Participation of the Unsymmetrical Disulfide of Coenzyme A and Glutathione in an Enzymatic Sulfhydryl-Disulfide Interchange

I. PARTIAL PURIFICATION AND PROPERTIES OF THE BOVINE KIDNEY ENZYME*

(Received for publication, March 14, 1966)

Simon H. Chang‡ and David R. Wilken§

With the technical assistance of Norma H. Best

From the Department of Biochemistry, Agricultural Experiment Station, Oklahoma State University, Stillwater, Oklahoma, 74074

SUMMARY

An enzyme that catalyzes the following sulfhydryl-disulfide interchange reaction has been purified 180-fold from bovine kidney.

\[
\text{GSH} + \text{CoASSG} \rightleftharpoons \text{GSSG} + \text{CoA-SH}
\]

In addition, it has been detected in most rat tissues studied. Its pH optimum is 8.2 and the equilibrium constant for the reaction is near unity at pH 6.9 and 25°C. Michaelis constants for reduced glutathione and CoASSG are \(3.3 \times 10^{-4}\) and \(4.5 \times 10^{-5}\), respectively. The molecular weight of the enzyme, determined by gel filtration, is approximately 12,000. Several unsymmetrical disulfides containing a glutathione residue are equally as effective as substrates. The enzyme, which becomes less active during storage, is largely reactivated by GSH. Its potential physiological significance is discussed.

EXPERIMENTAL PROCEDURE

All paper electrophoresis was conducted with a formic acid-acetic acid-water (3:12:85, v/v) mixture and acid-washed Whatman No. 3MM paper. Separations of reaction mixture components usually could be achieved within 45 min at 60 volts per cm.

CoASSG was synthesized from CoA and GSSG by a nonenzymatic sulfhydryl-disulfide interchange as described previously (2). Other unsymmetrical disulfides were synthesized by the same procedure, except that the appropriate sulfhydryl and disulfide compounds were substituted for CoA-SH and GSSG, respectively. Most unsymmetrical disulfides were separated from other products and residual reactants by preparative paper electrophoresis. Each unsymmetrical disulfide was located by spraying a portion of the paper with a solution of 10% ninhydrin in a mixture of ethanol-collidine-water (90:5:5). The remainder of the unsymmetrical disulfide was eluted with water. The unsymmetrical disulfide of cysteine and glutathione was separated from the other products and residual reactants by chromatography of the reaction mixture on a Dowex 1-acetate resin column (2 x 30 cm). A linear gradient (5), starting with 1 liter of water in the mixing flask and 1 liter of 2 M acetic acid in the reservoir, was used to elute the reaction products. The eluted products were detected by spraying an aliquot of each fraction on paper and spraying it with ninhydrin. Cysteine-glutathione was distinguished from other products eluted from the column by its mobility on electrophoresis. Fractions containing the cysteine-glutathione disulfide were pooled, lyophilized to remove excess acetic acid, and dissolved in water. The concentration of solutions of each of the unsymmetrical disulfides was estimated after performic acid oxidation (6) by a quantitative ninhydrin procedure (7). The average value for the color development given by two different performate-oxidized symmetrical disulfides
was used to calculate the concentration of the unsymmetrical disulfide. For example, the average color development obtained per mole of performate-treated homocystine and GSSG was used to estimate the concentration of the unsymmetrical disulfide of homocystine and glutathione. The structure of the unsymmetrical disulfide was established by performic acid oxidation and paper electrophoresis of the resulting sulfonic acids. When possible, these were detected by reaction with ninhydrin, and their mobilities were compared to the mobilities of known sulfonic acids prepared by performic acid oxidation of the appropriate sulfhydryl or disulfide compounds. Each of the unsymmetrical disulfides synthesized yielded the correct sulfonic acids and did not appear to be contaminated with other ninhydrin-reactive compounds.

In experiments measuring the $^{38}$S exchange between $^{38}$S-GSH and GSSG, the reaction products were separated by paper electrophoresis and located by spraying with ninhydrin. The purple spots each of formic acid and 30% hydrogen peroxide were added and GSSG, the reaction products were separated by paper electrophoresis of the resulting sulfonic acids. When possible, these were detected by reaction with ninhydrin, and their mobilities were compared to the mobilities of known sulfonic acids prepared by performic acid oxidation of the appropriate sulfhydryl or disulfide compounds. Each of the unsymmetrical disulfides synthesized yielded the correct sulfonic acids and did not appear to be contaminated with other ninhydrin-reactive compounds.

Assay of Transhydrogenase

Spectrophotometric Assay—In the spectrophotometric assay the reaction catalyzed by transhydrogenase was coupled to the reduction of GSSG by glutathione reductase and NADPH. The reaction was followed by measuring the decrease in absorbance of NADPH at 340 nm in a Beckman DB spectrophotometer. A molar absorptivity of $6.22 \times 10^3$ for NADPH was used (10). The standard reaction mixture contained 210 mM potassium phosphate (pH 7.6), 0.1 mM NADPH, 0.106% bovine serum albumin, 0.2 units of glutathione reductase, 0.5 mM CoASSG, 0.5 mM GSH, and transhydrogenase in a final volume of 1 ml. Either GSH or CoASSG was added last to initiate the reaction. Assays were conducted at 25°. One enzyme unit causes the formation of 1 μmole of GSSG per min under the standard spectrophotometric assay conditions. Specific activity is the number of units per mg of protein.

