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A Colorimetric Determination of Total Glutathione based on Its C-Terminal Glycine Residue and Its Application to Blood, Liver and Yeast

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An assay procedure for total glutathione in blood, liver and yeast was developed, based on the color reaction of the glycine residue, not the sulfhydryl group. Glycine, which showed a color reaction similar to that of glutathione, was first eliminated by reaction with sodium nitrite under acidic conditions, then deaminated glutathione was reacted with benzoyl chloride in sodium hydroxide solution. The reaction product(s) was extracted with ethyl acetate containing 5% (v/v) ethanol, after acidification of the reaction mixture with phosphoric acid. An aliquot of the organic phase was evaporated to dryness under reduced pressure, and the dried residue was treated to develop the color by the addition of acetic anhydride, *p*-dimethylaminobenzaldehyde and pyridine. The absorbance was measured at 458 nm after reaction at 40° for 1 hr. Beer's law was obeyed in the range from 0.05 to 1.0 μmol of the reduced form or from 0.025 to 0.5 μmol of the oxidized form in the cuvette. This procedure is applicable to the determination of total glutathione in amino acid mixtures, whole blood, rat liver cytosol and yeast heat extract.

Keywords——glutathione; determination; colorimetry; C-terminal glycine; 458 nm

Introduction

Glutathione, in reduced (GSH) or oxidized (GSSG) form, is widely distributed in living cells, from microbes to higher organisms, and is known to have important biological functions.[1] It is present at about 1 mmol per liter of whole blood[2] and 7 μmol per gram of wet tissues.[3] Various analytical methods have been estabilshed for GSH, utilizing column chromatography,[4] colorimetry,[5] gas chromatography,[6] fluorometry,[7] spectrophotometry,[8] titration,[9] measurement of radioactivity,[2a] amperometry.[10] enzymatic reaction[11] and high performance liquid chromatography.[2b,3,12] Most of the methods cited above are based on the chemical or physical properties of the sulfhydryl group of GSH . Current methods for the determination are based on high performance liquid chromatography and enzymatic reaction,[11e] but colorimetric methods[5c,d] are still frequently used, because the former methods are often laborious and expensive.

Recently, we reported a simple and specific colorimetric determination of glycine.[13] In this method, glycine was first converted to hippuric acid through benzoylation. In the course of the study, we noticed that peptides having a C-terminal glycine residue show a color reaction similar to that of glycine, and we considered that the color reaction might be applicable to the determination of glutathione.

In the present paper, a unique colorimetric method using only two test tubes and familiar reagents for the assay of GSH and GSSG is described; it is applicable to amino acid mixtures, whole blood, liver homogenate and yeast heat extract.

Materials and Methods

Reagents and Instruments

GSH and GSSG were kindly supplied by Yamanouchi Pharmaceutical Co. Ltd. (Tokyo). All other reagents (analytical grade) were purchased from Wako Pure Chemical Industries Ltd. (Osaka). Specimens

of human blood were supplied by the Medical School of this University. A standard solution for the amino acid analyzer contained 17 usual amino acids (Lys, His, Arg, Asp, Glu, Thr, Ser, Gly, Ala, Val, Pro, Leu, Ileu, Met, Tyr, Phe, Cys) and ammonia at the concentration of 2.5 mM.

Visible absorption spectra were taken on a Hitachi 124 spectrophotometer. Evaporation of organic solvents under reduced pressure and benzoylation were carried out as described in our previous paper.[13]

Methods

a) **Determination of Glutathione in an Aqueous Solution**——Sample solutions containing GSH and/or GSSG were treated by the procedure described previously.[13] A sample solution (0.1 to 0.5 ml) was mixed with 1 N NaOH (1.0 ml) and benzoyl chloride (50 μl) in a 20-ml test tube. After 30 min of vigorous stirring at 40°, the mixture was acidified with 0.3 ml of 43% phosphoric acid, and extracted with 5 ml of benzene–*n*-hexane (1 : 1, v/v). The organic layer containing benzoic acid was removed thoroughly by aspiration and evaporation. The reaction product(s) in the aqueous layer was extracted into 7 ml of ethyl acetate containing 5% (v/v) ethanol. An aliquot (5 ml) of the ethyl acetate layer was transferred to another test tube and was evaporated to dryness at about 40° under reduced pressure.

