Structural Studies of Bovine Liver Rhodanese

I. ISOLATION AND CHARACTERIZATION OF TWO ACTIVE FORMS OF THE ENZYME*

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KENNETH M. BLUMENTHAL† AND ROBERT L. HEINRIKSON‡

From the Department of Biochemistry, University of Chicago, Chicago, Illinois 60637

SUMMARY

Crystalline bovine liver rhodanese, prepared by ammonium sulfate and pH precipitation, has been shown to be comprised of two fully active components present in approximately equal amounts which are separable by polyacrylamide gel electrophoresis and by ion exchange chromatography. The two rhodanese forms, designated A and B on the basis of their order of elution from columns of DEAE-Sephadex, are not in equilibrium nor do they represent free enzyme and an enzyme-substrate complex. Furthermore, the two rhodanese species are identical with respect to kinetic parameters, amino acid composition, NH₂-terminal amino acid, sulfhydryl content, tryptic peptide maps, and molecular weight. Both forms exhibit equal activity toward β-mercaptopyruvate and utilize this sulfur donor at an efficiency of about 1% that of thiosulfate. Although no chemical or physical basis for the difference between the two rhodanese forms has been found as yet, a new, milder method for the preparation of the enzyme yields a preponderance of rhodanese A (85 to 90%). This, considered together with the elution characteristics of rhodanese species A and B, suggests that rhodanese B may arise during the course of the purification by deamidation of the A form.

Rhodanese (thiosulfate: cyanide sulfur transferase, EC 2.8.1.1) is the name given by Lang (1) to the enzyme which catalyzes the formation of thiosulfate from cyanide and thiosulfate. Sorbo was the first to crystallize (2) and partially characterize (3) bovine liver rhodanese, and subsequent studies by a number of investigators have provided information with regard to the mechanism and the chemical nature of the enzyme. The molecular weight of rhodanese is 37,000 (2). Volini, DeToma, and Westley (4) demonstrated that the enzyme exists in monomer-dimer equilibrium and that the monomeric subunits are identical having a molecular weight of about 18,000. There are 2 cysteine residues per monomer, one of which has been implicated in the catalytic function of the enzyme (5-7).

Alkylation of the native enzyme at pH 8.0 with iodoacetate yields totally inactive monooxarboxymethylsulfenyl rhodanese (5, 8). DeToma and Westley (8) have isolated a number of tryptic peptides from this derivative, including that containing the essential cysteine residue.

An investigation of the homogeneity of crystalline rhodanese was undertaken in this laboratory as a first step in the sequence analysis of the enzyme. It was discovered that the crystalline enzyme prepared by repeated ammonium sulfate and pH fractionation procedures (9) may be resolved by ion exchange chromatography into two fully active, homogeneous components. The present communication describes the isolation and characterization of these two forms of bovine liver rhodanese, together with a new method for the purification of the enzyme which yields essentially one rhodanese component. A preliminary account of part of this work has been published (10).

EXPERIMENTAL PROCEDURE

Materials—Crystalline rhodanese was prepared from bovine liver by a modification of the procedure of Horowitz and DeToma (9). The protein, precipitated at pH 4.5 and in 1.8 M ammonium sulfate (Step 3, 20 to 30% pure), was dissolved in 5 mM Tris-sulfate, pH 7.5, containing 10 mM Na₂SO₃ (Buffer A), and ammonium sulfate was removed by dialysis against the same buffer. A large amount of contaminating protein precipitated during the dialysis and was removed by centrifugation. The pH of the rhodanese solution was adjusted to 7.5 and the solution was dialyzed further. This solution was then added to a slurry of DEAE-Sephadex A-50 prepared as described in a later section. The ratio of exchanger to protein was 20 to 1 by weight. After stirring for 5 min, the resin was allowed to settle, and the supernatant was decanted and discarded. Rhodanese was preferentially eluted from the exchanger by washing with several volumes of 5 mM Tris-sulfate, pH 7.5, containing 50 mM Na₂SO₃ (Buffer A), each volume being equal to two-thirds that of the settled resin bed. The enzyme obtained in this way was 60 to 80% pure as judged by specific enzyme activity. These preparations could be brought to 100% purity by repeated crystallization from 2.5 M ammonium sulfate, pH 7.9, as described earlier (9). Bovine liver (5 pounds) routinely yielded 200 mg of crystalline enzyme.

