Metabolic Interrelations of Sulfur in Proteins, Thiosulfate, and Cystine*

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SUMMARY

Examination of the reactions of protein disulfide sulfur led to the implication of three mechanisms in the removal of sulfur from cysteiny1 residues. (a) Persulfide formation in alkali with subsequent loss of the reactive persulfide sulfur; (b) organic thiocyanate formation by direct attack of CN⁻, followed by elimination of SCN⁻ by OH⁻ or by appropriately located intraprotein nucleophiles; and (c) sulfite reduction of disulfide bonds with formation of S-sulfocysteiny1 residues, followed by elimination of thiosulfate. Spectral as well as kinetic evidence was obtained for persulfide formation from a variety of disulfide compounds. The ease of persulfide formation correlated with the ability of base to abstract a proton from the carbon atom β to the disulfide bond.

Evidence was obtained indicating that sulfur eliminated from cystine or cystinyl residues in vivo occupies a metabolic branch point. Incorporation into cystine of the labeled outer sulfur atom of thiosulfate, the corresponding labeled sulfur in the form of tetrathionate, and labeled elemental sulfur occurred in vivo. Unlabeled sulfide failed to suppress the level of radioactivity incorporated and the inner atom of thiosulfate failed to be incorporated. Evaluation of the time course of incorporation as well as the chemical reactivities of the various sulfur species led to the proposal of protein-coated elemental sulfur as a retained intermediate in mammalian sulfur metabolism.

The major metabolic end product of the sulfur in cysteine and methionine is inorganic sulfate. The ingestion of either of these amino acids by mammals, or the injection of Na₂S₄O₆ or Na₂S₂O₃ leads to a prompt urinary excretion of an equivalent amount of sulfur, 80 to 90% as inorganic sulfate (2). The transsulfuration pathway whereby methionine is converted to cysteine has been studied in detail. Similarly, Fridovich and Handler (3) and Macleod et al. (4) have investigated in detail the oxidation of sulfur at the oxidation level of the outer sulfur atom of thiosulfate. A related goal was to evaluate the physiological significance of the observation that the outer sulfur atom of thiosulfate may have a central position in degradative metabolism.

* This investigation was supported by Research Grants GB-3846 and GB-5666 from the National Science Foundation and by a United States Public Health Service Fellowship 1-F2-AM-33,701 from the National Institute of Arthritis and Metabolic Diseases. A preliminary report has been presented (1).

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§ Research Career Development Awardee 2-K3-GM-18,449 of the National Institutes of Health.
in these studies with the present knowledge regarding the reactivity of organic disulfides.

**METHODS**

**Preparation of A and B Chains of Insulin**—Sulfonated A and B chains of insulin were prepared by the technique of Cecil and Loening (14), using sulfite to reduce the interchain but not the intrachain disulfide bonds of insulin. Insulin was prepared in a concentration of 5 mg per ml in 0.04 M HCl. To each milliliter of this insulin solution, 2.5 ml of freshly prepared 0.2 M Na$_2$SO$_3$ were added, and the mixture was incubated for 4 min at $37^\circ$. Removal of the excess sulfite was performed in a 5-ml electrodeialis cell from the Chemical Rubber Company, Cleveland, Ohio, for 45 min at 100 volts, 50 ma, against distilled water. The rate of flow of the water was 2 liters per hour, maintained by a Vibrostatic pump from the Chemical Rubber Company. At the end of 2 hours, the current had dropped to less than 10 ma.