Because five variables, including the transhydrogenase-catalyzed reaction, contributed to the total rate of change of absorbance in the spectrophotometric assay, appropriate controls were required. The other variables were (a) the direct reduction of CoASSG by glutathione reductase, (b) the presence of approximately 1% GSSG in the GSH used, (c) a nonenzymatic oxidation of GSSG to GSH during reaction velocity measurements, and (d) the nonenzymatic sulfhydryl-disulfide interchange reaction between GSH and CoASSG. The relative contribution of the first variable to the total disappearance of NADPH was a function of the phosphate concentration (Fig. 1, A). It was mini-

For the purpose of simplicity, the term transhydrogenase will be used in this paper to indicate the enzyme catalyzing the GSH:CoASSG sulfhydryl-disulfide interchange reaction, which is similar to other transhydrogenases. Other transhydrogenases will be explicitly indicated.

The amount of glutathione reductase necessary for the standard spectrophotometric transhydrogenase assay was determined by conducting a glutathione reductase assay in 60 mM potassium phosphate (pH 7.6), 0.106% bovine serum albumin, 0.1 mM NADPH, 0.4 mM GSH, and glutathione reductase in a final volume of 1 ml. Disappearance of NADPH was measured at 340 μM. One unit causes the oxidation of 1 μmole of NADPH per min at 25°.

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mized without adversely affecting the transhydrogenase activity (Fig. 1, •) by the use of 0.21 M phosphate buffer.

Different control assays were required depending on whether GSH or CoASSG was added last to initiate the transhydrogenase reaction. When GSH was added last, the reaction rate was recorded for approximately 1 min prior to GSH addition. The difference between this rate and the rate obtained after GSH was added corrected for the first variable. This difference could be taken as the rate due to transhydrogenase. This assay did not correct for rate contributions from the second, third, or fourth variables. These were relatively small at a pH of 7.6 or below and at a relatively low GSH concentration (0.5 mM), as in the standard assay mixture. This assay was adequate for estimating enzyme during purification. More precise rate measurements were obtained in other experiments by correcting the rate obtained after addition of GSH to a complete reaction mixture by the rate obtained in a similar reaction mixture not containing transhydrogenase. When CoASSG was added last to initiate the reaction it was added approximately 1 min after GSH to allow time for the GSSG in the GSH (variable b) to be reduced. The rate obtained after addition of CoASSG, corrected by the rate obtained in a similar assay but done in the absence of transhydrogenase, was taken as the transhydrogenase-catalyzed reaction corrected for all the above variables. The transhydrogenase reaction rate was essentially the same whether GSH or CoASSG was used to initiate the reaction.

Fig. 2 shows that the reaction rate was linear with time for approximately 1 min at several enzyme concentrations. Fig. 3 shows that the reaction rate was linear with protein concentration within the range tested.

Electrophoretic Assay—In some experiments the transhydrogenase-catalyzed reaction was followed by estimation of the reaction products after their separation by electrophoresis. In these experiments the reaction was stopped by acidification with the acid electrophoresis buffer described above. Aliquots were subjected to electrophoresis on acid-washed Whatman No. 3MM paper at 60 volts per cm for 45 min. CoA and CoASSG were detected by their light absorption with a 253.7 nm ultraviolet light. These areas were cut from the paper, eluted with water, and adjusted to a final volume of 1 ml. The quantity of either compound was estimated from its absorbance at 260 nm. The composition of the reaction mixtures will be given with the individual experiments.

RESULTS

Transhydrogenase Distribution in Rat Tissue

Table I shows the specific activity of the transhydrogenase in extracts of several rat tissues. The highest specific activities were observed in brain and pancreas and the lowest detectable activity was in heart.

Purification of Transhydrogenase

Despite the fact that brain, pancreas, and liver had higher specific activities than kidney in the rat, the enzyme has been partially purified from bovine kidney. This is because it was

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Table I: Distribution of transhydrogenase in rat tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Pancreas</td>
<td>28.0</td>
</tr>
<tr>
<td>Brain</td>
<td>14.6</td>
</tr>
<tr>
<td>Liver</td>
<td>9.1</td>
</tr>
<tr>
<td>Kidney</td>
<td>5.4</td>
</tr>
<tr>
<td>Lung</td>
<td>5.2</td>
</tr>
<tr>
<td>Muscle</td>
<td>4.5</td>
</tr>
<tr>
<td>Heart</td>
<td>2.9</td>
</tr>
</tbody>
</table>
more readily available than the former two tissues at a local abattoir and because the difficulties encountered in early fractionation attempts with liver were not encountered with kidney. All fractionation procedures were conducted at 0–4°C unless otherwise specified.