Iodoacetic acid-1³C (13.4 mCi per mmole) was purchased from New England Nuclear and was diluted to a specific radioactivity of 1.94 mCi per mmole with unlabeled iodoacetic acid obtained from The Matheson Company, Inc., East Rutherford,

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New Jersey. Trypsin (bovine, type XI, dicyclohexylcarbodi-
imide-treated, once crystallized, lyophilized, lot #78B-S170) and
dimethylaminanaphthalene-5-sulfonyle chloride were obtained
from Sigma. Polyamide layers for thin layer chromatography
were purchased from the Gallard-Schlessinger Chemical Manu-
facturing Corporation, Carle Place, New York. Crystalline
denized urea was prepared according to the method of Benesch,
Lardy, and Benesch (11). Ammonium S-mercaptoptovaruate
was synthesized according to the procedures of Kun (12). Ellman's
reagent (5,5'-dithiothreitol/2-nitrobenzonic acid) was obtained
from Calbiochem. Sephadex G-25, G-75, and DEAE-Sephadex
A-50 were purchased from Pharmacia. Hemoglobin, chymo-
trypsinogen, and ribonuclease A standards for molecular weight
determinations were obtained from Worthington. All other
reagents were of the purest commercially available grade.

Enzyme Assay—Rhodanese activity was measured colori-
metrically by the rate of formation of thiocyanate from cyanide
and thiosulfate (2, 13).

Protein Determinations—Protein concentrations were deter-
mined by a modification of the biuret method (14). Effluent
fractions from column chromatographic procedures were mon-
tored spectrophotometrically for protein content by the ab-
 sorbance of the solutions at 280 nm.

Amino Acid Analyses—Amino acid analyses were performed
by automated ion exchange chromatography according to the
general procedures of Spackman, Stein, and Moore (15). Pro-
tein samples were hydrolyzed in 6 N HCl for 22, 48, and 72 hours
at 110°. Special precautions recommended for the preparation
and hydrolysis of samples were observed (16, 17). Analyses
were performed on a BioCal RC-200 amino acid analyzer (Bio-
Cal Instrument Corporation, Richmond, California).

Ion Exchange Chromatography—DEAE-Sephadex A-50 (10 g)
was washed 6 times with 350-ml portions of 0.5 M Na2SO4 and
was finally equilibrated in Buffer A by washing with six 400-
ml portions of the solution. Because of the low ionic strength
of the buffer employed, it was most critical that the equilibration
be complete. Columns of this material were routinely washed
with 4 to 6 volumes of Buffer A. If any further swelling oc-
curred, as evidenced by diminution of the flow rate, the resin
was removed from the column and washed with an additional
liter of buffer. When the elution was used in the batch
purification step described above, the supernatant buffer was
decanted, and the protein solution was added directly to the
settled material.

Polyacrylamide Gel Electrophoresis—Purified preparations
of rhodanese were subjected to electrophoresis in polyacrylamide
gels, pH 8.9, as described by Davis (18). Electrophoresis was

Radioactivity Determination—The extent of alkylation of rhodanese by ¹⁴C-iodoacetic acid was determined by counting
2- to 50-µl samples of the carboxymethylated protein dried on
aluminum planchets in a Nuclear-Chicago gas flow counter
(model 18IA) with an efficiency of about 35%. The radio-
activity of peptides separated by high voltage paper electrophoresis was measured in a Packard Tri-Carb scintillation
counter (model 574). Ninhydrin-positive spots from electropho-
retograms were cut out, immersed in 15 ml of scintillation
fluid (0.5% 2,5-diphenyloxazole and 0.025% p-bis-(2-hydroxy-
azoxy)benzene in toluene), and counted at 4°. The efficiency
of this procedure is about 18% for compounds containing ¹⁴C.

Preparation of ¹⁴C-Monocarboxymethylcysteinyl Rhodanese—
Approximately 50 mg (2.8 µmoles of monomer) of crystalline
rhodanese were dissolved in 10 ml of desalted 0.1 M glycine,
PH 9.0. After dialysis to remove ammonium sulfate, enzyme-
bound sulfur was discharged by the addition of 30 µl of desalted
0.125 M KCN. Immediately thereafter, 1.0 ml of a solution
containing 29 µmoles of ¹⁴C-iodoacetic acid in 10 mM NaOH
was added, and the reaction was allowed to proceed at 4° until
the residual activity had fallen to less than 5% of the zero
value (about 45 min). Excess reagent was destroyed by the
addition of 50 µl of 2-mercaptoethanol, and the reaction by-
products were removed by exhaustive dialysis against 0.1 mM
N-ethylmorpholine acetate, pH 8.0. The specific radioactivity
of the protein thus prepared was 1.0 × 10⁶ cpm per µmole,
indicating an incorporation of one carboxymethyl group per
mole of rhodanese monomer.