Conditions of color development were as reported previously[13] except that 0.2% *p*-dimethylaminobenzaldehyde solution in pyridine was used in the present procedure instead of 0.4% solution. To the dried residue, 1.0 ml of acetic anhydride and 2.0 ml of the 0.2% solution were added in turn, and the solution was vigorously mixed with a Vortex mixer. After color development at 40° for 1 hr, the absorbance at 458 nm was measured against a blank solution containing acetic anhydride and the 0.2% solution.

b) **Determination of Glutathione in an Aqueous Solution containing Glycine and in Amino Acid Mixture**——As mentioned in our previous paper,[13] di- or tripeptides having a C-terminal glycine residue show a color reaction similar to that of glycine. In order to determine only glutathione in the presence of glycine, some procedure to eliminate glycine was required. A sample solution (1.0 ml) containing glutathione and glycine or glutathione and the amino acid mixture was added to 1 M $NaNO_2$ (0.25 ml) and glacial acetic acid (0.5 ml) in a 20-ml test tube, which was allowed to stand at 0° for 10 min. The test tube was heated at 100° for 10 min, then the solution was evaporated to dryness at about 40° under reduced pressure. Next, 2 N NaOH (1.0 ml) and benzoyl chloride (50 μl) were added to the dried residue, and the solution was treated in the manner described above, except that 0.5 ml of 43% phosphoric acid was used for the acidification of the reaction mixture.

c) **Determination of Glutathione in Whole Blood**——Heparinized blood (0.5 ml) was mixed with water (1.7 ml) and 30% metaphosphoric acid (0.8 ml) in a 10-ml test tube, and the mixture was heated at 100° for 1 min. After cooling, it was centrifuged at $1000 \times g$ for 5 min and the supernatant was filtered through a filter paper. An aliquot (1.5 ml) of the filtrate was transferred to a 20-ml test tube, and was added to 1 M $NaNO_2$ (0.25 ml) and glacial acetic acid (0.5 ml). The mixture was treated as described above, and a 5-ml aliquot of the extract was subjected to the color development.

d) **Determination of Glutathione in Liver Cytosol**——Rat liver was homogenized in 3 volumes of 1.15% KCl with a Potter-Elvejhem homogenizer with a Teflon pestle. The homogenate was centrifuged at $9000 \times g$ for 30 min, and the supernatant was further centrifuged at $105000 \times g$ for 60 min. The supernatant (cytosol, 0.5 ml) was treated in the manner described in procedure c).

e) **Determination of Glutathione in Yeast**——Fresh baker's yeast was suspended in an equal volume of water, and the suspension was heated at 100° for 10 min. After cooling to room temperature, it was centrifuged at $3800 \times g$ for 15 min. An aliquot (50 μl) of the supernatant was treated in the manner described in procedure c).

Results

Optimal Reaction Condition for Elimination of Glycine

a) **Effect of Reaction Time with $NaNO_2$**——An aqueous solution (1.0 ml) containing 0.28 μmol of GSH and 0.7 or 7 μmol of glycine was reacted with 1 M $NaNO_2$ (0.25 ml) at 0° for various times from 0 to 1.5 hr. After being heated at 100° for 10 min, the sample solution was treated in the manner described in procedure b). The results are shown in Fig. 1, and indicate that a reaction time at 0° of 10 min is sufficient for complete elimination of glycine. Heating at 100° for 10 min was essential for completion of the elimination reaction.

b) **Effect of the Amount of $NaNO_2$**——An aqueous solution (1.0 ml) containing 0.28 μmol each of GSH and glycine was reacted with various amounts of 1 M $NaNO_2$ (0.25 to 1.0 ml) at 0° for 10 min. After being heated at 100° for 10 min, the sample solution was treated in the manner described in procedure b). The results are shown in Fig. 2, which indicates that 0.25 ml of 1 M $NaNO_2$ is sufficient for complete elimination of glycine.