Step 1: Crude Extract—Bovine kidney (400 g), trimmed of fat and connective tissue, was homogenized for 2 min with 2 volumes of cold water in a Waring Blender. The homogenate was filtered through cheesecloth and centrifuged at 14,000 × g for 30 min. The cloudy supernatant solution (crude extract) was used as the enzyme source. A 1-ml aliquot of the crude extract was clarified of cold water in a Waring Blendor. The homogenate was filtered approximately 200-ml portions) was heated in a 1-liter stainless steel beaker in an 80°C water bath until the temperature reached 58°C. The beaker was transferred to a 58°C water bath for 1 min and then to an ice-water slurry until the mixture reached 5°C. The heat-treated solutions were combined and centrifuged for 30 min at 14,000 × g.

Step 2: Heat Treatment at 58°C—The crude extract (approximately 200-ml portions) was heated in a 1-liter stainless steel beaker in an 80°C water bath until the temperature reached 58°C. The beaker was transferred to a 58°C water bath for 1 min and then to an ice-water slurry until the mixture reached 5°C. The heat-treated solutions were combined and centrifuged for 30 min at 14,000 × g.

Step 3: Ammonium Sulfate Fractionation and Concentration—The supernatant solution from the heat step was adjusted, with stirring, to 40% saturation with ammonium sulfate (226 g per liter) during a 20-min period and it was stirred for an additional 10 min. The mixture was centrifuged at 14,000 × g for 30 min. The enzyme in the supernatant solution was precipitated by adjusting the ammonium sulfate concentration to 100% saturation (418 g per liter) during 20 min, stirred for an additional 10 min, and centrifuged for 30 min at 14,000 × g. The supernatant solution was removed as completely as possible by decantation and the tubes were then allowed to drain well while in an inverted position. The precipitate was dissolved in water to a final volume of 200 ml.

Step 4: Acetone Fractionation—The solution containing transhydrogenase was brought to 58% acetone by adding 1.38 volumes of acetone at -15°C. During the addition of the acetone the temperature of the solution was gradually decreased from 0°C to -10°C to -15°C by adding Dry Ice to the acetone cooling bath in which the stainless steel beaker containing the enzyme was placed. The mixture was stirred continuously by a magnetic stirring bar during the addition of acetone and for an additional 10 min. It was then centrifuged at -10°C for 1 min at 7,000 × g. The supernatant solution was adjusted to 90% acetone at -15°C to -20°C by the addition of 2.2 volumes of cold acetone (-15°C), stirred for an additional 10 min, and centrifuged as above. The precipitate was dissolved in water to a final volume of about 40 ml. The yellow solution was dialyzed for 5 to 12 hours against three changes of water (1 liter each) in a dialysis bag which had been soaked previously in 1 mM EDTA. The solution containing transhydrogenase was concentrated to approximately 10 ml (not to dryness) by lyophilization, and the thawed preparation was centrifuged if insoluble material was visible.

Step 5: DEAE-cellulose Chromatography—The solution which contained enzyme was placed on a DEAE-cellulose column (50 x 1.8 cm) equilibrated with potassium phosphate buffer, pH 7.6. The enzyme was eluted with a 5 to 200 mM potassium phosphate linear gradient, pH 7.6 (5), starting with 500 ml of each solution. Fractions of 5 ml each were collected. The enzyme appeared in the first protein peak while most of the protein was retained on the column. Fig. 4 shows the early part of the protein elution pattern. Protein was detected by measuring the absorbance of each fraction at 280 nm and the enzyme activity was detected by the spectrophotometric assay. Fractions containing the enzyme were pooled and divided into several tubes for storage at -12°C. Table II summarizes the results of the purification of the transhydrogenase.

### Table II

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activitya</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Recovery</th>
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<tbody>
<tr>
<td>1. Crude extractb</td>
<td>670</td>
<td>24,000</td>
<td>2.8</td>
<td>1.0</td>
<td>100</td>
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<tr>
<td>2. Heat at 58°C</td>
<td>360</td>
<td>14,300</td>
<td>2.5</td>
<td>0.9</td>
<td>54</td>
</tr>
<tr>
<td>3. 40 to 100% (NH₄)₂SO₄</td>
<td>365</td>
<td>8,860</td>
<td>4.1</td>
<td>1.5</td>
<td>64</td>
</tr>
<tr>
<td>4. Dialyzed to 90% acetone</td>
<td>68</td>
<td>48</td>
<td>143</td>
<td>51</td>
<td>10</td>
</tr>
<tr>
<td>5. DEAE-cellulose chromatography</td>
<td>30</td>
<td>5.7</td>
<td>513</td>
<td>184</td>
<td>4.5</td>
</tr>
</tbody>
</table>

*Based on spectrophotometric assay.

b Crude extract from 400 g of kidney.

-10°C to -15°C by adding Dry Ice to the acetone cooling bath in which the stainless steel beaker containing the enzyme was placed. The mixture was stirred continuously by a magnetic stirring bar during the addition of acetone and for an additional 10 min. It was then centrifuged at -10°C for 1 min at 7,000 × g. The supernatant solution was adjusted to 90% acetone at -15°C to -20°C by the addition of 2.2 volumes of cold acetone (-15°C), stirred for an additional 10 min, and centrifuged as above. The precipitate was dissolved in water to a final volume of about 40 ml. The yellow solution was dialyzed for 5 to 12 hours against three changes of water (1 liter each) in a dialysis bag which had been soaked previously in 1 mM EDTA. The solution containing transhydrogenase was concentrated to approximately 10 ml (not to dryness) by lyophilization, and the thawed preparation was centrifuged if insoluble material was visible.