Reduction and Carboxymethylation—¹⁴C-Monocarboxymethyl-
cysteinyl rhodanese was reduced and carboxymethylated ac-
cording to the method of Crestfield, Moore, and Stein (16).
Fifteen min after the addition of iodoacetate, 100 µl of 2-mer-
captoethanol were added and the solution was dialyzed ex-
haustively against 0.1 mM N-ethylmorpholine acetate, pH 8.0.
The dicarboxymethylated protein invariably precipitated during
the dialysis.

Hydrolysis of Reduced, Carboxymethylated, ¹⁴C-Carboxymethyl-
cysteinyl Rhodanese by Trypsin—The dialyzed protein sus-
pension was placed in a vessel maintained at 40° in a pH-stat
(Radiometer Type TTTT). Trypsin was dissolved in 1 mM HCl
at a concentration of 0.5 mg per ml, and 100-µl aliquots of this
solution were added to the preparation of carboxymethylated
protein. The amount of trypsin added to 50 mg of protein
never exceeded 0.2 mg. The pH of the reaction mixture was
maintained at 8.0 by titration with 0.1 M NaOH. Hydrolysis
was judged to be complete when addition of more trypsin failed
to result in uptake of base and when the insoluble protein had
been solubilized. This generally took about 2 hours, but the
reaction mixtures were always allowed to stand overnight at
room temperature.

Peptide Mapping—Peptide maps were obtained by high
voltage paper electrophoresis of the trypptic digest from approx-
imately 4 mg of reduced carboxymethylated protein. Sam-
ple were spotted on sheets of Whatman No. 3 MM chromatog-
raphy paper (57 cm in length), and electrophoresis was carried
out in pyridine acetate buffer, pH 6.5 (pyridine:acetate acid:
water, 25:1:225), for 45 min at 2050 volts (52 volts per cm).
After complete air drying, descending chromatography was run
overnight in pyridine:isomyl alcohol:water, 35:55:30. The positions of the peptides were detected by immersing the papers
in a solution of ninhydrin-CdCl₂ reagent (21). After drying
for 15 min at room temperature, the papers were placed in a forced air oven at 70° for 10 min in order to complete the color development.

Peptides containing tryptophan were detected by immersing the dried, ninhydrin-stained maps in a solution of Ehrlich's reagent (22) and allowing the papers to dry in air.

RESULTS

One of the final steps in the procedure for purification of rhodanese devised by Horowitz and DeToma (9) involves precipitation of the enzyme at pH 4.5 and in 1.8 M ammonium sulfate (Step 3) followed by selective dissolution of contaminating proteins by rapid adjustment of the pH to 4.9. Because of losses of rhodanese in the pH adjustment, this stage of the purification was replaced by a step involving absorption to and batchwise elution from DEAE-Sephadex as described under "Experimental Procedure." It was found that the purity of Step 3 rhodanese was increased from about 20% to nearly 80% by this procedure. Repeated crystallization (Step 6 (9)) was employed to bring the enzyme to 100% purity as judged by the criterion of specific enzyme activity.

In order to further ascertain the purity of the rhodanese preparation prior to the undertaking of chemical studies, a sample containing 100 μg of enzyme was subjected to polyacrylamide gel electrophoresis at pH 8.9. Two major protein bands of about equal staining intensity and with similar rates of migration were clearly resolved. No other bands were observed.

Isolation of Rhodanase A and Rhodanase B—An attempt was made to isolate the two major components on a preparative scale utilizing column chromatography on DEAE-Sephadex. A column (2 x 23 cm) of the exchanger prepared as described above (cf. "Experimental Procedure") was washed with Buffer A until both the pH and the absorbance at 280 nm of the eluent and effluent were identical. A suspension containing about 60 mg of crystalline rhodanese in 2.5 M ammonium sulfate, pH 7.9, was centrifuged, and the pellet was dissolved in 6 ml of Buffer A. This material was judged to be 100% pure on the basis of activity and contained two electrophoretically distinct protein components. The solution was freed of ammonium sulfate either by gel filtration on a column (5 x 75 cm) of Sephadex G-25 equilibrated with Buffer A, or by dialysis against the same buffer. Fractions containing protein were pooled, and the solution was added directly to the DEAE-Sephadex column. Protein was eluted from the column with a linear gradient of Na₂SO₄. The mixing chamber contained 500 ml of Buffer A, and the reservoir contained 500 ml of 5 mM Tris-sulfate buffer, pH 7.5, containing 20 mM Na₂SO₄. Fractions of 10 ml were collected at a flow rate of 20 ml per hour. As may be seen in Fig. 1, two protein peaks were clearly resolved, both of which were coincident with rhodanese activity. The specific enzyme activity of the two components was the same and indicated a purity of 95 to 100%. The faster moving component was designated rhodanese A and the slower rhodanese B. When purified rhodaneses A and B were reconcentrated separately on DEAE-Sephadex as described above, each was eluted as a single, homogeneous peak at the expected position in the gradient. Better resolution of the two forms may be achieved by eliminating the gradient and by developing the column with Buffer A alone.