**Thiosulfate Analysis**—Thiosulfate was assayed by the cupric ion-catalyzed method of Sörbo (15).

**Persulfide Analysis**—The analyses were carried out by a modification of the procedure of Cavallini et al. (16). To the alkaline incubation mixture was added 0.2 ml of 1.0 M KCN, and incubation was continued at $37^\circ$ for 10 min. In all cases, incubation with CN$^-$ for less than 5 min was adequate to give complete conversion of the persulfide labile sulfur to SCN$^-$. If necessary, the pH of the reaction mixture was adjusted by addition of 0.025 M phosphate 0.025 M borate buffer (pH 9.0), or 0.2 M glycine buffer (pH 10.0), or by addition of 1.0 M HCl to pH 7 so that elimination of SCN$^-$ from the organic thiocyanate formed after CN$^-$ addition did not occur. Volume was then adjusted to 1.0 ml with H$_2$O, and SCN$^-$ was determined by adding 0.4 ml of 15% formaldehyde, 0.1 ml of 0.125 M Na$_2$SO$_4$, and 1.5 ml of the ferric nitrate reagent of Sörbo (17) diluted 50% with H$_2$O. Absorbance was measured at 460 nm with a Bausch and Lomb spectronic 20 spectrophotometer, after centrifugation to clarify the solution if necessary. Direct spectrophotometric observation of persulfide formation was done using a Perkin-Elmer model 202 recording ultraviolet and visible spectrophotometer.

**Sulfide Analysis**—Sulfide was measured with a silver sulfide membrane electrode from Orion Research, Inc., Cambridge, Massachusetts.

**Model Disulfides**—The following compounds were utilized in demonstrations of the mechanism of persulfide formation: L-cystine, pyridoxal-5-P, and crystalline bovine serum albumin from Nutritional Biochemicals; N-acetyl-L-cysteine from Pierce Chemical, Rockford, Illinois; GSSG, A grade, from Calbiochem; crystalline bovine insulin from Sigma; and oxidized N-lipoic acid from Aldrich. N,N'-Diacetyl-L-cysteine was prepared by perborate oxidation of a 0.02 M solution of N-acetyl-L-cysteine in 0.025 M tetraborate-0.025 M phosphate buffer (pH 8.0). To this was added 0.2 ml of 0.05 M perborate in the same buffer for each milliliter of N-acetyl-L-cysteine solution. The course of oxidation at room temperature was followed by the disappearance of organic sulfhydryl groups detectable with Ellman reagent (5,5'-dithiobis-2-nitrobenzoic acid) from Calbiochem until more than 95% of the N-acetyl cysteine had been oxidized. The excess perborate was destroyed by adding 20 μg of twice recrystallized catalase from Sigma.

**Preparation of Protein-associated Sulfur**—Elemental sulfur was prepared by adding 1.0 ml of 1.0 M HCl to 0.5 ml of outer labeled thiosulfate solution. The colloidal sulfur formed was permitted to precipitate overnight. The precipitate was then washed with distilled water and dried with a stream of N$_2$. After the sulfur had been permitted to crystallize overnight, it was dissolved in 1 ml of ethanol. Forty milligrams of crystalline bovine albumin (Sigma) were dissolved in 2 ml of H$_2$O, and the ethanoic sulfur was added to this solution dropwise with constant agitation. A gel-like precipitate was separated, dried with a stream of N$_2$, and redissolved in 1.0 ml of deionized H$_2$O. Some colloidal sulfur was present; this was permitted to aggregate and was removed by centrifugation. To the supernatant was added 0.2 ml of 3.0 M trichloracetic acid. The precipitate formed was sedimented and the supernatant discarded. The tube containing the precipitate was rinsed with 0.2 ml of H$_2$O to remove excess trichloracetic acid. The precipitate was then dissolved in 1.0 ml of 0.15 M NaCl to give a clear solution which contained 80 to 90% of the albumin as determined by measuring absorption at 280 μm and 10 to 20% of the radioactive sulfur.

**Assay for Release of Radioactive Sulfur from Protein**—Assay for release of alkaline-solubilizable sulfur was performed by adding an aliquot of the 0.15 M NaCl solution of protein-associated sulfur to H$_2$O (total volume, 1.0 ml). To this was added 0.1 ml of 1.0 M NaOH and the solution was incubated at $37^\circ$. At various intervals, the incubations were terminated by addition of 0.1 ml of 3.0 M trichloracetic acid and the precipitates were recovered by centrifugation.

