Microbial Transsulfuration: the Mechanism of an Enzymatic Disulfide Elimination Reaction

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In mammalian tissues the formation of cysteine from homocysteine, a process which has been called "transsulfuration," has been shown to be mediated by two separable enzymes (1-3). The first catalyzes a condensation between homocysteine and serine to yield the unsymmetrical thioether L-cystathionine. The latter is then cleaved, by a γ elimination catalyzed by the second enzyme, to cysteine, α-ketobutyrate, and ammonia. The cleavage enzyme also catalyzes the decomposition of homoserine to α-ketobutyrate and ammonia.

The ability of microorganisms to grow without exogenous methionine has generally been attributed to their capacity to carry out a reciprocal pair of reactions, in which cystathionine is formed from cysteine and homoserine, and cleaved, by a β elimination, to homocysteine, pyruvate, and ammonia. Some evidence has been reported that microorganisms also possess the two enzymes present in animal tissues (4, 5). If this were correct, sulfur could be reversibly transferred from cysteine to homocysteine by a process mediated by four different enzymes, each catalyzing an essentially irreversible reaction. This scheme is based on studies of the nutritional requirements and metabolite accumulations of Neurospora mutants blocked in methionine biosynthesis (4, 6). At the enzyme level it remains largely hypothetical.

We have begun a study of microbial transsulfuration by undertaking to isolate the cystathionine cleavage enzyme which catalyzes γ elimination to yield cysteine and α-ketobutyrate. To facilitate obtaining this enzyme free from the hypothetical second cleavage enzyme, we chose as enzyme source a Neurospora mutant that can grow when supplied with methionine or homocysteine, but not with cystathionine, which it accumulates (4). The mutant was expected to lack an enzyme which cleaves cystathionine to homocysteine and pyruvate.

This expectation has not, in fact, been realized. Our present evidence suggests that wild type Neurospora may contain only one cleavage enzyme, which catalyzes a heterogeneous decomposition of cystathionine. We have not detected any modifications in the physical or catalytic properties of the enzyme from the mutant strain, or any difference in the amount of it present.

These aspects of the work, which are mentioned here to place the enzyme we have studied in perspective, will be described in detail at another time.

In examining the properties of the enzyme we found that it could decompose, besides thioethers and hydroxyamino acids, certain disulfides, but not the corresponding mercaptans (8). The nature and mechanism of the reaction by which disulfides are decomposed is the subject of this report.

After this work was nearly completed, we learned of the paper by Cavallini et al. (9), who had studied a reaction in which cystine was decomposed by an enzyme from liver. These authors had followed a different experimental path toward conclusions similar to ours, but had not been able to obtain proof for the nature of the reaction. This proof can now be supplied, through the isolation and identification of a derivative of a reactive intermediate which is formed in the course of the reaction.

We have also established the identity of the enzyme with that which cleaves cystathionine, the coenzyme role of pyridoxal-P, the specificity with respect to disulfide and mercaptan, chain length, and optical configuration, and have documented several additional spontaneous reactions of the intermediate.

EXPERIMENTAL PROCEDURE

Biological Materials—Two strains of Neurospora mutant H98, locus me-2 (4, 6), have been used, and they will be distinguished by a parenthetical symbol. Although we have not detected any physical or catalytic differences between enzymes purified from the two strains, they have shown differences in certain other properties. Mutant H98(II) was received directly from Dr. N. H. Horowitz, and H98(201) reached us from the same source by a single enzyme, from L-cystathionine. (When cystathionine containing equal parts of L- and D-allo isomers is used, pyruvate and homocysteine make up 40% of the products. Although unlikely, the possibility must therefore be considered that the small amounts of these products formed from L-cystathionine actually arise from another isomer which contaminates it.) We have also found no differences, from wild type, in the cystathionine cleavage enzyme that can be extracted from mutant 4894 at locus me-7 (6). This mutant is thought to be blocked in the condensing reaction which involves homoserine and cysteine. We could not confirm a report that it lacks the homologous cleavage reaction to cysteine (8). Some possible explanations for these results are: that we have missed another cleavage enzyme present in the cell; that we have missed some cellular factor which can modify the proportions of products formed by a single enzyme; or that cystathionine is not an intermediate in the major pathway of methionine biosynthesis.

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1 Our efforts were initially directed to the γ-elimination reaction, because of our interest in a closely related enzymatic elimination of phosphate (7). We wished to investigate the point of divergence in the mechanisms of the reactions by which the phosphate ester of homoserine yields threonine, whereas homoserine itself (or cystathionine) yields α-ketobutyrate.
2 Enzyme from both wild type and mutant (H98) liberates about 10% of pyruvate + homocysteine, and 90% of α-ketobutyrate.

M. Flavin and C. Slaughter, unpublished results.
way of Dr. N. E. Murray and the Dartmouth Stock Center (FGSC 201). 4

Cells were cultured as previously described (10), media being supplemented with 50 mg per liter of dl-methionine.

Cystathionine Cleavage Enzyme Assay—Assays are based on the rate of formation of one of the products, mercaptan or α-keto acid, formed from the various substrates.