Samples of rhodanese A, rhodanese B, and the solution subjected to DEAE-Sephadex column chromatography were subjected to polyacrylamide gel electrophoresis. Rhodanese A migrated to a position coincident with the slower moving component of the mixture, and the mobility of rhodanese B corresponded to that of the faster moving component. These relative electrophoretic mobilities are as would be predicted on the basis of the chromatographic behavior of the two forms on DEAE-Sephadex.

In order to establish that rhodaneses A and B are present in the liver of one individual and do not arise due to population differences, crystalline enzyme was prepared from a single bovine liver and a solution of the protein was subjected to chromatography on DEAE-Sephadex as described above. Both forms A and B were resolved from crystalline rhodanese isolated from a single liver exactly as in the case of enzyme prepared from several livers.

Determination of Molecular Weights of Rhodaneses A and B—Evidence has been presented (4) that rhodanese exists both as a monomer of molecular weight 18,000 and as a dimer. This raised the possibility that the two forms which had been resolved might merely represent monomeric and dimeric forms of the enzyme. Accordingly, an approximation of the molecular weight of rhodaneses A and B was obtained by gel filtration on a column of Sephadex G-75. The column (2.0 x 45 cm) was calibrated with hemoglobin, chymotrypsinogen, and ribonuclease A standards. Samples containing approximately 1.5 mg of rhodanese A or rhodanese B were passed through this column and the molecular weight was estimated from a plot of the log of molecular weight versus the elution volume (23). Both forms had identical elution volumes corresponding to a dimeric molecular weight of 35,000.

A more precise determination of the molecular weights of rhodaneses A and B was obtained by electrophoresis in SDS-polyacrylamide gels. Gels run in 0.1% SDS but without the previous incubation treatment of Weber and Osborn (19) showed rhodanese dimer as the only species present (Table I). The same results were obtained after prior incubation in 1% 2-mercaptoethanol and 1% SDS for 2 hours at 37° followed by dialysis.
Incubation showed primarily dimer and a small amount of zoic acid. The reagent was dissolved in 0.1 M sodium phosphate, pH 7.0, containing 0.1% SDS and 8% 2-mercaptoethanol. In Experiment B samples were previously incubated and dialyzed overnight prior to electrophoresis as described by Weber and Osborn (19). Samples in Experiment C were dissolved directly in 10 mM sodium phosphate buffer, pH 7.0, containing 1.0% SDS and 8% 2-mercaptoethanol. Parallel runs were made in each case with standard mixtures containing bovine serum albumin, carboxypeptidase A, diisopropyl fluorophosphate-treated trypsin, and lysozyme, and the calculations were made as described earlier (19).

### Table I

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Concentration of SDS in electrophoresis buffer</th>
<th>Molecular weight</th>
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<tr>
<td></td>
<td>%</td>
<td>Rhodanese A</td>
</tr>
<tr>
<td>A</td>
<td>0.1</td>
<td>35,400</td>
</tr>
<tr>
<td>B</td>
<td>0.1</td>
<td>35,100</td>
</tr>
<tr>
<td>C</td>
<td>1.0</td>
<td>35,300</td>
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<td></td>
<td></td>
<td>17,800</td>
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</table>

*In Experiment C, a faint band was resolved from each form with a molecular weight corresponding to monomeric enzyme.

and electrophoresis. Samples run in 1% SDS without prior incubation showed primarily dimer and a small amount of apparently monomeric material.

**Sulfhydryl Analysis**—Rhodaneses A and B were analysed for sulfhydryl content by reaction with 5,5'-dithiobis(2-nitrobenzoic acid). The reagent was dissolved in 0.1 M K$_2$HPO$_4$ buffer, pH 8.1, at a concentration of 3.75 mM. A standard curve was obtained with a freshly prepared solution of 10 mM cysteine. Reaction mixtures contained 100 μl of reagent, 1.0 ml of 4% SDS, and varying amounts of cysteine in a total volume of 3.0 ml. The extent of reaction was determined spectrophotometrically by measuring the absorbance of the solutions at 412 nm.