Assay for persulfide labile sulfur was performed by incubation of an aliquot of the protein solution in 0.5 M NaOH, total volume 0.1 ml. After increasing periods of incubation at $37^\circ$, the mixture was neutralized with 0.5 ml of 1.0 M HCl, and 0.5 ml of 0.2 M KCN was added. Incubation was continued at $37^\circ$ for 5 min; then 0.1 ml of 3.0 M trichloracetic acid was added and the precipitate separated.

Assay for the release of cyanide-labile disulfide sulfur was performed by adding an aliquot of the protein solution to H$_2$O (total volume, 0.1 ml). To this was added 0.1 ml of 0.2 M KCN and the solution was incubated at $37^\circ$. As with alkaline incubation, 0.1 ml of 3.0 M trichloracetic acid was added to terminate the reaction and the precipitates were recovered by centrifugation.

Aliquots of the supernatants of all the samples were counted in scintillation fluid consisting of 250 ml of anisole from Eastman Chemical, 1500 ml of p-dioxane (spectral grade), 250 ml of dimethyl-dithylenyglycol, 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, and 8 mg of 2,5-diphenyloxazole in a Packard liquid scintillation spectrometer.

**Isolation of Labeled Hair, Incorporation in Vivo**—Albino male rats from Sprague-Dawley weighing 90 to 120 g were anesthetized with ether; they were then shaved with electric clippers over the dorsum from the neck to tail and laterally on both sides of the front and back legs. Depilation was completed by applying calcium thioglycolate (Neo), which was washed off after 5 min or long enough to remove the hair stubble. Hair growth resumed in the form of a wave of growth generally beginning laterally. By 2 days it was long enough so that milligram quantities of hair could be pinched readily from less than 10% of the shaved area. The rats were placed either on a normal rat chow diet or on the complete synthetic amino acid diet (originated by J. R. Thompson) which General Biochemicals, Inc., Chagrin Falls, Ohio, modified to reduce the methionine concentration to 113.6 g/100 pounds of diet (lot 671497) (18). In most cases, the rats were given 50 mg of neomycin sul-
fate (from Eli Lilly and Company) per 100 ml of water to be drunk ad lib. On the basis of their consumption of water they obtained 60 to 120 mg per kg of neomycin a day. This dose is effective in significantly decreasing the enteric bacterial flora (19). After 2 days the rats were injected with 1 ml of 0.15 M NaCl containing 4.0 mg of L-serine from Sigma and various amounts of compounds labeled with \(^{14}C\) or \(^{35}S\). Outer labeled thiosulfate was obtained from New England Nuclear. During the period of use, the specific activity had a range of 18 mCi per mmole to 4.5 mCi per mmole. Inner labeled thiosulfate, 23 mCi per mmole, was obtained from Nuclear Chicago (source, The Radiochemical Centre, Amersham, England). Uniformly labeled \( ^{14}C\)-L-cystine, lot 6701, 180 mCi per mmole, and \( ^{35}S\)-L-cystine were obtained from Schwarz BioResearch. During the period of use, the \( ^{35}S\)-cystine had a range of specific activity of 43 to 5.3 mCi per mmole. Injections were intraperitoneal. Mouse hair (20) and human hair (21) have been demonstrated to take up small amounts of injected \( ^{35}S\)-cystine rapidly into growing hair. Thus, rat hair was used to "isolate" \( ^{35}S\)-cystine. Hair was plucked at various times after injection and washed in a Buchner funnel three times each with water, ethanol, and ether, and then dried at 90\(^\circ\) overnight. Weighed samples of hair for scintillation counting were placed in glass vials to which were added 5 ml of toluene containing 2,5-diphenyloxazole (0.43\%, w/v) and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (0.01\%, w/v) (22) both from Packard Instrument Company. Addition of 50 mg of unlabeled hair to 50 mg of labeled hair had no effect on the number of counts recorded within \( \pm 5\% \). Samples of labeled hair of 5 to 55 mg gave the same number of counts per min per mg of hair within \( \pm 3\% \). Samples were counted in a deep well Packard liquid scintillation spectrometer. Radioactivity was measured for sufficient time to reduce the standard error to \( 3\% \) or less.