In following the enzyme purification, the substrate was α-cystathionine (L-homo-γ-thiolactone), and the assay was for mercaptan (i.e., for the sum of the cysteine + homocysteine liberated). This assay is based on the reaction between alkyl mercaptans and an aromatic disulfide (ArSSAr),4 which liberates a strongly colored aromatic mercaptan (Equation 1). ArSSAr is incorporated directly into the reaction mixture, and a kinetic assay is possible because Reaction 1 goes instantaneously to completion at the

\[ \text{2 Alkyl-SH + ArSSAr} \rightarrow \text{alkyl-SS-alkyl} + 2 \text{ArSH} \] 

pH used. 5 Because large amounts of protein alone cause a slow liberation of ArSH from its disulfide (11), a companion blank incubation without substrate is also added, except with Step 4 enzyme. A unit of enzyme is the amount liberating 1 μmole of ArSH in 1 minute under standard conditions, at 25°C. The procedure is to add, to a 1-cm light path cuvette: water to yield a final volume of 1 ml, 100 μmole of potassium phosphate (pH 7.3), 0.1 μmole of pyridoxal-P, 2 μmole of cystathionine, 0.2 μmole of ArSSAr, and 0.0005 to 0.005 units of enzyme. The liberation of ArSH is followed spectrophotometrically at 1-minute intervals, for 3 or 4 minutes, at λ_{250} (E = 12,000).

It will be apparent from subsequent discussion that this assay can also be used when the substrate is a disulfide, even though in this case mercaptan is not an immediate product of the reaction. Hydroxymethyl mercaptan substrates require the use of an assay for α-keto acid.

Lactic dehydrogenase is used to measure the rate of liberation of α-keto acid. The crude Neurospora enzyme fractions decompose DPNH, and must be inactivated before measuring α-keto acid in a second incubation with lactic dehydrogenase. This assay does not give true initial rates,3 and has not been afforded that the same enzyme catalyzes decomposition of thiocysteine and disulfides. Steps 1A, 1B, and 1C are performed as previously described (10), except that the cells are blended with glass beads instead of Dry Ice (Step 1A). Step 2 is a 30 to 47% saturated ammonium sulfate precipitation at pH 6.2, and Step 3 involves heating the redisolved precipitate, without removing residual salt, for 6 minutes at 60°C. The dialyzed supernatant protein from the heat step has been used in most experiments. In this fraction the enzyme has been purified 25-fold, in 30% yield. An additional 20-fold purification has been obtained by elution from columns of either carboxymethyl cellulose (Step 4a), or diethylamine cellulose (Step 4b), in the general manner previously described (10). Both steps give poor yields; both appear to be based on a selectively anionic character of the enzyme at pH 6.3.

Chemical Preparations—Homocysteine solutions are prepared just before use by heating the commercially available lactone hydrochlorides for 3 minutes at 100°C with two equivalents of alkali. Homocysteine is much more resistant to air oxidation than cysteine in neutral solution.

The mixed disulfide of thioglycolate and cystine (12) could be readily prepared by mixing, at room temperature, equimolar amounts of cystine and thioglycolic mercaptan, at pH 11 in a stream of helium. Electrophoresis, either immediately or 90 minutes after mixing, reveals the same proportions of products, which consist of the two mercaptans and three possible disulfides. After cold storage without anaerobic precautions the mercaptans disappear, leaving another equilibrium mixture of roughly equivalent amounts of the three disulfides (see Column 2, Fig. 4) (13). The mixed disulfide can be isolated from the mixture by elution with dilute acetic acid after electrophoresis, or from Dowex 1-acetate with an acetic acid gradient. Electrophoresis now reveals a very slight, but detectable, dismutation back into the two symmetrical disulfides. Thioglycolic disulfide was prepared by aeration of a slightly alkaline solution of mercaptan in the presence of 10^-3 M CuCl_2.

Electrophoretic separations were made on Whatman No. 1 paper with a high voltage apparatus (Frankfurt model, Hormuth-Vetter, Heidelberg) at 40 volts per cm. The buffer was 1% aqueous pyridine (volume per volume) adjusted to the desired pH with glacial acetic acid. Amino acids were located by spraying with ninhydrin; disulfides, with KMnO_4 in 50% aqueous methanol, followed by nitroprusside reagent (14).

Chemical Materials and Reagents—Crystalline muscle lactic dehydrogenase was obtained from Boehringer. Its specific activity was 500 μmole per mg × minute, when assayed in 1 ml volume containing 100 μmole of potassium phosphate (pH 7.3), 2 μmole of pyruvate, and 0.1 μmole of DPNH.

Mercaptoamino acids were obtained from California Corporation or Cyclo Chemical Corporation. No information is available on the purity of the mesocysteine. Disulfides were dissolved to make 0.01 M solutions with the aid of four equivalents of HCl; ArSSAr (Aldrich) was used as a 0.004 M solution in 0.05 M potassium phosphate, pH 7.0.

Lanthionine was a mixture of L- and meso isomers from California Corporation. In most experiments we have used a preparation of cystathionine from Light and Company, containing equal parts of the L- and D-allo isomers. Samples of L-cystathionine were obtained from Dr. M. D. Armstrong,6 and from Cyclo Chemical Corporation, and have also been isolated from Neurospora mutant H98(H) (4). 2

6 We are also indebted to Dr. Armstrong for samples of four other thioethers: S-carboxymethyl(homo)cysteine and S-carboxamidomethyl(homo)cysteine.