Rhodanese A and rhodanese B were transferred to solution in 0.1 M K$_2$HPO$_4$, pH 8.1, by gel filtration on a column of Sephadex G-25 (1.5 × 40 cm). Reaction mixtures contained 100 μl of reagent, 1.0 ml of 4% SDS, and 0.75 mg of protein in a total volume of 3.0 ml. The reactions were monitored as described above, and the results obtained are shown in Table II. Each of the rhodanese forms gives a value of four free sulfhydryl groups per dimer, in good agreement with the values given by Srbob (7) and by Wang and Volini (5).

**Amino Acid Composition**—The results of amino acid analyses of homogeneous, reduced, carboxymethylated rhodanese A and B subjected to acid hydrolysis for 22, 48, and 72 hours are presented in Table III. Corrected compositions for those amino acids stable to acid hydrolysis were derived by averaging the three values obtained at the times designated in Table III. The corresponding values for carboxymethylcysteine, serine, threonine, and methionine were corrected for decomposition by extrapolation to zero time and the value obtained in the 72-hour analysis was used for isoleucine which is liberated more slowly during the hydrolytic procedure. Tryptophan was determined spectrophotometrically by the procedure of Beaven and Holiday (24). Numbers of residues of amino acids are based upon the presence of 12 residues of alanine per rhodanese monomer. The analyses reveal the amino acid compositions of rhodanese A and B to be identical. It should also be noted that the number of residues obtained (cysteine, 2; histidine, 3; methionine, 2; and tryptophan, 4) agree well with other analyses obtained for rhodanese monomer (25). The total of lysine, 8, plus arginine, 8, would lead one to expect a total of 17 tryptic peptides.

**End Group Analyses**—End group analyses were performed on rhodanese A and rhodanese B as described by Gray (26) by reaction with dimethylaminonaphthalene-5-sulfonyl chloride. A solution of rhodanese (0.1 μmole) in 0.5 ml of 8 M urea containing 0.5 M NaHCO$_3$ was treated with 0.5 μl of a solution of DNS-Cl in acetone (20 mg per ml). Reaction mixtures were incubated for 24 hours at room temperature. The protein which precipitated in both cases during the course of reaction was isolated by centrifugation, washed twice with 3.0 ml portions of acetone and was finally suspended in and dialyzed exhaustively against 0.1 M acetic acid to remove urea, NaHCO$_3$, and fluorescent by-products. Half of the suspension of each dialyzed sample was taken to dryness and suspended in 0.20 ml of 6 N HCl. The tubes were sealed under reduced pressure (16) and placed in an oven at 110°C for 18 hours during which time all solid material dissolved. The hydrolysates were evaporated to dryness and dissolved in 30 μl of deionized water. These solutions were analyzed for DNS-amino acids by thin layer chromatography on polyamide layers as described by Woods and Wang (27). Inspection of the chromatograms from the two forms under ultraviolet light revealed in each case a single spot corresponding to DNS-lysine.

**Tryptic Peptide Maps**—As may be seen in Fig. 2, tryptic peptide maps of reduced carboxymethylated rhodanese A and B are not significantly different. The shaded area in Fig. 2 was the only radioactive spot and was thus identified as containing the active site cysteinyl peptide of rhodanese. Spots marked T were identified as tryptophan-containing peptides on the basis of their reaction with the Ehrlich reagent. The area marked Y consistently gave a yellow product upon reaction with ninhydrin. The highly positively charged spot designated L has been shown by amino acid analysis to contain only lysine and probably represents the lysyl residue at the amino terminus of the protein. The small differences seen in the neutral zone are not reproducible.

**Determination of Enzyme-bound Sulfur in Rhodanese A and B**—The possibility that rhodanese A and B might differ only in

### Table II

<table>
<thead>
<tr>
<th>Enzyme species</th>
<th>-SH groups</th>
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<tr>
<td>Rhodanese A</td>
<td>3.85</td>
</tr>
<tr>
<td>Rhodanese B</td>
<td>4.10</td>
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</table>

