A New Method for Determination of Hepatic Glutathione Reductase Activity. (28223)

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Hepatic glutathione reductase activity has been determined by following the disappearance of reduced TPN* or by measuring the appearance of GSH. For instance, Mize and Langdon(1) used the decrease in optical density at 340 mμ to measure TPNH utilization. An iodine titration method similar to the one described by Tunnicliffe(2) was used by Mann(3) and by Rall and Lehninger(4) to determine the formation of GSH. Racker (5) also determined GSH concentration, but used the colorimetric nitroprusside method developed by Grunert and Phillips(6). In this report, a new spectrophotometric method for determining GSH is described.

Brown(7) observed that nitrous acid increased the yellow color of 3-hydroxykynurenine and used this observation to develop a spectrophotometric method for the assay of kynurenine hydroxylase activity(7,8). During studies on the status of kynurenine hydroxylase activity in mitochondria of hepatoma(9), we observed that GSH reacted with sodium nitrite to yield an ultraviolet absorbing product. The product had an absorption maximum distinct from that of 3-hydroxykynurenine (367 mμ(7)), and no ultraviolet absorbing compound was formed with GSSG. Thus studies were undertaken to ascertain whether this reaction could be used to determine glutathione reductase activity.

The purpose of this report is (a) to describe the spectral characteristics of the product, (b) to describe conditions for its formation, and (c) to demonstrate its use as a method for determining glutathione reductase activity.

Materials and methods. Chemicals were purchased from the following sources: GSH, GSSG and DL-homocysteine thiolactone · HCl, California Corp. for Biochemical Research, Los Angeles; glucose-6-phosphate dehydrogenase, Sigma Chemical Co., St. Louis; TPN, Pabst Laboratories, Milwaukee; L-cysteine, Merck and Co., Inc., Rahway.

Scannings of the ultraviolet spectra were performed in a Beckman DB spectrophotometer with a Sargent Recorder Model SR attached. The 18,000 *g* supernatant fractions of rat liver homogenates were prepared using an International Refrigerated Centrifuge Model PR-2.

When glutathione reductase activity was determined, female Holtzman rats were sacrificed by cervical fracture, and the livers were homogenized in 0.25 M sucrose (9 ml/g of liver). Following centrifugation, 1 ml of the supernatant fraction was added to 2 ml of a reaction mixture and incubated at 37°C for 5 minutes. The reaction mixture was essentially identical to that of Mize and Langdon (1) except that (a) TPNH was replaced by TPN, 3.0 μmoles; glucose-6-phosphate, 10.0 μmoles; and glucose-6-phosphate dehydrogenase, 0.4 Kornberg unit; and (b) GSSG was increased from 1.63 μmoles to 5.0 μmoles. Reactions were stopped with 1 ml of 16% TCA. In all experiments, 2 methods were used to determine GSH concentration: (a) the nitroprusside-ammonium sulphate method described by Chinard and Hellerman(10), and (b) the nitrous acid procedure to be described.

Results. The ultraviolet absorption spectra of GSH, GSSG, and sodium nitrite are shown in Fig. 1-A. Among these, only sodium nitrite showed absorption at wave lengths above 320 mμ. When GSSG and GSH were reacted with sodium nitrite, curves as shown in Fig. 1-B were obtained. Below 320 mμ, the curve of the GSSG + $NaNO_2$ was virtually identical to that of GSSG alone, while above 320 mμ the curve of the combination more closely resembled the curve of $NaNO_2$. From this it would appear that no appreciable reaction occurred between sodium nitrite and GSSG. Reaction between GSH and $NaNO_2$ yielded a

* The following abbreviations are used: TPNH, TPN, reduced and oxidized triphosphopyridine nucleotide; GSH, GSSG, reduced and oxidized glutathione; TCA, trichloroacetic acid; EDTA, (ethylenedinitrilo) tetraacetic acid; G-6-P, glucose-6-phosphate.

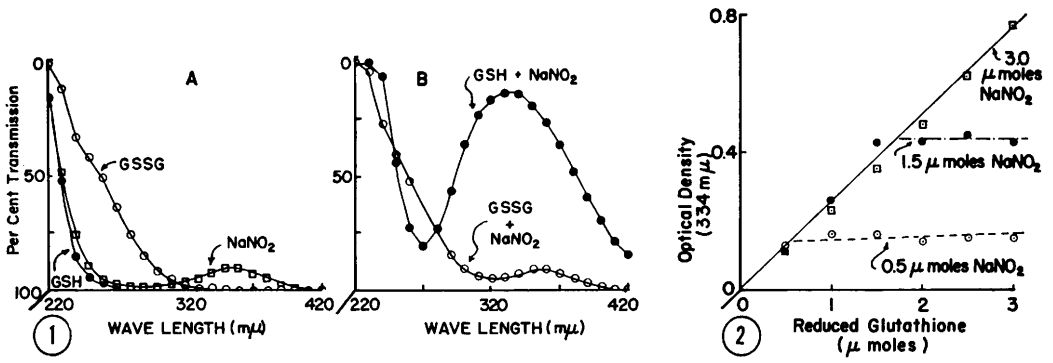

FIG. 1. Ultraviolet spectrum of reduced and oxidized glutathione before (A) and after (B) reaction with sodium nitrite. Conditions: pH 1.0; concentration, all 3 chemicals, 3.0 μmoles; instrument, Beckman Model DB Recording Spectrophotometer, 3.0 ml cuvette with 1 cm light path.