Isolation of Cystine from Hair—Samples of rat hair, up to 100 mg, were hydrolyzed in 0.5 ml of 6.0 \( \times \) HCl, in a boiling water bath for 6 hours. Aliquots of the hydrolysate were counted as thin layer samples on glass planchets with an open window Geiger-Müller tube. To the remainder of the hydrolysate was added a volume of 6.0 \( \times \) NaOH sufficient to neutralize the HCl. A standard method of cysteine isolation was then used (23). Isolation was carried to the stage of precipitation of the cysteine copper mercaptide. The precipitate was separated and washed three times with ethanol and then suspended in ethanol to an approximate concentration of 6 to 7 mg per ml. Aliquots were plated as thin layer samples for counting. Aliquots of the acid hydrolysates of hair were also chromatographed in acetone-butanol-H\( _2\)O (2:2:1, v/v/v) on Whatman No. 1 paper. This was dried and scanned with a 4-\( \pi \) scanner from Baird Atomic to determine location of radioactivity. Hair Follicular Protein Synthesis—Isolation of hair follicles was by the wax-sheet method of Rogers (24, 25), using male albino rats weighing 150 to 160 g. The wax was used was NoTweeze from Kemna, Inc., Indianapolis, Indiana. After isolation, protein determinations by the modified biuret method (26) gave an average value of 3 mg per ml. The brei of hair follicles was then added to the incubation medium containing an energy source as described by Rogers and Clarke (27). In addition, penicillin G (6000 units) from Abbott Laboratories, streptomycin (1.2 mg) from Pfizer Laboratories, and L-serine (160 \( \mu \)moles) were added. To this was added either 10 \( \mu \)Ci of \( ^{35}SSO_3^- \) (5 mCi per mmole) from New England Nuclear or 4 \( \mu \)Ci of \( ^{35}S\)-cystine (7.3 mCi per mmole) from Schwarz BioResearch. Reaction mixtures were shaken at 37\(^\circ\) with air as the gas phase for 20 min. The protein was then isolated and washed as described by Rogers (27). The dried protein was dissolved and plated on 2-inch stainless steel planchets and counted with an open window Geiger-Müller tube.

Isolation of Free Cysteine from Hair Follicle Incubation—Protein was precipitated and cysteine was separated from inorganic sulfur anions by paper electrophoresis using 0.02 M potassium hydrogen phthalate buffer at pH 4.75. At this pH, cysteine has a fractional positive charge. Ten microliters of supernatant from the reaction mixture were spotted halfway between the ends of a 3-cm wide strip of Whatman No. 1 paper. A potential of 20 volts per cm was applied to the paper for 55 min. After electrophoresis, the paper was sprayed with ninhydrin solution to locate the cysteine and counted with a 4-\( \pi \) counter to determine the location and amounts of radioactivity.

Preparation of Sodium Tetrathionate—Outer labeled thiosulfate was oxidized with 0.2 m I\( _2 \) in aqueous KI until residual I\( _2 \) color was present. The product was chromatographed in acetone-butanol-H\( _2\)O (2:2:1, v/v/v). Scanning with a 4-\( \pi \) counter revealed a single peak with an \( R_F \) of 0.24, which is compatible with the \( R_F \) of 0.27 for tetrathionate obtained separately.

Degradation of Cystinyl Sulfur

Effect of Nucleophilic Attack on Interchain Disulfide Bonds of Insulin—Nucleophilic attack on insulin by sulfite at pH 7 gave rise to a mixture of A and B chains. After electrodialysis to remove small amounts of injected \( ^{35}S\)-cystine rapidly into growing