* Per molecule of molecular weight 36,000.
TABLE III

Amino acid compositions of reduced and carboxymethylated rhodanases A and B

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<tr>
<th>Amino acid</th>
<th>Recovery*</th>
<th>Corrected composition†</th>
<th>Relative molar quantities‡</th>
<th>No. of residues per molecule§</th>
<th>Recovery</th>
<th>Corrected composition†</th>
<th>Relative molar quantities‡</th>
<th>No. of residues per molecule§</th>
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<tr>
<td></td>
<td>22 hours</td>
<td>48 hours</td>
<td>72 hours</td>
<td></td>
<td>22 hours</td>
<td>48 hours</td>
<td>72 hours</td>
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<tr>
<td>Lysine</td>
<td>26.5</td>
<td>20.9</td>
<td>26.8</td>
<td>20.7</td>
<td>7.94</td>
<td>8</td>
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<td>Histidine</td>
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<td>Arginine</td>
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<td>S-Carboxymethylcysteine</td>
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<td>3.60</td>
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<td>6.75</td>
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<td>Aspartic acid + asparagine</td>
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<td>44.7</td>
<td>44.7</td>
<td>44.7</td>
<td>13.23</td>
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<tr>
<td>Threonine</td>
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<td>22.2</td>
<td>28.3</td>
<td>7.63</td>
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<td>Serine</td>
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<td>25.2</td>
<td>30.8</td>
<td>10.95</td>
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<td>Glutamic acid + glutamine</td>
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<tr>
<td>Methionine</td>
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<td>5.4</td>
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<tr>
<td>Leucine</td>
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<td>Tyrosine</td>
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<td>Tryptophan*</td>
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<td>23.5</td>
<td>23.4</td>
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<td>7</td>
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</table>

* Samples containing 0.5 mg of crystalline homogeneous rhodanases A and R were hydrolyzed for the times designated (cf. "Experimental Procedure") in 2.0 ml of 6 N HCl. The concentration of amino acids was calculated as described by Moore and Stein (17).

† An average value for the three analyses is presented for those amino acids which are stable to acid hydrolysis. Figures for serine, threonine, methionine, and S-carboxymethyleysteine were corrected for decomposition by extrapolation of the values obtained at 22, 48, and 72 hours back to zero time. The value for isoleucine, which is liberated more slowly during the hydrolytic procedure, is that obtained after 72 hours.

‡ Calculated by dividing the corrected composition by 3.4 mmoles, which is the equivalent of 1 residue of amino acid.

§ Determined spectrophotometrically (24).

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the amount of enzyme-bound sulfur was deemed remote since the two forms had been isolated from solutions containing at least 1.0 mm NaS₂O₃. Nevertheless, the amount of enzyme-bound sulfur which could be discharged from each form by CN⁻ was determined. Varying quantities of crystalline rhodanases A and B were dissolved in 0.1 M Tris-HCl, pH 8.0, and the solutions were subjected to gel filtration on a column (1.5 × 45 cm) of Sephadex G 25 equilibrated with the same buffer. The protein in a volume of 4.1 ml was then incubated at 25° for 45 cm) of Sephadex G-25 equilibrated with the same buffer. The protein in a volume of 4.1 ml was then incubated at 25° for 10 min with 0.10 ml of 0.25 M KCN followed by addition of 1.5 ml of the dilute ferric nitrate reagent (2) employed in the rhodanase assay. After centrifugation to remove precipitated protein, the absorbance of each supernatant solution was measured at 460 nm. A standard curve relating color yields at 460 nm to SCN⁻ concentration was prepared with a KSCN solution of known concentration. No differences in the amount of sulfur bound to rhodanases A and B were observed, and each form was found to contain 0.675 mole of sulfur bound per mole of monomeric enzyme.

**Determination of Kₙθ for Rhodanases A and B**—The Kᵣ values for thiosulfate of rhodanases A and B were determined essentially as described by Mintel and Westley (29), except that the reactions were run for 30 sec at 25°. The re-
action mixtures contained enzyme-saturating concentrations of KCN, varying nonsaturating concentrations of sodium thiocyanate, and rhodanese A or rhodanese B at a concentration of $2.5 \times 10^{-10}$ M. A Lineweaver-Burk (30) plot of the initial rates of reaction observed at various concentrations of thiocyanate is presented in Fig. 3. The experimentally derived value of $K_m$ for both forms of the enzyme was $6.30 \times 10^{-3}$ moles per liter, in good agreement with the constant of $6.67 \times 10^{-3}$ moles per liter reported earlier (29). These findings show that the $K_m$ and $V_{max}$ values for both rhodanese A and rhodanese B are indistinguishable.

Rhodanese-catalyzed Reactions of $\beta$-Mercaptopyruvate and Cyanide—The partial resolution of two rhodanese activities, one specific for thiocyanate as the sulfur donor and the other capable of transferring the sulfur atom of both thiosulfate and $\beta$-mercaptopyruvate to cyanide, was reported by Fiedler and Wood (31) in 1956. Accordingly, the activities of highly purified preparations of rhodanese A and B toward $\beta$-mercaptopyruvate were measured in order to verify that this compound was a specifically with regard to deamidation.