4 N. E. Murray, personal communication.
5 The abbreviation used is: 5,5'-dithio-ditoluic acid, ArSSAr (diaryl disulfide); in the reduced form, ArSH (aryl mercaptan) (11). Where not otherwise explicit, the words disulfide or mercaptan are added after the names of divalent sulfur compounds.

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**Analytical Determinations**

Alkyl mercaptans and α-keto acids were determined by the procedures described under "Cystathionine Cleavage Enzyme Assays." In the presence of cyanide, the former were determined with nitroprusside (15) in 1-cm cuvettes at \( \lambda_{460} \), the reagent volumes being reduced for a final volume of 1 ml.

Iodide was determined by nitrous acid oxidation to iodine; the stable color could be measured at \( \lambda_{480} \). A deproteinized aliquot containing 0 to 3 \( \mu \)moles was added to a cuvette with enough water to yield a final volume of 1 ml, followed by 0.12 ml of 0.1 M sodium nitrite and 0.08 ml of \( \text{N} \) sulfuric acid.

Anomia was determined in acid-deproteinized solutions by diffusion, followed by direct nesslerization. Diffusion was accomplished from small rotating bottles (Scientific Industries, Inc., Springfield, Massachusetts) onto glass plugs inserted in the stoppers, which had been dipped in dilute sulfuric acid.

Inorganic sulfide was trapped in cadmium acetate in Warburg vessel center wells. The CdS was dissolved in acid in the diffusion bottle, and \( \text{H}_2\text{S} \) allowed to diffuse for 5 to 10 minutes into alkali on the center plug. Losses occur after longer periods of diffusion. The sulfide was then determined with \( \text{ArSSAr} \), from which it liberates two equivalents of \( \text{ArSH} \) (see Equation 10). The alkaline sulfide must be rapidly stirred into the neutral solution of the color reagent, because local alkali will itself liberate \( \text{ArSH} \) from the disulfide.

**Elemental Sulfur**—Elemental sulfur was determined by reduction to sulfide with excess mercaptoan (10). A negative result should rule out the presence of sulfur whether as atomic sulfur, linear or cyclic inorganic polysulfide, or organic polysulfide. A positive result does not establish the form of sulfur present. However, in all cases where we have identified significant amounts of sulfur, a heavy opalescence in the reaction mixture indicated that it was largely in inorganic form. These analyses were done after long incubations, or under other conditions in which the intermediate organic polysulfide (discussed below) had decomposed.

An aliquot of an enzymatic incubation mixture is placed in a small aeration vessel, and deproteinized by heating for 2 minutes at 100° at pH 5.5. The vessel is then attached, containing an air stopcock, to a glass train leading into a receiver with 5 ml of 0.1 M NaOH. To the aeration vessel are added water to yield a final volume of 2 ml, 200 \( \mu \)moles of potassium phosphate (pH 7.3), and a large excess (50 \( \mu \)moles) of \( \beta \)-mercaptopropionate. The \( \text{H}_2\text{S} \) liberated, as sulfur is reduced by the mercaptan, is flushed continuously, with helium, into the receiver. Aliquots from the trap are assayed at intervals for sodium sulfide, as described above, and flushing is continued (usually overnight) until the evolution of \( \text{H}_2\text{S} \) comes to a stop.

A satisfactory sulfur reference standard was not available; crystalline and colloidal sulfur appeared to react slowly or incompletely, and suspensions of flowers of sulfur were difficult to pipet. However, several experiments of the types described below have given consistent results.

**Dialkyl Disulfides**—These were determined by cyanolysis according to Reaction 2, followed by nitroprusside assay of the liberated mercaptaan. When excess iodoacetate was present, it

\[
\text{RSSR} + \text{CN}^- \rightarrow \text{RS}^- + \text{RSCN} \quad (2)
\]

was necessary first to destroy it by incubation with alkaline piperidine, which rapidly displaces iodide. The procedure is to pipet a 0.1-ml aliquot of an enzyme incubation mixture (which need not be deproteinized) into a cuvette, and to mix in 0.01 ml of 2 M NaOH and 0.02 ml of 2 M aqueous piperidine; after 5 minutes 0.05 ml of 2 M NaCN is added, and then, after 10 to 15 minutes, 0.02 ml of 30% metaphosphoric acid, followed by the other nitroprusside reagents. L-Cystine was used as a reference standard. A correction must be made if alkyl hydrogen disulfide is also present (see below).

Cyanolysis, in this assay and that described below, has given less than theoretical and somewhat unrepeatable color values. In Table V it can be seen that as cystine is replaced by mixed disulfide, the total disulfide value somewhat exceeds theoretical. Presumably the mixed disulfide yields more color (which was also observed to fade more rapidly) than the reference standard. Inorganic sulfide gives an extremely transient nitroprusside color, and does not interfere with this determination. (Uncyanolysed alkyl hydrogen disulfide also gives an extremely transient color.)