Step 5: DEAE-cellulose Chromatography—The solution which contained enzyme was placed on a DEAE-cellulose column (50 x 1.8 cm) equilibrated with potassium phosphate buffer, pH 7.6. The enzyme was eluted with a 5 to 200 mM potassium phosphate linear gradient, pH 7.6 (5), starting with 500 ml of each solution. Fractions of 5 ml each were collected. The enzyme appeared in the first protein peak while most of the protein was retained on the column. Fig. 4 shows the early part of the protein elution pattern. Protein was detected by measuring the absorbance of each fraction at 280 nm and the enzyme activity was detected by the spectrophotometric assay. Fractions containing the enzyme were pooled and divided into several tubes for storage at -12°C. Table II summarizes the results of the purification of the transhydrogenase.

**Reaction Products**

To determine the nature of the transhydrogenase reaction products, a mixture containing CoASSG, 0.5 μmole; GSH, 2.5 μmole; potassium phosphate, 8 μmole; and 0.1 unit of transhydrogenase (by the spectrophotometric assay) was prepared in a final volume of 0.16 ml with a final pH of 6.7. A similar reaction mixture was prepared except that no transhydrogenase was added. After a 5-min reaction period, a 15-μl aliquot from each mixture was added to separate 20-μl portions of the acid electrophoresis buffer to stop the reaction. The reaction products and remaining reactants were separated by paper electrophoresis. Each compound was detected either by its ultraviolet absorption or by its ninhydrin reactivity. The mobility of each product was compared to that of known compounds. A reproduction of

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**Fig. 4. Fractionation of transhydrogenase on DEAE-cellulose.**

The dialyzed and concentrated protein which precipitated between 58 and 90% acetone was chromatographed on DEAE-cellulose. Protein (●) was eluted by a linear gradient between 5 and 200 mM potassium phosphate buffer, pH 7.6. Fractions of 5 ml were collected. Protein was detected by measuring the absorbance of each fraction at 280 nm, and transhydrogenase activity (○) was detected by assaying each fraction spectrophotometrically as described in "Experimental Procedure." Only the early portion of the protein elution pattern is shown. The transhydrogenase usually was eluted between 20 and 30 mM phosphate.

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Coenzyme A from Coenzyme A-Glutathione Disulfide
Electrophoretic separation of the products of the transhydrogenase-catalyzed reaction. GSH and CoASSG were incubated as described in the text either in the presence (Complete System) or absence (Nonenzymatic Control) of DEAE cellulose-purified transhydrogenase. Reaction products in aliquots of the reaction mixtures as well as known GSH, GSSG, CoA-SH, and CoASSG were separated by electrophoresis and detected as described in "Experimental Procedure." The reproduction of the electrophoretogram shows that GSSG and CoA-SH were the only new components detected after incubation of GSH, CoASSG, and transhydrogenase.

The electrophoresis paper is shown in Fig. 5. The nonenzymatic control shows only the unchanged substrates. The complete reaction mixture shows the residual substrates plus two reaction products having the mobilities of oxidized glutathione and coenzyme A.

**Stoichiometry and Equilibrium Constant**

Fig. 6 shows the results of an electrophoretic assay in which 0.5 μmole of CoASSG, 2.5 μmoles of GSH, 8 μmoles of potassium phosphate (pH 7.6), and 0.02 unit of transhydrogenase (assayed spectrophotometrically) were incubated in a volume of 0.16 ml and a final pH of 6.9 for various periods of time. Aliquots (15 μl) were acidified with 15 μl of the acid electrophoresis buffer and the reaction products were separated by electrophoresis. The results show that as CoASSG disappeared an equal amount of CoA appeared. No nonenzymatic reaction occurred in a control experiment. It was previously reported (Reference 2, Table II) that the complete utilization of CoASSG in a spectrophotometric assay was accompanied by the formation of an equal amount of GSSG. These results suggest that CoASSG and GSH react in equimolar amounts to form equimolar amounts of products, CoA and GSSG. At the longest time period shown in Fig. 6 the reaction had nearly reached equilibrium. From these data an approximation of the equilibrium constant was made after correcting the observed absorbance measurements for absorbance eluted from equal areas of the electrophoresis paper not containing CoA or CoASSG. The calculated value was 0.8.

**Reversibility**

The reversibility of the transhydrogenase-catalyzed reaction has been shown by an electrophoretic assay in which 0.7 μmole of CoA, 1.0 μmole of GSSG, 8 μmoles of potassium phosphate buffer (pH 7.6), and 0.035 unit of transhydrogenase (assayed spectrophotometrically) were incubated in a final volume of 0.16 ml and a final pH of 6.7. The reaction products in acidified aliquots were separated by electrophoresis. Fig. 7 shows that no nonenzymatic reaction occurred at this pH, but in the presence of enzyme CoA disappeared with a concomitant appearance of CoASSG.