Chromatography on DEAE-Sephadex of a preparation of rhodanese isolated as described above gave a preponderance of rhodanese B as described under “Results;” ○—○, as in Reference 9 except that pH adjustments in Step 3 were modified. The successive adjustments of pH to 6.0 and to 3.8 are made with a solution of 1.0 M glycine adjusted to pH 2.5 with H$_2$SO$_4$, rather than with 1.0 N HCl. In Step 3 the pH is adjusted to 7.6 with a solution of 1.0 M glycine, pH 9.8, in place of 1.0 M NH$_4$OH. The successive adjustments of pH to 6.0 and to 4.5 are carried out with 1.0 M glycine sulfate, pH 2.5, containing 1.8 M ammonium sulfate rather than with the corresponding solution of 1.0 M acetic acid. These modifications in the preparative procedure were designed to minimize conditions which might lead to alterations in the enzyme structure, specifically with regard to deamidation.

Dr. John Westley² in which all the buffers contain 1 mM Na$_2$SO$_3$ and 0.1 mM Zn(C$_2$H$_3$O$_2$) and the use of strong acids and bases for the pH adjustments is eliminated. In Step 2 of the method of Horowitz and DeToma (6), adjustment of the pH to 3.8 is made with a solution of 1.0 M glycine adjusted to pH 2.5 with H$_2$SO$_4$, rather than with 1.0 N HCl. In Step 3 the pH is adjusted to 7.6 with a solution of 1.0 M glycine, pH 9.8, in place of 1.0 M NH$_4$OH.

Chromatography on DEAE-Sephadex of a preparation of rhodanese isolated as described above gave a preponderance of the A form with an A to B ratio of 6 to 1 (Fig. 4). In view of the fact that a number of changes had been made in the preparative procedure, each of these was systematically evaluated in terms of the effect of its omission on the relative ratios of the A and B forms. The results of these experiments are presented in Fig. 4. It may be noted that the proportion of rhodanese B increases as

² Dr. John Westley, personal communication.
the protecting agents are removed. There does not appear to be any significant effect of thiosulfate upon the relative quantities of the A and B forms but the omission of the strong mineral acid pH adjustment and the inclusion of Zn\(^{++}\) favors production of rhodanese A. It is unlikely that the difference between the two rhodanese forms is that one contains zinc and the other does not since the inclusion of 5 \(\times\) 10\(^{-3}\) M EDTA in the preparative buffers has no significant effect on the ratio of the enzyme forms.

**DISCUSSION**

The findings reported herein support studies relating to the purification and characterization of bovine liver rhodanese which were undertaken preparatory to the sequence analysis of the enzyme. To date, crystalline and apparently homogeneous rhodanese has been prepared by fractional precipitation under varying conditions of pH and ammonium sulfate concentration. An extremely valuable preparative procedure from the point of view of structural studies has been recently published by Horowitz and DeToma (9) in which, by means of the precipitation techniques mentioned above, gram quantities of crystalline enzyme may be readily obtained. This material was judged to be homogeneous on the basis of a number of criteria including its specific enzyme activity. We have shown that rhodanese preparations isolated in this manner consist of two fully active enzyme components, present in about equal amounts, which may be resolved by polyacrylamide gel electrophoresis and by column chromatography (cf. Figs. 1 and 4). These components, designated rhodanese A and rhodanese B on the basis of the order of elution from columns of DEAE-Sephadex A-50, are present in enzyme preparations obtained from a single liver indicating that they do not arise due to variations in the population.

Thus far, attempts to establish the difference between rhodanese A and B have been unsuccessful. Both forms have a dimeric molecular weight of about 35,000 (Table I) and are identical with respect to amino acid composition (Table III). Each contains lysine as the amino-terminal residue, and analysis for free sulfhydryl groups yields a value of 4 per dimer for each form (Table II). While the tryptic peptide maps of rhodanese A and B are not identical, they are quite similar and are the same except for some irreproducible variations among the neutral peptides (Fig. 2). Similar examples of isozymes which have not been shown to differ structurally have been reported for venom phospholipase A from Crotalus adamanteus (32) and three of the four mushroom tyrosinases (33).

Cysteine and tryptophan have been implicated in the catalytic mechanism of rhodanese (5, 7, 34). Our findings establish that the tryptophan-containing tryptic peptides from rhodanese A and B exhibit identical electrophoretic and chromatographic behavior. The same is true for the tryptic peptides from each form containing the uniquely reactive cysteine residue.