**Alkyl Hydrogen Disulfide**—There is no specific color test for this compound. Having established its identity by other methods, we have, however, made use of the assay of Cavallini et al. (9) to estimate the amount of it present. The assay involves cyanolysis according to Equation 3, followed by a color test for

\[
\text{RSSH} + \text{CN}^- \rightarrow \text{RS}^- + \text{SCN}^- \quad (3)
\]

inorganic thiocyanate (17). We have confirmed that elemental sulfur reacts too slowly to give a positive test under the conditions used, because of its insolubility in water (18, 19); and have also found that inorganic sulfide will not yield thiocyanate if CuCl₂ is omitted (17). As a reference standard, we have used potassium tetrathionate (Reaction 4) (19, 20).

Alkyl hydrogen disulfides have been prepared (21), but are too unstable for this purpose.

\[
\text{SO}_2\text{O}_4^- + 3 \text{CN}^- + 2 \text{H}_2\text{O} \rightarrow \text{SO}_3\text{O}^- + \text{SO}_4^- + 2 \text{HCN} + \text{SCN}^- \quad (4)
\]

We have not been able to deproteinize incubation mixtures without destroying alkyl hydrogen disulfide. The procedure is to incubate an aliquot for 10 minutes at 25° with 50 \( \mu \)moles of NaCN in 0.3 ml volume; then add 0.6 ml of water and 0.1 ml of ferric nitrate reagent (17) containing enough trichloroacetic acid to precipitate protein. The stable supernatant color is determined at \( \lambda_{460} \). Preincubation with piperidine may result in bluish colors, and is unnecessary since iodoacetate does not interfere in this assay.

Since an equivalent of alkyl mercaptaan is also liberated in Reaction 3, the amount of RSSH must be subtracted from the RSSR to give the true value for the latter, when both are present.

**RESULTS**

Fig. 1 shows the formation, with time, of pyruvate or α-keto butyrate, from various substrates incubated with 400-fold purified Neurospora H98 cystathionine cleavage enzyme.

The upper two curves show that equal amounts of α-keto acid and mercaptaan are liberated from cystathionine throughout the incubation. The incorporation, into the reaction mixture of this experiment, of \( \text{ArSSAr} \), provides a cumulative marker for the amount of mercaptaan formed (Equation 1). By the same token, as they are liberated, the cysteine and homocysteine are oxidized by \( \text{ArSSAr} \) to the disulfides, which in turn are substrates.

The declining rate during the later part of the incubation reflects, in part, the fact that cystine, rather than cystathionine, is
now the principal substrate being decomposed. Under these conditions the disulfides also decompose with formation of equimolar amounts of α-keto acid and ArSH (see below and Fig. 3). Fig. 1 also shows the stimulation of the rate of cystathionine decomposition produced by ArSSAr, which may be due to removal of product inhibition by cysteine.

Some characteristics of the enzymatic decomposition of disulfides are illustrated in Fig. 2. Under sufficiently anaerobic conditions, such as those described in the legend to Fig. 2, L-cysteine will not replace L-cystine as a substrate, and partially inhibits keto acid formation when added together with the disulfide. L-Homocysteine is decomposed more slowly, and the D isomers are inert. L-Homocysteine also does not react; this can be shown more easily because it is resistant to air oxidation.

The principal evidence that a single enzyme catalyzes decomposition of the various substrates is that cystine, homo cysteine, homoserine, and lanthionine are decomposed at the same initial maximal velocities, relative to cystathionine, over a 400-fold range of enzyme purification. The same values for \( V_{\text{max}} \) and \( K_m \), shown in Table I, were obtained with enzyme from Steps 1C, 2, 3, and 4B. Comparisons between different enzyme fractions were made under identical conditions. The values reported were not determined precisely, because some of the substrates were optically heterogeneous. Substrates not decomposed, besides those already mentioned, include: D-serine, S-methyl-L-(homo)cysteine, S-carboxymethyl-L-homocysteine, and S-carboxamidomethyl-L-(homo)cysteine.

The pyridoxal-P requirement for decomposition of disulfides and thioethers is shown in Table II. Competition between substrate pairs, disulfide and thioether, or thioethers of different chain length (Table III), also indicates that a single enzyme is involved. Reciprocal inhibition of two enzymes is made less likely by the fact that addition to cystathionine of cystine, a slow reacting substrate of low \( K_m \) (Table I), reduces the rate of ArSH formation to that observed when the latter is present alone. This phenomenon may also be seen in the time course of cystathionine decomposition (Fig. 1).

Attention was now directed to the problem of the mechanism of the enzymatic decomposition of cystine. The remaining results will be shown to support the conclusion that the nature of this reaction is as shown in Equation 5.

\[
\text{Cystine} + H_2O \rightarrow \text{pyruvate} + \text{NH}_3 + \text{HSSCH}_2\text{CH(NH}_2\text{)COOH} \quad (5)
\]

The alkyl hydrogen disulfide proposed as a product of the enzymatic decomposition of cystine. The remaining reactions were assayed spectrophotometrically without deproteinization. The initial reaction velocities with saturating amounts of the substrates listed, and for the substrate concentrations giving half-maximal velocity, were compared under identical conditions, by ArSSAr assay, with enzyme Fractions 1C, 2, 3, and 4B. Over this 400 fold range of purification, no significant differences were found, with the exception of those for homoserine (lactic dehydrogenase assay). A second enzyme catalyzing dehydration of homoserine appears to be present in the original extract. A serine dehydrase activity was lost in Step 3.