**pH Optimum**

Incubations for the determination of the pH optimum were conducted in a depression of a spot plate which was partially immersed in a 25° water bath. In three separate spots were placed 2 μl of transhydrogenase, 6 μl of 50 mM GSH, and 12 μl of a solution which was 5 mM in CoASSG and 0.33 M in Tris-Cl buffer of different pH in different experiments. The GSH, CoASSG, and buffer were mixed with a stirring rod for 5 sec and then the enzyme was added. After a suitable time period, 20 μl of acid electrophoresis buffer were added to stop the reaction. The final pH of each reaction mixture was measured in a separate but identical reaction mixture which had not been acidified. The

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**Fig. 5**. Electrophoretic separation of the products of the transhydrogenase-catalyzed reaction. GSH and CoASSG were incubated as described in the text either in the presence (Complete System) or absence (Nonenzymatic Control) of DEAE cellulose-purified transhydrogenase. Reaction products in aliquots of the reaction mixtures as well as known GSH, GSSG, CoA-SH, and CoASSG were separated by electrophoresis and detected as described in "Experimental Procedure." The reproduction of the electrophoretogram shows that GSSG and CoA-SH were the only new components detected after incubation of GSH, CoASSG, and transhydrogenase.

**Fig. 6**. Direct assay for the formation of CoA-SH from CoASSG and GSH in the presence of DEAE-cellulose-purified transhydrogenase. The formation of CoA-SH (○) and concomitant disappearance of CoASSG (●) as a result of the action of transhydrogenase were determined by the electrophoretic assay described in "Experimental Procedure." The CoASSG concentration in a nonenzymatic control experiment is also shown (◇).
Fig. 7. Reversibility of the transhydrogenase-catalyzed reaction. The formation of CoASSG (○) from GSSG and CoA—SH in the presence of DEAE-cellulose-purified transhydrogenase and the concomitant disappearance of CoA—SH (●) were determined by the electrophoretic assay described in “Experimental Procedure.” The CoA—SH concentration in a nonenzymatic control experiment is also shown (●).

products of the reaction of a 30-μl aliquot of the acidified reaction mixtures were separated by paper electrophoresis. Fig. 8A shows the nonenzymatic and the enzymatic plus nonenzymatic disappearance of CoASSG as a function of time at various pH values. Fig. 8B shows the total reaction rate corrected for the nonenzymatic rate as a function of pH. The pH curve for the nonenzymatic reaction is also shown. At a pH value of 7.8 or below, the nonenzymatic reaction is slow at best; however, above this pH it increases rapidly. The transhydrogenase exhibits considerable activity at pH values at which little or no nonenzymatic reaction occurs and has an optimum pH near 8.2.

Specificity

Table III shows the results of experiments in which the rate of GSSG formation from GSH and several symmetrical or unsymmetrical disulfides was measured in the presence of transhydrogenase. The standard spectrophotometric assay conditions were used except that CoASSG was replaced by 0.1 μmole of the disulfide being tested. The initial reaction rate obtained with each disulfide was compared with the initial rate obtained with CoASSG. Three unsymmetrical disulfides, each of which contained a glutathione residue, were found to be as active as CoASSG. The initial reaction rate obtained with each disulfide was compared with the initial rate obtained with CoASSG. Three unsymmetrical disulfides, each of which contained a glutathione residue, were found to be as active as CoASSG. The initial reaction rate obtained with each disulfide was compared with the initial rate obtained with CoASSG. Two of these, composed of a glutathione residue and either thioethanolamine or pantetheine residues, are components of the CoASSG molecule. The third unsymmetrical disulfide, that composed of cysteine and glutathione, differed from the thioethanolamine-glutathione analogue only in that it contained an additional carboxyl group. In contrast to these results, the homologue of cysteine-glutathione disulfide, homocysteine-glutathione disulfide, was a relatively poor substrate for the enzyme. These data suggest that much of the CoA molecule is not required for substrate binding but that the distance between the sulfur and nitrogen atom in the thioethanolamine residue may be critical.

The relatively high activity observed with cystine as substrate was preceded by a short but detectable lag period, suggesting that the activity observed with cystine may have been due to the formation of cysteine-glutathione disulfide during the lag period (either enzymatically or nonenzymatically) followed by the enzymatic cleavage of the latter transhydrogenase substrate to form GSSG. A similar explanation for the activity observed with pantetheine or homocysteine as substrate is possible. However, under the conditions used the reaction rates with these substrates were so low as to make the detection of an initial lag period questionable. Little or no activity was observed with RNase, insulin, or lipoic acid.

Fig. 9 shows the results of an experiment in which the percent-
age of residual enzyme activity obtained with four substrates, CoASSG, pantetheine-glutathione, thioethanolamine-glutathione, and pantethine, was tested as a function of time of heat denaturation of the enzyme at 80°. The residual activity with each of the four substrates was essentially the same at each time period. Similar enzyme inactivation rates also were obtained with CoASSG and cysteine-glutathione in an experiment not shown. This suggests that the activity observed with these substrates is due to a single enzyme. The rate of enzyme inactivation obtained with homocysteine (not shown) yielded a curve which did not coincide with that obtained with CoASSG. However, the reaction rates observed with homocysteine were very low and thus were subject to greater error. The results with homocysteine are suggestive rather than conclusive that the activity observed with homocysteine is due to a contaminating enzyme.