RESULTS

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Fig. 1. Comparison of release of \( ^{35}SSO_3^- \) and residual \( S\)-sulfocysteynyl groups in a mixture of A and B chains of insulin. A mixture of A and B chains of insulin was prepared by sulfitolysis as described under "Methods." Release of thiosulfate from the \( S\)-sulfocysteynyl residues of insulin (O----O) was followed by incubation of 0.3-ml aliquots with 0.15 ml of 1.0 \( \times \) NaOH for up to 45 min at 37\(^\circ\). The samples were then neutralized with 0.15 ml of 1.0 \( \times \) HCl, and 0.2 ml of 0.2 M KCl and 0.1 ml of 0.1 M CuCl\( _2 \) were added. \( S\)-Sulfocysteynyl residues (O----O) were determined by adding 0.2 ml of 0.2 M KCN to exchange CN\( ^- \) for the sulfite moiety without neutralization or addition of CuCl\( _2 \). The solution was permitted to remain alkaline and elimination of SCN\( ^- \) occurred during an additional incubation for 5 min at 37\(^\circ\). In the absence of Cu\( ^{2+} \), the \( ^{35}SSO_3^- \) already released did not react with CN\( ^- \).
Interrelations of Organic and Inorganic Sulfur

FIG. 2. Formation of SCN⁻ from a mixture of A and B chains of insulin reduced by sulfitolysis. Incubation of 0.16 μmole of reduced insulin in 1.0 ml of 0.35 N NaOH (Δ-Δ), 0.1 N NaOH (●-●), 0.03 N NaOH (■-■), and 0.012 N NaOH (○-○) with varying concentrations of KCN was carried out at 37°C for 30 min. No SCN⁻ was formed in any sample in the absence of added KCN.

FIG. 3. Formation of SCN⁻ from insulin as a function of OH⁻ and CN⁻ concentrations. Incubation of 0.16 μmole of insulin in 1.0 ml of 0.35 N NaOH (Δ-Δ), 0.1 N NaOH (●-●), 0.03 N NaOH (■-■), and 0.012 N NaOH (○-○) with varying concentrations of KCN was carried out at 37°C for 30 min. No SCN⁻ was formed in any sample in the absence of added KCN.

Effects of base on the interchain disulfide bonds of insulin—Incubation of intact insulin with CN⁻ in 0.01 N or 0.03 N NaOH (Fig. 3) resulted in release of SCN⁻ directly related to cyanide concentration in a manner reminiscent of the release of SSO₃⁻ on alkaline incubation of S-sulfocysteinyl insulin. In 0.1 N and 0.35 N NaOH, however, more SCN⁻ was formed at the lower CN⁻ concentrations than would be expected if the OH⁻ acted simply to eliminate SCN⁻ from the organic thiocyanates. At 0.1 N and greater concentration of NaOH, the hydroxide ion also acts to convert disulfide bonds to reactive persulfides, which yield SCN⁻ rapidly in the presence of CN⁻. The amount of SCN⁻ formed under these conditions decreased with increase of cyanide concentration. Competition of the direct nucleophilic attack on the disulfide at increased cyanide concentrations decreased the amount of reactive persulfide formed and, thus, the rate of SCN⁻ formation.

The contribution of the persulfide route to SCN⁻ formation can be separated from that of the direct nucleophilic attack of cyanide on the disulfide bond. As shown in Fig. 4, incubation of insulin for varying periods of time in 0.5 N NaOH at 37°C resulted in formation of persulfide. The pH of the reaction mixture was reduced before addition of cyanide and incubation was then continued to measure persulfide content. Although cyanide could still attack the disulfide bond at the reduced pH, release of SCN⁻ from the organic thiocyanates failed to occur, as shown by the small amount of SCN⁻ in the zero time control. Lowering the NaOH concentration in the initial incubation to 0.08 N resulted in no persulfide formation measured as SCN⁻ even when the alkaline incubation was continued for 1 hour.

The decline in amount of persulfide present after 10 min of incubation in 0.5 N NaOH demonstrated the instability of the insulin persulfide. Measurement of sulfide formation (Fig. 5) showed that sulfide was a product of this decomposition. An initial lag in sulfide formation occurred during rapid accumulation of persulfide. The sulfide concentration then increased as the persulfide decomposed.

