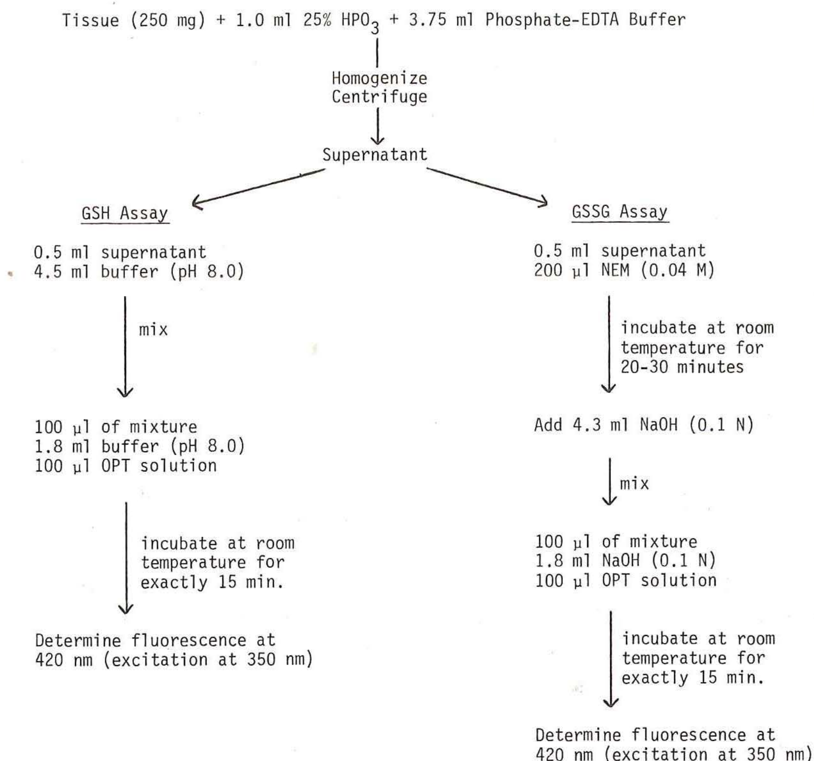

FIG. 3. Flow sheet.

are considerably higher than those found in earlier reports (see Table 8). One possible reason for this discrepancy may be due to the effect of NEM to inhibit the activity of glutathione reductase, when the enzyme was used for assay of GSSG. To avoid this problem, procedures were employed to remove excess NEM prior to addition of glutathione reductase (20,21). Güntherberg and Rost removed the excess NEM from intact erythrocytes by washing. They reported a GSSG concentration of 27 μg/ml of packed cells (25). Güntherberg and Rapaport (21) removed excess NEM from rat liver extracts by using columns of Sephadex G-10. The GSSG value reported by them, using the recycling assay of Owens and Belcher (10), was 21 μg/g of tissue. Wendell (20) used a cation-exchange resin to remove NEM and reported a GSSG concentration ~25 μg/g of rat heart. Tietz (8), using repeated ether extraction to remove NEM, reported GSSG values of 38 and 78 μg/g of tissue in rat kidney and liver, respectively. Most of the reported values for GSSG agreed reasonably well with one another. In contrast, the levels for GSSG reported here are considerably higher, ranging from 315 to 405 μg/g of liver. One explanation for the lower levels reported earlier may be the inability to effect complete removal of NEM from the

reaction mixture, since any inhibition of glutathione reductase by NEM would lead to low values of GSSG.

Hadley *et al.* (19), using glutathione reductase (without prior addition of NEM) and measuring disappearance of NADPH by fluorometry, reported GSH and GSSG values of 452 and 86 μg/g, respectively, in the Harding–Passey melanoma. Using the methods reported here, we have analyzed mammary glands and mammary tumors from BALB/c mice, as well as several samples of normal human breast tissue and infiltrating ductal carcinomas of the breast. A sampling of these data (to be published in full elsewhere) indicate a general agreement for GSH and GSSG levels in tumor tissues with the report of Hadley *et al.* (19).

In summary, the method presented here offers the advantage of measurement of both GSH and GSSG on the same tissue preparation with fewer steps and with specificity, reproducibility, and increased sensitivity, resulting in less tissue required for the assay. An outline of the suggested method is shown in Fig. 3; the amount of HPO_3 added should be appropriately increased or decreased to maintain the same proportion shown here for 250 mg.

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