**Catalysis of GSH-GSSG Exchange Reaction**

Fig. 10 shows the results of an experiment which indicated that the transhydrogenase catalyzes a GSH-GSSG exchange reaction. The time course of the enzymatic and nonenzymatic disappearance of 35S-labeled GSH and appearance of 35S-labeled GSSG is shown when 35S-labeled GSH and unlabeled GSSG were incubated in the presence or absence of transhydrogenase. The time course of the exchange reaction with boiled transhydrogenase (not shown in Fig. 10) was essentially the same as that observed in the absence of transhydrogenase. Although the nonenzymatic exchange reaction was relatively rapid, the initial rate of exchange was increased approximately 3.6-fold by 0.01 unit of transhydrogenase (measured spectrophotometrically).

**Table III**

Transhydrogenase disulfide specificity

DEAE-cellulose-purified transhydrogenase was assayed by the spectrophotometric method described in "Experimental Procedure" except that the indicated disulfide was tested at a final concentration of 0.1 mM in place of CoASSG. The initial reaction rates observed are reported as the percentage of the initial rate when CoASSG was the substrate. The initial rates of change in absorbance at 340 nm with CoASSG as substrate in Experiments 1, 2, and 3 were 0.157, 0.143, and 0.087 per min, respectively.

<table>
<thead>
<tr>
<th>Disulfide tested</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coenzyme A-glutathione</td>
<td>100</td>
</tr>
<tr>
<td>Pantetheine-glutathione</td>
<td>115</td>
</tr>
<tr>
<td>Pantethine</td>
<td>113</td>
</tr>
<tr>
<td>Thioethanolamine-glutathione</td>
<td>105</td>
</tr>
<tr>
<td>Cystine</td>
<td>64</td>
</tr>
<tr>
<td>Coenzyme A-cysteine</td>
<td>27</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>11</td>
</tr>
</tbody>
</table>

**Fig. 9.** Percentage of original transhydrogenase activity with different disulfide substrates remaining after partial heat inactivation. DEAE-cellulose-purified transhydrogenase was heated at 80° for the indicated time periods. CoASSG (○), pantetheine-glutathione (●), thioethanolamine-glutathione (▲), or pantethine (▲) was assayed spectrophotometrically as described in Table III and in "Experimental Procedure." The initial activity of each substrate in the presence of unheated transhydrogenase was taken as 100%.

**Fig. 10.** Time course of the GSH-GSSG exchange reaction catalyzed by transhydrogenase. Sodium phosphate (10 μmoles), pH 7.6, 1.5 μmoles of unlabeled GSSG, 1.5 μmoles of 35S-labeled GSH (0.021 μC per μmole), and 0.01 unit of DEAE-cellulose-purified transhydrogenase (when added) were incubated at 25° in a volume of 0.1 ml. In the nonenzymatic control experiment no transhydrogenase was added. The final pH of the reaction mixture was 6.8. Aliquots of 10 μl were removed at time intervals and the reaction was stopped by adding 20 μl of acid electrophoresis buffer. The reactants, in 20 μl of the acidified reaction mixture, were separated by electrophoresis and counted as described in "Experimental Procedure." A, enzymatic (○) and nonenzymatic (●) incorporation of 35S into GSSG; B, enzymatic (●) and nonenzymatic (●) disappearance of 35S-labeled GSH.
TABLE IV

Activation of transhydrogenase by GSH

In Experiment 1 an aliquot of DEAE-cellulose-purified transhydrogenase was assayed by the standard spectrophotometric assay described in "Experimental Procedure." In Experiment 2 the transhydrogenase was pretreated with 3.7 mM GSH in 0.2 M potassium phosphate, pH 7.6, at 25° for 5 min. This mixture was then diluted to 1 ml with the usual spectrophotometric assay reagents to yield a complete reaction mixture as described in "Experimental Procedure." In Experiments 3 and 4 the transhydrogenase was pretreated with 3.7 mM GSH in 0.2 M potassium phosphate, pH 7.6, at 25° for 5 min. This mixture was then conducted on the pretreated enzyme. Initial reaction rates are shown as the initial change in absorbance at 340 nm per min due to NADPH oxidation.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Initial rate</th>
<th>ΔA340/min</th>
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</thead>
<tbody>
<tr>
<td>1. Standard assay</td>
<td></td>
<td>0.048</td>
</tr>
<tr>
<td>2. Enzyme pretreated with GSH</td>
<td></td>
<td>0.180</td>
</tr>
<tr>
<td>3. Enzyme pretreated with water</td>
<td></td>
<td>0.045</td>
</tr>
<tr>
<td>4. Enzyme pretreated with CoASSG</td>
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<td>0.040</td>
</tr>
</tbody>
</table>