Spectral evidence of persulfide formation—When incubation of GS3G in 0.5 N NaOH was followed spectrophotometrically, an absorption peak at 340 μm was observed (Fig. 6). This absorption increased with time. Assay of 0.1 ml aliquots for persulfide
gave a parallel increase in ability to form SCN⁻. Addition of KCN to the sample abolished the absorption at 340 μm.

Incubation of 1.6 μmoles of insulin in 2.0 ml of 0.5 N NaOH resulted in a complex pattern of spectral changes throughout the ultraviolet region. The reference cell contained insulin in a solution of 0.5 N NaOH and 0.5 mM KCN to decompose any persulfide formed. Progressive changes occurred with an apparent maximum change in absorption at 370 μm. Incubation was at 37°, and the absorption reached a peak at 25 min. Addition of KCN to a concentration of 0.05 M in the sample cell abolished the difference in absorption within 2 min. Aliquots adjusted to pH 9.0 with 0.025 M phosphate-0.025 M borate buffer had the ability to form an equivalent amount of SCN⁻.

In a similar experiment, incubation of a mixture of A and B chains of insulin in 0.5 N NaOH gave no spectral changes in the ultraviolet. Aliquots under the same condition as with intact insulin failed to yield SCN⁻ as a function of time of incubation in NaOH.

Formation of Persulfide Related to Structure of Disulfide Compound (Table I)—Free cystine incubated in NaOH failed to yield significant persulfide. Modification of the amino group of cystine by acetylation or of the carboxyl group by esterification resulted in a structure that did yield persulfide. Similarly, persulfide was obtained from the formation of a Schiff base between cystine and pyridoxal-5-P. When cystine assumed an intrapeptide position, as in GSSG, the amount of persulfide liberation proceeds entirely by this mechanism, accumulation of maximum change in absorption at 370 μm. Incubation was at 22-24°, and the absorption reached a peak at 25 min. Addition of KCN to a concentration of 0.05 M in the sample cell abolished the difference in absorption within 2 min. Aliquots adjusted to pH 9.0 with 0.025 M phosphate-0.025 M borate buffer had the ability to form an equivalent amount of SCN⁻.

A plot of persulfide formation from bovine serum albumin related to structure of disulfide bonds, resulted in release of sulfur from the equivalent of only four of the disulfide bonds of albumin formed persulfide. Addition of KCN without neutralization, to measure SCN⁻ release following direct cyanide scission of disulfide bonds, resulted in release of sulfur from the equivalent of an additional five disulfide bonds.

Release of Sulfur from Albumin-associated Sulfur—Sulfur need not be part of cystinyl residues of protein to be released by incubation in alkali or cyanide. A solution of SÉS⁻ associated with bovine serum albumin released labeled sulfur on incubation in 0.5 N NaOH. Addition of CN⁻ after neutralization resulted in the above systems indicated that less than 10% of the SCN⁻ formed under these conditions came from reactions not involving persulfide. Only with Schiff base formation was the control high (40%). In this case, however, the observation can mean only that persulfide resulted immediately upon formation of the Schiff base, as expected (28).

Formation of SCN⁻ from Albumin—Although, as indicated above, protein structure can promote the release of some disulfide sulfur, it can also hinder release of sulfur from disulfide bonds. A plot of persulfide formation from bovine serum albumin related to time of incubation in NaOH was similar to that of insulin (Fig. 7). However, the maximum yield of 0.2 μmole of SCN⁻ indicated that the equivalent of only four of the disulfide bonds of albumin formed persulfide. Addition of KCN without neutralization, to measure SCN⁻ release following direct cyanide scission of disulfide bonds, resulted in release of sulfur from the equivalent of an additional five disulfide bonds.