FIG. 2. Relationship between concentration of reduced glutathione and sodium nitrite for formation of ultraviolet absorbing product. Reaction: 15 min at 50°C, pH 1.0.

product having an absorption maximum of 334 mμ.

Reaction between GSH and $NaNO_2$ occurred at room temperature (*ca* 25°C). However, the time required to reach a constant optical density was usually in excess of 3 hours. At 100°C instability of either the product or the reactants was encountered leading to low and variable optical density values. At 50°C constant optical density values were observed following incubation for 15 minutes. Thus in all subsequent work, GSH and $NaNO_2$ reacted for 15 minutes at 50°C.

Above pH 3.0 formation of the ultraviolet absorbing product was inhibited severely or completely. At pH 3.0 or lower, product formation proceeded with equal efficiency.

Fig. 2 indicates the stoichiometry between GSH and $NaNO_2$ for formation of the ultraviolet absorbing product. In all cases the data showed a mole for mole stoichiometry.

When sodium nitrite reacted with GSH in the presence of other compounds containing sulfhydryl groups, data as shown in Table I were observed. It was apparent that in the formation of the ultraviolet absorbing product, nitrous acid did not react specifically with GSH, but reacted with a wide variety of compounds having free sulfhydryl groups.

A comparison of the glutathione reductase activity of rat liver, as determined by the nitroprusside method(10), and the activity as determined by the nitrous acid method is shown in Table II. GSH standard curves were prepared using both methods, and reductase activity was determined by comparison with these curves. In this experiment and in others(9), reductase activities determined by the nitrous acid method were virtually identical to those determined by the nitroprusside method. Thus it appeared that in the absence of other sulfhydryl compounds or with suitable controls, the nitrous acid method could be utilized for determining glutathione reductase activity.

Routine assay of glutathione reductase activity was accomplished as follows: To an aliquot of the reductase reaction mixture, sufficient 16% TCA was added to give a final TCA concentration of 4%; proteins were removed by centrifugation; an aliquot of the

TABLE I. Reaction of Nitrous Acid with Other Sulfhydryl Compounds to Yield Ultraviolet Absorbing Products.

Additions	Concentration, μmoles	Optical density at 334 mμ
None		.022
Reduced glutathione	2.0	.525
L-cysteine·HCl	1.0	.161
	2.0	.322
L-ergothionine·HCl	1.0	.186
	2.0	.463
DL-Homocysteine Thiolactone·HCl	1.0	.025
	2.0	.033
Ring opened	1.0	.225
	2.0	.445

Conditions: $NaNO_2$, 3.0 μmoles; pH 1.0; temperature 50°C., 15 min.

TABLE II. Comparison of Nitrous Acid Method and Nitroprusside Method for Determination of Rat Liver Glutathione Reductase Activity.

	Glutathione reductase activity μmoles GSH/mg protein				
	Incubation time (min)				
Analytical method	1	2	3	4	5
Nitrous acid	.25 ± .02*	.55 ± .03	.77 ± .03	.93 ± .03	.94 ± .05
Nitroprusside	.28 ± .01	.56 ± .03	.85 ± .04	.96 ± .04	1.05 ± .03

Conditions: GSSG, 5.0 μmoles; TPN, 3.0 μmoles; EDTA, 10 μmoles; G-6-P, 10 μmoles; G-6-P dehydrogenase, 0.4 Kornberg unit; liver supernatant, 1.0 ml; total volume, 3.0 ml; 38°C. Liver homogenized in 0.25 M sucrose, centrifuged at 18,000 *g*, 10 min.
* Mean and standard error.

supernatant containing less than 2.5 μmoles GSH was mixed with 1 ml of 1 N HCl and 0.25 ml of 0.01 M $NaNO_2$; the volume was adjusted to 3.0 ml with water; the mixture was heated 15 minutes at 50°C and the optical density determined at 334 mμ. The concentration was determined by comparison with a GSH standard curve.

Summary. A reaction between sodium nitrite and reduced glutathione (GSH) yielded a product having an ultraviolet absorption maximum at 334 mμ. No ultraviolet absorbing product was formed when oxidized glutathione was reacted with sodium nitrite. Optimal conditions and stoichiometry of the reaction were described. The reaction was not specific for GSH; ultraviolet absorbing products were formed with a variety of sulfhydryl compounds. The reaction was used to determine glutathione reductase activity of rat liver, and yielded data which compared favorably with that obtained by an accepted method.

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Density Gradient Electrophoresis Studies on Hemagglutination Plaque Formation and Tumor Induction of SE Polyoma Virus.* (28224)

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Hemagglutination (HA), plaque formation (PF) as well as tumor induction (TI) are employed for titration of SE polyoma virus. Because of the relative simplicity, speed and precision of hemagglutination and of tissue culture methods it is important to know whether these 2 parameters measure the same particles which induce tumors. So far it has not been demonstrated whether these 3 biologic attributes of polyoma virus are associated with one or more kinds of physical particles.

Hemagglutination, tumor induction and tissue culture activity were not dissociated by heat treatment, ether treatment and first plaque isolations with the use of limiting dilu-

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