Enzyme Activation and Stability

Different enzyme preparations have been variable with regard to stability. It was observed that preincubation of some enzyme preparations with GSH restored part of the activity which had been lost during storage. Maximum reactivation was achieved when the enzyme was incubated in 0.2 M potassium phosphate, pH 7.6, and 3.7 mM GSH at 25° for 5 min prior to assay. The final assay conditions were the same as in the standard spectrophotometric assay. Table IV shows the results of experiments which show that the activation is due to GSH rather than to some other factor during the preliminary incubation period. Experiment 1 (Table IV) shows the rate of NADPH oxidation with untreated enzyme. Experiment 2 (Table IV) shows a 3.7-fold increase in activity when the enzyme was first incubated with GSH. Experiment 3 (Table IV) shows that no activation occurs during the preliminary incubation period in the absence of GSH. Experiment 1 (Table IV) indicates that incubation with 0.74 mM CoASSG prior to assay had no activating effect on the enzyme. The possibility that the observed activation was due to a conversion of GSH to GSSG during the enzyme incubation period was discarded by the manner in which the assays were conducted. CoASSG was always added last to initiate the reaction after any preformed GSSG had been converted to GSH. Little or no formation of GSSG from GSH during the preliminary incubation was observed in any of the experiments shown.

Activation by GSH was first observed in a 17-day-old preparation of enzyme. At this time the enzyme preparation retained 41% of its original activity. Activation by GSH yielded a preparation which had 98% of the activity of the freshly prepared enzyme. These data suggested that the DEAE-cellulose-purified enzyme was probably a mixture of active and inactive enzyme forms, and that a conversion of the active enzyme to an inactive form occurred at some stage during the isolation procedure and continued during storage after purification. Two enzyme purifications have been conducted to establish at which stage of the isolation procedure the property of activation by GSH might be acquired. In neither experiment was an appreciable activation by GSH observed (less than 8%) at any stage of purification. Thus, either the property of activation by GSH is not associated with any particular purification step used or it occurs in some enzyme preparations but not others for reasons as yet unknown. Two enzyme preparations which had been stored for some time have been re-examined for loss of activity on storage and for reactivation by GSH. One was found to be fully active after storage at -12° for 6 weeks but lost 70% of its activity on storage for 7 months. GSH activation of the enzyme stored for 7 months restored the activity to 89% of the original activity. Another enzyme preparation lost 70% of its activity on storage for 6 weeks but could only be reactivated by GSH to 50% of its original activity. Thus, the loss of enzyme activity on storage has been variable from one enzyme preparation to another and at least two types of inactivation of the enzyme occur, only one is reversed by GSH.

Michaelis Constants

The apparent maximum velocities of the transhydrogenase-catalyzed reaction were determined from extrapolations of Lineweaver-Burk (11) double reciprocal plots of data from experiments in which varying concentrations of CoASSG and several fixed concentrations of GSH were employed. Similarly, apparent maximum velocities were obtained from experiments in which varying concentrations of GSH and several fixed concentrations of CoASSG were used.
of CoASSG were used. Fig. 11 shows a Lineweaver-Burk plot of the reciprocal of the apparent maximum velocities determined from the above experiments plotted against the reciprocal of the GSH or CoASSG concentration. This technique was used because of technical difficulties with the spectrophotometric assay when a saturating concentration of GSH was used and because of the limited availability of CoASSG. The Michaelis constants calculated from these data were 3.5 x 10^{-4} M for GSH and 6.1 x 10^{-4} M for CoASSG. The enzyme preparation used to obtain these data could be and was reactivated 3-fold by incubation with GSH prior to performing the assays. In other experiments an enzyme preparation which could be reactivated less than 1.1-fold by GSH was used to determine Michaelis constants. The $K_m$ values obtained were 3.3 x 10^{-4} M for GSH and 4.5 x 10^{-4} M for CoASSG. These experiments show that reactivation of the inactive form of the transhydrogenase by GSH yields a form of active enzyme with Michaelis constants for either substrate which have not been changed appreciably.

Molecular Weight

The molecular weight of the enzyme was estimated with the use of the molecular sieve technique described by Whitaker (12). Sephadex G-100 was allowed to swell in 0.1 M potassium phosphate, pH 7.6. A 0.1 M potassium phosphate solution (1 ml), pH 7.6, which contained 5 mg each of catalase (mol wt 251,000 (13)), bovine serum albumin (mol wt 70,000 (14)), egg albumin (mol wt 45,000 (15)), and cytochrome $c$ (mol wt 13,000 (16)) in addition to 1.8 units of transhydrogenase was placed on the column and eluted with 0.1 M potassium phosphate, pH 7.6. Catalase was used to determine the void volume of the column, and the other proteins of known molecular weight were used to construct a standard curve relating the ratio of the elution volume to the void volume and the logarithm of the molecular weight. The elution pattern of the proteins and the standard curve are shown in Fig. 12, A and B, respectively. The molecular weight of the transhydrogenase calculated from the standard curve was 12,300. The close correspondence of the elution pattern for transhydrogenase and cytochrome $c$ prompted testing cytochrome $c$ and reduced cytochrome $c$ (reduced by ascorbic acid) for transhydrogenase activity. Neither cytochrome $c$ nor reduced cytochrome $c$, equal in concentration to that of transhydrogenase (as judged by 280 nm absorbance), could replace transhydrogenase in forming GSSG in the spectrophotometric assay.