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**Table I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Approximate relative ( V_{\text{max}} )</th>
<th>Approximate ( K_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Cystathionine</td>
<td>100</td>
<td>( 4 \times 10^{-4} )</td>
</tr>
<tr>
<td>Cystathionine (L- + D-allo-)</td>
<td>100</td>
<td>( 6 \times 10^{-4} )</td>
</tr>
<tr>
<td>Lanthionine (L- + meso-)</td>
<td>110</td>
<td>( 5 \times 10^{-4} )</td>
</tr>
<tr>
<td>Mesocystine</td>
<td>47</td>
<td>( 5 \times 10^{-5} )</td>
</tr>
<tr>
<td>L-Homocysteine</td>
<td>17</td>
<td>( 5 \times 10^{-5} )</td>
</tr>
<tr>
<td>L-Homocystine</td>
<td>10</td>
<td>( 5 \times 10^{-4} )</td>
</tr>
<tr>
<td>D,L-Homoserine</td>
<td>10</td>
<td>( 10^{-2} )</td>
</tr>
</tbody>
</table>

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enzymatic reaction is unstable under most conditions, decomposing into elemental sulfur and mercaptan (Reaction 6). It has not been isolated as such. The evidence which we shall now present is of two kinds. First, the products which can be identified, under various reaction conditions, have been shown to be those which would be predicted to result from spontaneous reactions of the intermediate under each set of conditions. Second, the intermediate has been trapped as it is formed, with iodoacetate, and the resulting stable derivative has been isolated and identified.

The first observation was of the appearance during the reaction of a progressive opalescence indicative of the separation of elemental sulfur. Qualitatively, the appearance of sulfur lags behind that of keto acid, more so at pH 8.2 (100 minutes) than at 7.3, but it invariably precipitates if the incubation is continued. When ArSSAr is present in the reaction mixture, the precipitate appears sooner, and the formation of equimolar amounts of pyruvate and ArSH, shown in Fig. 3, may be explained by the intermediate having been trapped as it is formed, with iodoacetate, 20 pmoles (pH 8.2); L-cystine, 1.2 pmoles; iodoacetamide, 20 pmoles (pH 8.2); and ArSSAr, 2 μmoles (pH 7.3); = α-keto acid formed, = ArSH formed.

Reactions 5 and 7, which provide the basis for the ArSSAr assay of the disulfide reaction (Tables I to III). The top two

```
RSSH ⇌ RSH + S  
```

(6)

The formation of equal amounts of pyruvate and ArSH, with a lag in sulfur precipitation, could also be explained by reaction 7a, but the subsequent fate of the

```
RSSH + ArSSAr → RSSAr + S + ArSH  
```

(7a)

trisulfide might be less clear.

* This interpretation rests on the qualitative observation of a sulfur precipitate in the mixture, since the assays used would not distinguish between the compounds on either side of the arrows in Equation 6.
Incubations in double side arm Warburg vessels at 30° in 2 to 3 ml final volume containing, in addition to components indicated, 10⁻⁴ M pyridoxal-P and 0.02 m potassium phosphate, pH 7.3 (Experiments 1, 2a, 3), or 0.01 m potassium pyrophosphate, pH 8.2 (Experiments 2b to d). Enzyme: Step 3 from H98 (201). Gas phase: Experiment 1, air; Experiment 2, helium; Experiment 3, hydrogen. Center well: Experiments 2 and 3, 0.3 ml m cadmium acetate. One side arm contained the enzyme, which was tipped in after gassing for 25 minutes; the other contained L-cystine (Experiment 3), sulfhydryl trapping agent (Experiment 2), or 0.3 ml of 1.5 m trichloroacetic acid (Experiment 1) which was tipped in to terminate the incubation. In Experiment 1 the ammonia blank was enzyme incubated without substrate, and the reference standard was enzyme incubated with ammonium sulfate. Determinations of pyruvate, ammonia, sulfur, sulfide, and ArSH are described in the text. Cysteine (Experiment 2) was determined as nonvolatile sulfhydryl by ArSSAr assay, immediately after opening the Warburg vessel, and without deproteinization.

<table>
<thead>
<tr>
<th>Table IV</th>
<th>Disulfide reaction products in presence of iodoacetate</th>
</tr>
</thead>
</table>

Aerobic incubations in stoppered test tubes at 30° in 10 ml final volume containing, in addition to components indicated, 2 × 10⁻⁴ M pyridoxal-P and 0.01 m potassium pyrophosphate, pH 8.2. Enzyme: Step 3 from H98 (201). Iodide was determined after trichloroacetic acid deproteinization of aliquots. The blank was the zero time value, reference standard was sodium iodide added to a deproteinized aliquot from Experiment 1e. Dialkyl disulfide and alkyl hydrogen disulfide were determined in aliquots without deproteinization, and Experiment 1e was used as blank. Reference standards for dialkyl disulfide were the zero time values for Experiment 1a and 1b, for alkyl hydrogen disulfide, a companion incubation with 10 μmoles of potassium tetrathionate instead of cystine.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Additions</th>
<th>Iodoacetate</th>
<th>Enzyme</th>
<th>Incubation time</th>
<th>Pyruvate</th>
<th>Iodide</th>
<th>Alkyl hydrogen disulfide</th>
<th>Dialkyl disulfide</th>
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<td>20</td>
<td>0.96</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>20</td>
<td>2.6</td>
<td>23</td>
</tr>
<tr>
<td>lb</td>
<td>20</td>
<td>0.96</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>20</td>
<td>2.6</td>
<td>23</td>
</tr>
<tr>
<td>lc</td>
<td>100</td>
<td>0.96</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>20</td>
<td>2.6</td>
<td>23</td>
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</table>