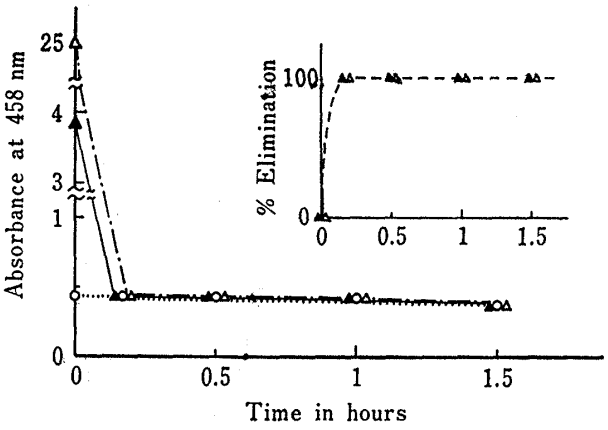

Fig. 1. Influence of the Reaction Time on the Elimination of Glycine

An aqueous solution (1.0 ml) of GSH (0.28 mM) (○···○), of GSH (0.28 mM) and glycine (0.7 mM) (▲——▲), or of GSH (0.28 mM) and glycine (7 mM) (△—·—·△) was reacted with 1 M $NaNO_2$ (0.25 ml) at 0° for 10 min, followed by heating at 100° for 10 min. Glutathione in each sample solution was determined as described in the text.

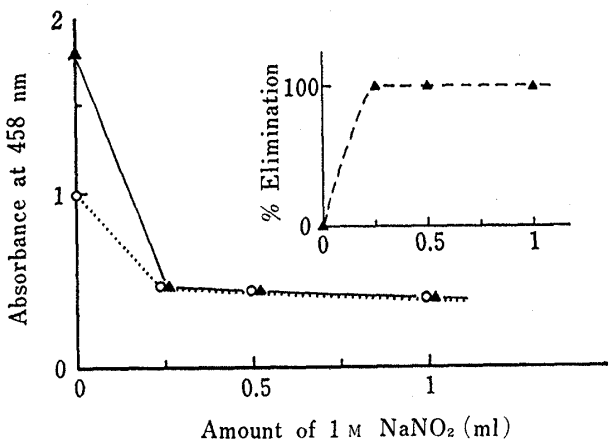

Fig. 2. Influence of the Amount of 1 M $NaNO_2$ on the Elimination of Glycine

An aqueous solution (1.0 ml) of GSH (0.28 mM) (○···○), or of GSH (0.28 mM) and glycine (0.28 mM) (▲——▲) was reacted with various amounts of 1 M $NaNO_2$ at 0° for 10 min, followed by heating at 100° for 10 min. Glutathione in each sample solution was determined as descrived in the text.

c) **Effect of the Amount of Glycine**——An aqueous solution (1.0 ml) containing glycine in the range from 0.2 to 200 μmol was reacted with 1 M $NaNO_2$ (0.25 ml) at 0° for 10 min. After being heated at 100° for 10 min, the sample solution was treated in the manner described in procedure b). Glycine in the range from 0.2 to 10 μmol was completely eliminated through this procedure, and 99.9% or 93.4% of glycine was eliminated in the case of 100 or 200 μmol of glycine, respectively.

Absorption Spectra

The absorption spectra of the colored solution obtained from authentic GSH with or without treatment with $NaNO_2$ are shown in Fig. 3. The shapes of both spectra are essentially identical, but the treatment with $NaNO_2$ resulted in a decrease in color intensity by 50% relative to that obtained without the treatment. The shape of the spectrum of GSSG was the same as that of GSH, and the absorbance of GSSG was twice as intense as that of GSH. The absorption maxima of glutathione were observed at 408, 432 and 458 nm, with a shoulder at 489 nm.