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<table>
<thead>
<tr>
<th>Compound</th>
<th>Time of incubation</th>
<th>Disulfide present</th>
<th>Persulfide formed</th>
<th>Conditions of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Cystine</td>
<td>180 min</td>
<td>2.0 μmole</td>
<td>0.025 μmole</td>
<td>0.4 ml of 0.05 M cystine in 0.2 N NaOH + 0.2 ml of 1.0 N NaOH</td>
</tr>
<tr>
<td>L-Cystine + pyridoxal-5-phosphate</td>
<td>90 min</td>
<td>2.0 μmole</td>
<td>0.4 μmole</td>
<td>0.2 ml of 0.01 M cystine in 0.2 N NaOH + 0.4 ml of 1.0 N NaOH + 0.2 ml of 0.02 M pyridoxal-5-P</td>
</tr>
<tr>
<td>N,N'-Diacetyl-L-cystine</td>
<td>150 min</td>
<td>1.0 μmole</td>
<td>0.12 μmole</td>
<td>0.2 ml of 0.005 M N,N'-diacetyl-L-cystine in 0.025 M phosphate-0.025 M borate buffer (pH 8) + 0.6 ml of 1.0 N NaOH</td>
</tr>
<tr>
<td>L-Cystine diethylester di-HCl</td>
<td>210 min</td>
<td>1.0 μmole</td>
<td>0.35 μmole</td>
<td>0.1 ml of 0.01 M cystine diethylster di-HCl in 0.2 M glycine buffer (pH 10.0); 0.8 ml of 0.2 M glycine buffer added at end of incubation</td>
</tr>
<tr>
<td>Glutathione</td>
<td>15 min</td>
<td>1.0 μmole</td>
<td>0.4 μmole</td>
<td>0.2 ml of 0.005 M GSSG in 0.5 N NaOH</td>
</tr>
<tr>
<td>Insulin</td>
<td>10 min</td>
<td>0.32 μmole</td>
<td>0.21 μmole</td>
<td>0.2 ml of 5 x 10⁻⁸ M insulin in 0.5 N NaOH + 0.05 ml 1.0 N HCl + 0.65 ml of 0.025 M phosphate-0.025 M borate buffer (pH 9.0) added at end of incubation</td>
</tr>
</tbody>
</table>

* Times of incubation were selected to give maximum yield of persulfide for each compound. All incubations were at 37°.

* Only the two interchain disulfide bridges are considered.

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Fig. 6. Glutathione persulfide formation as a function of time as measured by increase in ultraviolet absorption. GSSG (40 μmoles) was incubated in 2 ml of 0.5 N NaOH against a blank containing these components and in addition 0.1 ml of 1.0 N KCN. Readings were taken at various times during incubation at 22-24°. After 90 min, 0.1 ml of 1.0 N KCN was added. The absorbance after 5 min of additional incubation is indicated by the dashed line.
Fig. 7. A comparison of SCN⁻ release from bovine serum albumin as a function of time of incubation in NaOH with and without neutralization before addition of KCN. A mixture of 0.1 ml of 6 × 10⁻⁴ M bovine serum albumin and 0.1 ml of 1.0 N NaOH was incubated for up to 45 min at 37°. Samples were neutralized with 0.1 ml of 1.0 N HCl, 0.6 ml of 0.05 M phosphate-0.05 M borate buffer and 0.1 ml of 0.2 M KCN were added (pH 9.0 to 9.5), and incubation was continued for 5 min at 37° (O--O). Samples incubated with 0.7 ml of H₂O added in place of the HCl and phosphate-borate buffer (O---O).

Fig. 8. Accumulation of radioactive sulfur in hair after injection of various quantities of ³⁵S-cystine or ³⁵SSO₄²⁻. The maximum amount of radioactivity per mg of hair in rats was plotted against the number of microcuries of ³⁵S-cystine (O--O) or ³⁵SSO₄²⁻ (O---O) per 100 g of rat weight injected intraperitoneally 2 days after depilation. The slope of the line thus reflects the specific activity of cystine in hair as related to the amount of radioactivity injected, corrected for the size of the animal. The slope of the line for ³⁵S-cystine was 126; that for ³⁵SSO₄²⁻ was 3.5.