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Additions</th>
<th>Pyruvate</th>
<th>Iodide</th>
<th>Alkyl hydrogen disulfide</th>
<th>Dialkyl disulfide</th>
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<td>1a</td>
<td>20</td>
<td>0.96</td>
<td>0</td>
<td>0.3</td>
<td>20</td>
</tr>
<tr>
<td>1b</td>
<td>20</td>
<td>0.96</td>
<td>0</td>
<td>0.3</td>
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<tr>
<td>1c</td>
<td>100</td>
<td>0.96</td>
<td>0</td>
<td>0.3</td>
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</table>

that, in these incubations, analyses for sulfur, sulfide, and mercaptan are negative (Table IV, Experiments 2b to 2d). Iodoacetate was chosen for further attempts to account for the missing sulfur.

In a control incubation, without iodoacetate, the amount of pyruvate formed is equal to the amount of cystine (dialkyl disulfide) disappearing, as shown in Experiment 1b, Table V. This incubation was carried out at a higher pH (8.2), where the liberation of elemental sulfur from intermediary alkyl hydrogen disulfide is delayed. The reaction was stopped as soon as the first opalescence of inorganic sulfur appeared, when it was assumed that a major portion of the alkyl hydrogen disulfide had not yet decomposed. It was accordingly necessary to determine and subtract the amount of the latter, to obtain the true value for residual unreacted cystine shown in Table V (see "Experimental Procedure"). This determination, by the thioionite method of Cavallini et al. (9), also confirms that Reaction 6 has not yet gone far toward completion. The amount of alkyl hydrogen disulfide accumulating is nearly equal to the amount of pyruvate formed.

In the companion incubation with iodoacetate (Experiment 1a, Table V) three differences are apparent. First, there is very little accumulation of alkyl hydrogen disulfide. This confirms the results of Experiment 2b, Table IV, since the assays for elemental sulfur and mercaptan do not differentiate between the components on either side of the arrows in Equation 6. Second, pyruvate without inorganic sulfur compounds, and with apparent disappearance of dialkyl disulfide. Additional side reactions must be involved.

of these incubations. It is slow in the forward direction, due to sulfur insolubility (see "Experimental Procedure"). The observed sulfide recoveries have been low under these conditions, perhaps due to difficulty in excluding oxygen during the long incubation required. However, good equivalent stoichiometry is obtained when ArSSAr is incorporated into the medium (Table IV, Experiment 3). This allows complete decomposition of the 2 added disulfides, as predicted by Equation 11 (sum: 1, 5, 6).

Cystine + ArSSAr + 2 H₂O → 2 pyruvate + 2 NH₃ + 2 S + 2 ArSH (11)

In attempting to assess the exact proportions of different products formed in the heterogeneous decomposition of cystathionine, a number of sulfhydryl reagents were used to block the further decomposition of the mercaptan products (i.e. after air oxidation to disulfides). The next insight into the nature of the disulfide reaction came, fortuitously, from observations of the products formed in the presence of these reagents.

Fig. 3 shows the time course of pyruvate liberation in the presence of 0.01 m N-ethylmaleimide, iodoacetate, or iodoacetamide. Although the higher pH, at which the latter two are effective sulfhydryl traps, itself causes some decrease in the reaction rate, the trapping agents themselves have no significant effect on the rate. They do cause the reaction to stop after liberation of one equivalent of pyruvate. More interesting is that iodoacetamide sometimes allows liberation of 2 equivalents of
iodide is liberated in amounts equimolar with the pyruvate formed. Liberation of iodide is dependent on the presence of cystine in the reaction mixture (Experiment 1c, Table V). As the incubation is continued, the micromoles of pyruvate and iodide formed slowly and equally increase, and come to exceed the micromoles of cystine originally added. Third, there is no disappearance of dialkyl disulfide. Cystine must accordingly have been replaced by an exactly equal amount of one or more new disulfides.

The identification of the new dialkyl disulfide(s) accumulating in iodoacetate incubations was now undertaken. The remainder of the incubation mixture of Experiment 1a, Table V, was deproteinized and concentrated under reduced pressure, and an aliquot was subjected to paper electrophoresis, along with a number of known compounds for comparison. The results are shown in the photograph in Fig. 4. All components were added at duplicate spots on opposite sides of the paper. At the end of the electrophoretic run, the paper was then divided, so that one half could be sprayed for amino acids, and the other for disulfides. Known compounds were applied at Positions 1, 2, and 4, at the origin, the transverse line near the bottom of the paper. At the pH used, neutral amino acids migrate slowly toward the cathode (bottom of the paper).