Standard Curve and Sensitivity

The standard curve for GSH treated with $NaNO_2$ is shown in Fig. 4. The curve was linear up to 1.0 μmol of GSH, and the coefficient of variation was calculated to be 2.2% for GSH. As little as 0.05 μmol of GSH could be detected by the present procedure. The standard curve for GSSG gave just twice the slope obtained for GSH, and was linear up to 0.5 μmol (data not shown). The molar absorption coefficient at 458 nm was calculated to be 6.46×10^3 $M^{-1} \cdot cm^{-1}$ for GSH.

Glutathione in an Aqueous Solution containing Glycine and in Amino Acid Mixture

Determination of glutathione was carried out in an aqueous solution containing GSH and glycine or GSH and the amino acid mixture. As summarized in Table I, values obtained by the present procedure were not affected by the presence of these additives.

Recovery Tests

With the aim of applying this procedure to biological samples, recovery tests were carried out. Various amounts of GSH were added to whole blood, liver cytosol and yeast heat extract, and the total amount of GSH was measured by each procedure described in "Materials

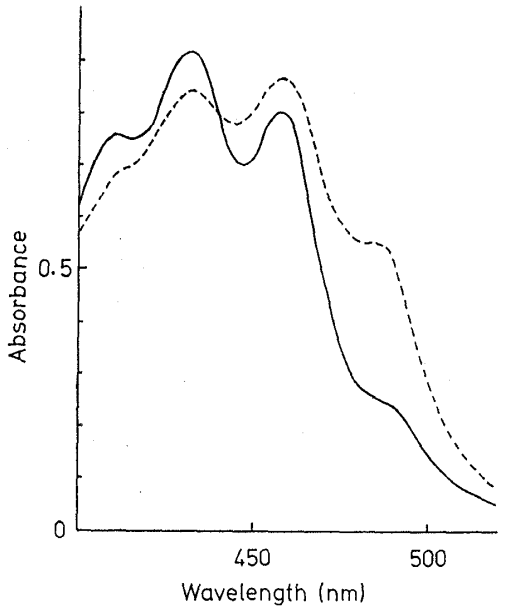

Fig. 3. Absorption Spectra of GSH with and without $NaNO_2$ Treatment

An aqueous solution of GSH (0.42 mM, 1.0 ml) was treated in the manner described in procedure b) (----), and another GSH solution (0.21 mM, 1.0 ml) was treated in the manner described in procedure a) (——).

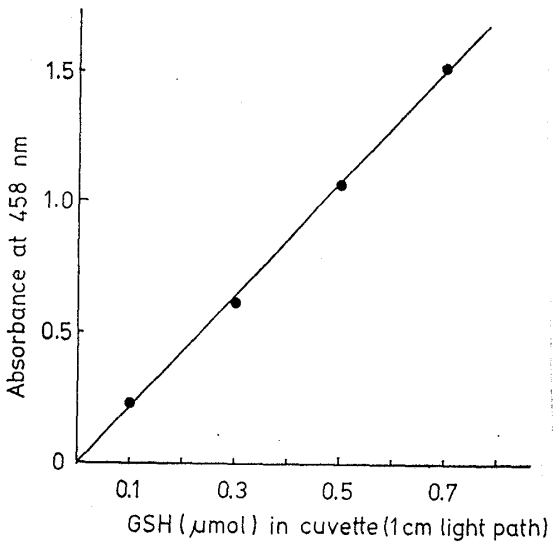

Fig. 4. Standard Curve for Glutathione after $NaNO_2$ Treatment

A 1.0 ml portion of aqueous GSH solution at various concentrations was measured by procedure b).