Discussion

Several enzymes which apparently catalyze sulfhydryl-disulfide interchange reactions have been described in the literature. Racker (17, 18) detected glutathione: homocysteine transhydrogenase in liver and yeast and partially purified the enzyme from bovine liver. That this enzyme and the transhydrogenase which reduces CoASSG are different enzymes has not been demonstrated conclusively. They probably are different enzymes, however, since GSH: homocysteine transhydrogenase has been shown to be unstable to fractionation with ammonium sulfate, in contrast to the transhydrogenase which reduces CoASSG, and the rate of heat denaturation of the two enzymes appears to be different. The relatively low activity with homocysteine or homocysteine-glutathione disulfide compared to CoASSG may be due to contamination of the DEAE-cellulose-purified transhydrogenase from kidney by GSH: homocysteine transhydrogenase. Katzen, Tietze, and Stetten (19) have demonstrated that GSH: insulin transhydrogenase catalyzes the reduction of all three disulfide bonds in insulin. Under assay conditions similar to those used by Katzen and Stetten (20), little or no reduction of insulin was observed with the transhydrogenase which reduces CoASSG (Table III). Thus two different enzymes are indicated. A microsomal enzyme has been shown to catalyze a sulfhydryl-disulfide interchange reaction in the reactivation of a "denatured" form of RNase (21, 22). The transhydrogenase that reduces CoASSG has been tested at several concentrations for its ability to substitute for or enhance the activity of the solubilized microsomal enzyme (prepared according to Goldberger, Epstein, and Anfinsen (23)) which renatures reduced RNase. In neither type of experiment did the transhydrogenase cause a significant increase in RNase reactivation over that obtained in appropriate control experiments. Therefore, it appears that transhydrogenase and the microsomal enzyme are different enzymes.

* Unpublished experiments of S. Chang, N. Best, and D. R. Wilken.
The transhydrogenase described in this paper is potentially important in one or more of the following physiological functions. The simplest function to envision is the salvage of the coenzyme form of the vitamin pantothenic acid which might be "lost" to the cell as CoASSG. CoASSG would not be expected to replace CoA—SH in reactions requiring the sulfhydryl form of the coenzyme. For example, CoASSG is inactive in the phosphotransacetylase-catalyzed reaction (2, 24). The CoASSG might be formed in a cell either by a presumably nonenzymatic oxidation of CoA—SH and GSH or by an interchange reaction between GSSG and CoA—SH. In either case, the formation of CoASSG would represent a loss to the cellular economy in terms of the loss of a vitamin and the energy expended in the synthesis of the coenzyme form of the vitamin. The transhydrogenase may have an even broader role in the maintenance of the free CoA—SH cellular level. The disulfide specificity studies indicated that pantetheine-glutathione and cysteine-glutathione disulfides, both of which are analogues of intermediates in CoA biosynthesis, are substrates for the transhydrogenase. If glutathione unsymmetrical disulfides of these normal CoA precursors cannot act as substrates in CoA synthesis, the transhydrogenase may function to salvage the CoA precursors from their unsymmetrical glutathione disulfide forms. The transhydrogenase may also function as part of a mechanism involved in controlling the level of the functional sulfhydryl form of pantetheine derivatives, including CoA—SH and acyl carrier protein. That the transhydrogenase may exist in an inactive as well as an active form adds to the attractiveness of this possibility. Experiments to test these possible functions of the enzyme are currently being conducted.

Only limited information is available at present concerning the mechanism of the transhydrogenase-catalyzed reaction. However, that the enzyme is sensitive to CMB1 and catalyses a GSSG exchange reaction is consistent with the formation of an enzyme-bound glutathione intermediate.

Pihl, Eldjarn, and Bremer (25) have presented evidence that partially purified glutathione reductase from liver does not catalyze a direct reduction of several unsymmetrical disulfides containing a glutathione residue, including the unsymmetrical disulfide of thioetherolamine and glutathione. While these results support the transhydrogenase mechanism, a further examination of the disulfide substrate specificity of the transhydrogenase (Table III) also obtained information on the disulfide specificity of yeast glutathione reductase (not shown in Table III). The results obtained confirm those of Pihl et al. (25) with thioetherolamine-glutathione disulfide and extend them to a wider variety of unsymmetrical disulfides containing a glutathione moiety. Of the eight disulfides listed in Experiments 1 and 2 of Table III, only CoASSG was reduced by glutathione reductase and NADPH at an initial rate greater than 1% of the rate observed with GSSG as substrate.6 The rate observed with CoASSG was 4 to 7% of the rate obtained with GSSG. Since the assays were conducted under conditions (high phosphate concentration) in which the rate of reduction of CoASSG by the yeast glutathione reductase preparation was purposely reduced, CoASSG may be a relatively good substrate compared to GSSG under other assay conditions. Whether the reduction of CoASSG by the commercial preparation of yeast glutathione reductase is due to a non-specificity of the reductase or to a contaminating reductase specific for CoASSG remains to be established. In either case the relatively high activity observed with CoASSG compared to the low activity exhibited by several of its analogues suggests a high degree of substrate specificity.

Acknowledgments—The authors would like to thank Dr. K. E. Edner and Dr. F. R. Leach for their helpful and stimulating discussions of this research.

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