Known cystine, applied at Position 4, can be seen to give a reaction for both amino acid and disulfide (the nitroprusside spot, on the right, has been streaked somewhat from placing a glass plate over the wet paper for prompt photography). With the nitroprusside reagent, cystine also appears as a circle at the
origin, perhaps because of its insolubility. Also at Position 4, S-carboxymethylcysteine (the product of reaction of iodoacetate with cysteine), appears as a ninhydrin-positive, disulfide-negative component. The same derivative of homocysteine was applied at Position 1; it has almost the same mobility as cystine-thioglycolic mixed disulfide. Also at Position 1, thioglycolic disulfide appears as a ninhydrin-negative, disulfide-positive component. A crude preparation of cystine-thioglycolic mixed disulfide has been added at Position 2, where it appears as a ninhydrin- and disulfide-positive component, with a mobility intermediate between those of the cystine and thioglycolic disulfide which are also present (see "Experimental Procedure").

The iodoacetate incubation mixture was applied at Position 3. It contains no S-carboxymethylcysteine, and no residual cystine. The latter has been replaced by two new disulfides. One reacts also with ninhydrin, and has the same mobility as cystine-thioglycolic mixed disulfide. The other, present in smaller amounts, corresponds to thioglycolic disulfide.

**DISCUSSION**

A Neurospora cystathionine cleavage enzyme has been found to catalyze pyridoxal-P-dependent elimination reactions from a number of amino acids besides cystathionine. Substrates include amino acids containing an -OH substituent in the y position, or an -SR or -SSR in either the b or y position. Mercaptoamino acids are not decomposed.

Preliminary evidence that the enzymatic decomposition of L-cystine involves elimination of an unstable alkyl hydrogen disulfide has been obtained by identifying the products formed under three different reaction conditions. In each case, the products are those that would be predicted from the spontaneous reactions that RSSH would be expected to undergo under each set of conditions. First, in short anaerobic incubations, disappearance of 1 mole of cystine leads to the formation of 1 mole each of pyruvate, ammonia, cysteine, and elemental sulfur. This accords with decomposition of RSSH into mercaptan and sulfur. Second, in the presence of a diaryl disulfide, each mole of cystine consumed leads to the formation of 2 moles each of pyruvate, ammonia, elemental sulfur, and aryl mercaptan. This accords with oxidation of RSSH by ArSSAr. Third, in the presence of iodoacetate, cystine consumed is replaced by an intermediate between those of cystine and thioglycolic disulfide which are also present (see "Experimental Procedure").

More conclusive evidence for the intermediary formation of RSSH has been obtained by isolating the new disulfides formed in the last incubation, and identifying the major one as the mixed disulfide of cystine and thioglycolate.

The presence, in the iodoacetate incubation mixture, of a smaller amount of thioglycolic disulfide, offers a clue to the slow evolution of more than one equivalent of pyruvate and iodide (Table V). This can now be interpreted as resulting either from partial spontaneous dismutation of mixed disulfide into substrate and thioglycolic disulfide (Equation 14), which might be unlikely in the presence of iodoacetate, or from enzymatic attack on the mixed disulfide, and further reaction with iodoacetate (Equations 15, 16). Either path would yield stoichiometrically the mixed disulfide of cystine and thioglycolate.

Since intermediary alkyl hydrogen disulfide accumulates under some conditions for a time, before evolving elementary sulfur, we were interested to determine the rate of its reaction with iodoacetate. The reaction in Experiment 1b, Table V, was stopped after 90 minutes by chilling (attempts to deproteinize destroyed RSSH). Incubation of an aliquot for 2 minutes at 25°C with 0.01 m iodoacetate completely destroyed the ability to form SCN-. The alkyl hydrogen disulfide therefore seems to be comparable to cysteine in nucleophilic character. However, after cold storage, although the thioenolate reaction persisted, it could no longer be discharged by prior treatment with iodoacetate. Hydroxylaminase assays did not support the possibility of cyclization of alkyl hydrogen disulfide to a dithiodiolactone (22, 23). Another possibility, which has not been investigated, would be air oxidation to cysteine tetrasulfide (Equation 18).

\[
\text{HOOCCH}_2\text{SSCH}_2\text{CH(NH}_2\text{)COOH} + \text{I}^- \rightarrow \text{RSSCHR} + \text{H}_2\text{O} (18)
\]

The enzymatic decomposition of cysteine appears, from these results, to involve a elimination of disulfide from its mono-Schiff base with pyridoxal-P, followed by hydrolysis of the aminocarboxyl-Schiff base to pyruvate and ammonia. From the standpoint of the mechanism of enzyme action, the proposed mechanism (Fig. 5) resembles those postulated for elimination 

\[
\text{HOOCCH}_2\text{SSCH}_2\text{CH(NH}_2\text{)COOH} + \text{I}^- \rightarrow \text{RSSCHR} + \text{H}_2\text{O} (18)
\]

The proposed stoichiometry is shown in Equation 13 (sum: 5, 12).