TABLE I. Assay of Glutathione in an Aqueous Solution containing Glycine and the Amino Acid Mixture

	GHS added (mM)	Glycine added (mM)	Amino acid mixture added (mM)	GSH determined (mM)
A	0.2	0.2	—	0.194
	0.2	0.5	—	0.196
	0.2	1.0	—	0.196
	0.2	5.0	—	0.199
B	0.2	—	0.625	0.201
	0.2	—	1.25	0.189

An aqueous solution of GSH (0.2 mM, 1.0 ml) containing various amounts of glycine (column A) or the amino acid mixture (column B) was reacted with 1 M $NaNO_2$ (0.25 ml) at 0° for 10 min, followed by heating at 100° for 10 min. Glutathione concentration was determined as described in the text.

and Methods." The results are summarized in Table II, which indicates that there was 99.2 ±5.4% recovery of GSH added to whole blood, 100.5±5.7% recovery of GSH added to liver cytosol, and 99.3±3.5% of recovery of GSH added to the yeast heat extract. In these experiments, the assay procedure was started within 5 min after the addition of GSH to the above samples to avoid the GSH thiol exchange reaction.[5d]

Comparison of Results obtained by the Present Method and by the 5,5'-Dithio-bis(2-Nitro Benzoic acid) (DTNB) and N-1-Naphthyl Ethylene Diamine (NED) Methods

In order to evaluate the analytical values obtained by the present method, total glutathione in biological samples was determined by the method of Ellman (the DTNB method)[5c] and by the method of Saville (the NED method),[5d] both of which are still widely employed, as well as by the present method. The results shown in Table III demonstrate that the values obtained by the present method are in good agreement with those obtained by the DTNB and NED methods.

Table II. Recovery Tests

Species	GSH added (mM)	GSH determined (mM)	Recovery %	Recovery Mean±SD
Blood	0	0.485		99.2±5.4
	0.8	1.32	104.4	
	1.6	1.96	91.9	
	2.4	2.86	98.8	
	3.2	3.74	101.6	
Liver	0	0.470		100.5±5.7
	0.8	1.24	96.1	
	1.6	2.16	105.8	
	2.4	2.76	95.2	
	3.2	3.83	105.0	
Yeast	0	6.02		99.3±3.5
	16	21.7	98.0	
	32	39.3	104.1	
	48	53.7	99.4	
	64	67.3	95.8	

Various amounts of GSH were added to the samples, and GSH was measured by the procedure described in the text.
All values are means from duplicate determinations.

Table III. Comparison of Results obtained by the Present Method and by the DTNB and NDE Methods

Biological sample	GSH determined (mM)		
	Present method	DTNB method	NED method
Whole blood	0.485	0.489	0.545
Liver cytosol	0.470	0.495	0.488
Yeast extract	6.02	6.18	6.06

Total glutatione in each sample was determined by the present, DTNB and NED methods.
All values are means from duplicate determinations.

Discussion

Almost all the techniques for the determination of glutathione so far reported are based on the reactivity of the thiol group. Biological samples, however, usually contain some nonprotein thiols other than glutathione, such as cysteine, homocysteine, lipoate, ergothioneine, coenzyme A and cysteinylglycine, but their contents in biological samples amount to less than one-tenth of that of glutathione. Exceptionally, human serum may contain as much as 0.61 μM GSH, 0.23 μM cysteinylglycine and 0.71 μM unidentified thiol compounds.[14] Hence, if glutathione in biological samples containing relatively large amounts of other thiol compounds is determined by a method based on the thiol group, glutathione content will be overestimated. On the other hand, the amounts of peptides having C-terminal glycine other than glutathione and cysteinylglycine are negligible compared to the amount of glutathione. Therefore, the present method is applicable to the determination of glutathione in biological samples. Furthermore, this method should be applicable as a quantitative or qualitative test for peptides having C-terminal glycine other than glutathione.

The reaction product of the color reaction of glycine was isolated and identified by us,[15] as will be reported elsewhere. The reaction products of glutathione, however, have not yet been elucidated.

In conclusion, the method presented here is suitable for the estimation of total glutathione, which exists mainly as GSH in mammalian tissues,[2b] plants[12b] and a microorganism,[1a] and may be useful in the field of clinical biochemistry.

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