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Note

Rapid separation of reduced and oxidized glutathione following its reaction with N-ethylmaleimide*

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While investigating the mechanism of action of thiol protein-disulfide oxidoreductase (EC 1.8.4.2) it was necessary to reliably determine small quantities of reduced glutathione (GSH) and glutathione disulfide (GSSG). N-Ethylmaleimide (NEM) was used to inhibit the conversion of GSH and GSSG[1] during isolation. NEM in the reaction mixture inhibits glutathione reductase, which is often used to measure GSSG concentrations and as a result can lead to erroneous estimates of GSSG content. Several methods including column chromatography[2–5] and extraction procedures[6] have been used to remove NEM prior to enzyme assay. Hissin and Hilf[7] have suggested that low values of GSSG obtained using glutathione reductase may be due to the inability to completely remove NEM from the reaction mixture. A number of chromatography procedures use either Dowex or Amberlite ion-exchange resins which do not completely resolve NEM, the product of the reaction ofGSH and NEM (GS-NEM), and GSSG[2–5].

We report a rapid one-step column chromatography procedure that separates unreacted NEM, GS-NEM, and GSSG. The procedure also affords a quantitative recovery of all three compounds.

MATERIALS AND METHODS

QAE-25 Sephadex was purchased from Sigma (St. Louis, Mo., U.S.A.) and N-ethylmaleimide from Aldrich (Milwaukee, Wisc., U.S.A.); all other reagents were of analytical grade. [2-^{3}H]Glycine GSH (0.25 mCi/mg) was purchased from New England Nuclear (Boston, Mass., U.S.A.) and purified by the procedure of Furano[8]. GSSG was formed by reacting GSH with homogeneous thiol:protein oxidoreductase.

QAE-25 Sephadex was swollen in 0.05 *M* potassium phosphate buffer (prepared from a mixture of the mono- and dibasic salts, $I = 0.077$), pH 6.2; then 8.5 ml of gel were packed into a column (11 × 1 cm) and equilibrated with 100 ml of the same buffer.

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RESULTS AND DISCUSSION

To inhibit the conversion of GSH to GSSG by oxidative processes during the chromatography, excess NEM was used. The [2-^{3}H]glycine GSH (80 nmoles) and labeled GSSG (20 nmoles) were dissolved in 0.7 ml of 0.1 *M* phosphate at pH 7.0 containing 300 nmoles of NEM and allowed to react for 15 min at room temperature. The reaction mixture was then placed on the QAE-25 Sephadex column and washed with one column-volume of 0.05 *M* phosphate buffer, pH 6.2, in order to elute the unreacted NEM. The column was then eluted with a linear gradient of phosphate buffer, pH 6.2 from 0.05 *M* to 0.5 *M* using a total volume of 100 ml. Fractions of 2 ml were collected and assayed for NEM, GS-NEM and GSSG. NEM was measured by monitoring the absorption at 305 nm[9] and GS-NEM and GSSG were determined by counting 0.5-ml aliquots of each fraction in a Beckman LS-100C liquid scintillation system. Fig. 1 shows the elution profile for the separation of the three reaction components. NEM is eluted from the column with 0.05 *M* phosphate buffer, while the GS-NEM and GSSG are eluted at 0.06 *M* and 0.18 *M* phosphate, respectively. The fact that all three reaction components are clearly resolved from each other allows for an accurate estimate of GSSG using glutathione reductase. In principle this method should also facilitate the assay of these components by other analytical methods.

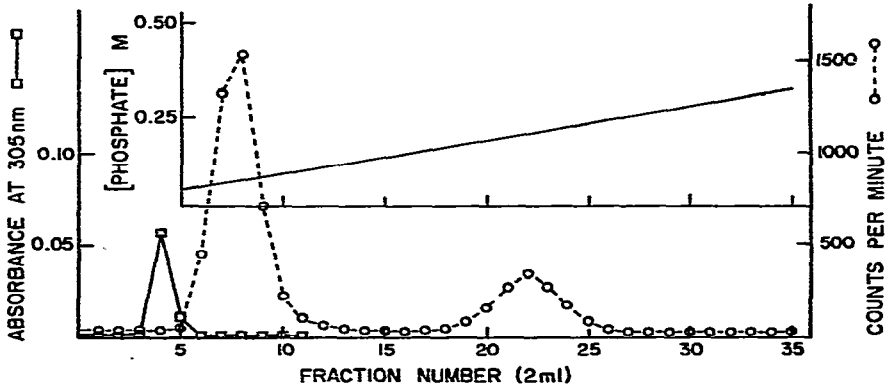

Fig. 1. Chromatography of NEM, GS-NEM and GSSG on a column of QAE-25 Sephadex (11 × 1 cm). □, Absorption at 305 nm, ○ counts/min of 0.5-ml aliquots of each fraction (2 ml). The compounds in their order of elution from the column are: NEM, GS-NEM and GSSG.

The quantitative recovery of the three components is shown in Table I. The fact that 96% of the theoretical amount of NEM, 97% of GSH as GS-NEM, and 110% GSSG are recovered indicates that there is no selective loss of any of these components under the conditions used for chromatography. The recovery of 110% of GSSG is caused by oxidation of GSH to GSSG during the N-ethylmaleimide incubation. This was demonstrated by using an equivalent amount of GSH and NEM as described above without the addition of GSSG. It should also be noted that there is a quantitative recovery of total radioactivity (*i.e.* the decrease of 550 cpm in GS-NEM appears as 520 cpm in GSSG).

TABLE I

RECOVERY DATA FOR THE COMPOUNDS STUDIED

	NEM (nmoles)	*GS-NEM (total cpm)*	*GSSG (total cpm)*
Theoretical	220	18,700	5,000
Measured value	212	18,150	5,520
Percentage found	96%	97%	110%

In conclusion, this note describes a rapid separation of NEM, GS-NEM and GSSG from one another and also affords a quantitative recovery of the three components. The avoidance of strongly acidic solutions and organic solvents which denature certain proteins[2–6] is also a distinct advantage of this procedure.

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