Cystine + iodoacetate + H_2O → pyruvate + NH_3 + I^- + HOOCCH_2SSCH_2COOH + H^+ (13)
of other electron-captive $\beta$ substituents in model reactions of amino acids and pyridoxal (24, 25). The elimination is depicted as unconcerted, although this has not yet been tested.$^{11}$

It is too soon to interpret the multiple reactions catalyzed by cystathionine cleavage enzyme in terms of the mechanism of action of the enzyme, but the results suggest some relevant questions. Accessible protein sulfhydryl groups are obviously not necessary for catalysis. Except for hydroxynoaminoc acids, the enzyme does not show the expected preference for $\beta$ carbon chain length ($\gamma$ elimination) in the coenzyme-bound portion of the substrate. Although the inhibition by cysteine might only reflect thiazolidine formation (26, 27), the specific decomposition of the disulfide forms also suggest that structural features in the "free" ends of these substrates, and of thiocysteine, should be taken into consideration. Mesocysteine reacts 3 times faster than L-cysteine (Table I), but yields only one equivalent of pyruvate,$^3$ suggesting that the $\beta$ configuration is preferred in the free end, although $\gamma$ is required in that which is bound. More puzzling is the contradictory tentative evidence that $\beta$-allo-cystathionine preferentially undergoes $\beta$ elimination,$^3$ which implies a preference for $\beta$ configuration in the coenzyme-bound end of the thioether.$^{12}$

The reaction resembles the probable mechanism of the base-catalyzed and autocatalytic decomposition of cystine (28–30), in which evolved pyruvate may play a role similar to that of pyridoxal-P (31). Intermediary formation of alkyl hydrogen disulfide has not been demonstrated in the nonenzymatic reaction. However, alternative mechanisms that have been proposed for the latter can be ruled out for the enzymatic reaction. An ionic cleavage between sulfur atoms, following labilization of a hydrogen linked to carbon $\alpha$ to sulfur (32), would not yield pyruvate or the other products identified, nor would hydrolytic cleavage to mercaptan and sulfenic acid (28).

The related base-catalyzed formation of lanthionine from cystine (29, 30, 33) is of interest from the standpoint of cystathionine biosynthesis. Lanthionine is decomposed faster than cysteine by the enzyme, but its postulation as an intermediate would appear superfluous, and would require that cysteine and elemental sulfur be primary products. If the latter were correct, accumulation of cysteine-thioglycine mixed disulfide could only occur through reversal of Reaction 6; this reversal could not take place in the presence of iodoacetate. All the evidence indicates that formation of alkyl hydrogen disulfide must precede that of free $\gamma$-mental sulfur.

The results are also incompatible with intermediary formation of $\text{S}^\circ$, either directly through the action of adjacent active centers, or secondarily through further enzymatic decomposition of alkyl hydrogen disulfide. It is still possible that the latter, or other disulfides, do or may accumulate (cystine-thioglycine mixed disulfide, cysteine tetrathiolide) are very slowly attacked by the enzyme.

The enzyme rapidly decomposes cysteine at low concentrations.

$^{11}$ The evidence for an unconcerted $\gamma$-elimination step in the isomerization of P-homoserine to threonine was equivocal (7). The apparent incorporation of more than one atom of tritium-labeled solvent hydrogen into residual, unchanged substrate could have reflected enrichment in the $\alpha$ position due to subsequent discrimination against tritiated substrate. Degradation of P-homoserine to determine whether $\beta$ hydrogen had undergone exchange was not achieved.

$^{12}$ Liver cystathionine cleavage enzyme catalyzes $\gamma$ elimination from the L isomer, and $\beta$ elimination from L- allo (34).

The question of the physiological importance of this reaction depends on whether appreciable amounts of the disulfide exist inside the cell. Cavallini et al. (9) have shown some alternative reactions which the alkyl hydrogen disulfide might then undergo, without separation of elemental sulfur.

Through reversal of Reaction 10, the stoichiometry of Reaction 9 could also be achieved if the actual substrate were a catalytic amount of cysteine, decomposed according to Reaction 10. We have shown that the disulfide reaction cannot be mediated by

\[
\text{Cysteine} + \text{H}_2\text{O} \rightarrow \text{pyruvate} + \text{NH}_3 + \text{S}^\circ + 2 \text{H}^+ \quad (10)
\]

the decomposition of a catalytic amount of cysteine. However, in many cases of "cystine desulfhydrase" reactions (Reaction 19), the converse situation, in which a catalytic amount of disulfide would mediate the decomposition of cysteine, has not been ruled out. Although cysteine and cystine do not seem to be catabolically equivalent, at least in the whole organism (35), the extreme susceptibility to air oxidation of the former suggests that the desulfhydrase reactions might merit reinvestigation, in some cases (2), to determine whether the actual substrate is the disulfide.$^{13}$

**SUMMARY**

A cystathionine cleavage enzyme purified 400-fold from a mutant strain of *Neurospora* has been found to decompose, besides thiocysteines and hydroxynoaminoc acids, the $\lambda$ and meso forms of cystine and homocystine. This reaction, which requires pyridoxal phosphate, is specific for disulfides; the corresponding mercaptans are not attacked.

When cystine is incubated with the enzyme and a diaryl disulfide, the two disulfides are quantitatively decomposed to two equivalents each of pyruvate, ammonia, and elemental sulfur, and ary1 mercaptan. If diaryl disulfide is replaced by iodoacetate, the products are one equivalent each of pyruvate, ammonia, and cysteine-thioglycine mixed disulfide. The isolation of this derivative of the intermediate alkyl hydrogen disulfide establishes the reaction as a $\beta$-disulfide elimination.

**REFERENCES**


$^{13}$ Since this paper was submitted for publication, this supposition has been shown, in one case, to be correct (56).
Microbial Transsulfuration: the Mechanism of an Enzymatic Disulfide Elimination Reaction
Martin Flavin