The possibility that rhodanese A and B coexist in some kind of equilibrium state and are interconvertible seems highly remote in view of the fact that once either form has been purified it does not give rise to the other. Moreover, the two forms do not represent free enzyme and enzyme-sulfur complex; both rhodanese A and B have been shown to contain equal quantities of enzyme-bound sulfur. Attempts to distinguish between rhodanese A and B on the basis of kinetic differences with respect to the sulfur donor have also been unsuccessful. The kinetic parameters \(K_m\) and \(V_{max}\) toward thiosulfate are the same for each form of the enzyme. Furthermore, both forms are able to utilize \(\beta\)-mercaptopyruvate as substrate with equal efficiency. Fiedler and Wood (31) observed the presence of two enzyme species catalyzing the thiosulfate to cyanide trans-sulfuration reactions in rat liver acetone powders. These two activities could be partially resolved by paper electrophoresis at pH 7.4 but were still quite similar in charge at this pH. They reported at the same time that one of these two species was able to catalyze the transfer of sulfur from \(\beta\)-mercaptopyruvate to cyanide, thus confirming their earlier observation (35) that \(\beta\)-mercaptopyruvate can serve as a sulfur donor in reactions catalyzed by impure rhodanese preparations. However, Sörbo (36) later found that with highly purified bovine liver rhodanese, only one electrophoretic component was present at pH 5.4 and this was devoid of \(\beta\)-mercaptopyruvate to cyanide trans-sulfuration activity. It seemed possible that one of the two rhodanese forms may have been lost in his purification procedure, thus accounting both for the homogeneity of his preparation and the absence of activity with \(\beta\)-mercaptopyruvate. Whether or not rhodanese A and B correspond to the enzyme species observed by Fiedler and Wood (31), the results of the present study clearly indicate that both forms are able to catalyze the transfer of sulfur from \(\beta\)-mercaptopyruvate to cyanide with an efficiency of about 1% that observed with thiosulfate.

Whatever the difference is between rhodanese A and B, it would appear that one arises from the other during the isolation of the enzyme since the relative ratios of the two forms are dependent upon the preparative procedures employed. With the method of Horowitz and DeToma (9), we have observed ratios of the A to B forms which vary between 1 and 0.3. However, when this general procedure is modified as described in the present communication, the ratio of rhodanese A to the B form may be increased to about 6 (Fig. 4). These modifications involve the elimination of strong acid and base in the pH adjustments and the inclusion of Zn\(^{++}\) and thiosulfate in the buffers and would be expected to provide a milder means of rhodanese purification. Since rhodanese A is the predominant form isolated under these conditions, it would seem highly probable that this is the species which is native to the liver cell.

It is possible that the two rhodanese forms represent conformational isomers with identical covalent structures, but this seems unlikely in view of the evidence which suggests that they are not interconvertible. If rhodanese B is formed as a result of some structural alteration of rhodanese A occasioned during the isolation of the enzyme, at least two factors, proteolysis and deamidation, must be considered as being possibly involved. The arguments against the production of the B form due to endoproteolytic cleavages in rhodanese A are fairly conclusive. Reduced and carboxymethylated derivatives of both forms give identical amino acid compositions and molecular weights. Rhodanese monomer is small enough that even differences in composition of a single residue may be easily detected with most of the amino acids. The reduced and carboxymethylated derivatives were dialyzed exhaustively and subjected to gel filtration in 50% acetic acid prior to amino acid analysis. Some samples of the insoluble alkylated proteins were solubilized by citraconylation (37), followed by gel filtration at neutral pH. In all cases, the molecular weights and compositional analyses were identical, and there was no evidence for the existence of contaminating peptide material.

While the evidence is by no means conclusive, deamidation appears at present to be the most likely explanation for the production of the B form from rhodanese A. The chromatographic and electrophoretic mobilities of the two activities are consistent...
with this hypothesis. Moreover, the elimination from the preparative procedure of conditions known to facilitate deamidation leads to an increase in the relative yield of the A form. If deamidation is involved in the A to B conversion, then a specific and unusually labile amide linkage must be cleaved which is not essential for catalysis.

The results of this investigation emphasize the importance of the careful evaluation of product homogeneity in protein preparative procedures in which extreme conditions of pH are employed. Although the elucidation of the difference between rhodaneses A and B must await detailed structural analysis of the two forms, the difference does not appear to be related in any way to the function of the enzyme.

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