Incubation of labeled precursors with isolated hair follicles excluded the possibility that the outer sulfur atom of thiosulfate was being incorporated directly into cystine of hair. Hair follicles failed to incorporate radioactive sulfur from ³⁵SSO₄²⁻ under conditions that gave incorporation of ³⁵S-cystine into protein at a level of radioactivity 6- to 7-fold greater than the zero time control.

Electrophoresis in phthalate buffer (pH 4.75) of aliquots from follicles incubated with ³⁵SSO₄²⁻ showed no radioactive material other than thiosulfate.

Immediate Cystine Biosynthesis—Intraperitoneal injection of ³⁵SSO₄²⁻ into weanling rats provided evidence relating to the physiological significance of the mitochondrial cystine biosynthesis observed previously (13). Regrowing hair rapidly took up ³⁵S-cystine injected intraperitoneally (Fig. 8). Injection of tracer quantities of ³⁵SSO₄²⁻ resulted in oxidation and excretion of 40 ± 10% of the labeled sulfur atoms as sulfate. Of the retained outer sulfur atom, only a small amount was incorporated initially into the cystine of hair (Fig. 8). A plot of the amount of radioactivity per milligram of hair against the dose amounts of radioactivity per weight of rat revealed that the injected quantities of both thiosulfate and cystine were true tracer amounts (Fig. 9). Confirmation of this fact was obtained by showing that addition of 4 mg of Na₂SO₄·5H₂O to the 0.4 mg of outer labeled thiosulfate injected failed to lower the level of incorporation of radioactive sulfur into hair. Comparison of the slopes of the two lines in Fig. 9 indicated that the labeled outer sulfur atom of thiosulfate was only 3% as effective a source of radioactivity in hair as was labeled cystine. Assuming that synthesized and injected cystine enter the same metabolic pool, only 3% of the labeled outer sulfur atom of thiosulfate was incorporated immediately into cystine. These data might have indicated repression of cystine biosynthesis by the normal dietary levels of cystine and methionine. However, rats placed on a diet containing a limited amount of methionine as the only organic sulfur source failed to show increased cystine biosynthesis.
After restriction of dietary methionine for 2 (Table II, Experiment 2) through 5 days, the proportionate increases in specific radioactivity of the hair after injection of 36SSO32- was followed by repeated depilation to remove the previously labeled hair. The decline in radioactivity in radioactivity (Fig. 11) following 5S-cystine injection reflected the slight radioactivity observed was cystine.

**Long Term Cystine Biosynthesis**—The decline in radioactivity after injection of labeled compounds was followed by repeated depilation to remove the previously labeled hair. The decline in radioactivity (Fig. 11) following 5S-cystine injection reflected pulse labeling of the cystine pool. The decline in radioactivity appearing in hair was slower after the injection of 5S-cystine, suggesting continued 5S-cystine synthesis from a relatively large pool of sulfur. Since thiosulfate itself was rapidly absorbed and metabolized, the outer sulfur atoms of thiosulfate must have been retained in some intermediate form or forms not readily oxidized to sulfide but available for cystine biosynthesis.

The presence of 5S-polythionates ("OzSS2SO2-") where z was 1...
to 4) and radioactive elemental sulfur associated with proteins in rat serum was readily detectable after intraperitoneal injection of $^{35}$SO$_4^{2-}$. Such radioactive materials can be identified either by electrophoresis in phthalate buffer (pH 4.75) or by chromatography as described under "Methods" for identification of tetrathionate.$^1$ Injected tetrathionate, formed by oxidation of $^{35}$SO$_4^{2-}$ with IO$_3^-$ gave essentially the same levels of $^{35}$S cysteine in hair as did an equivalent amount of $^{35}$SO$_4^{2-}$ (Table II, Experiment 4). Elemental radioactive sulfur in ethanol was added to a solution of bovine serum albumin to form protein-associated sulfur. Again the route of injection was intraperitoneal. Despite the slow absorption expected for this colloid containing foreign protein, incorporation of radioactive sulfur into the cystine of hair was observed (Table II, Experiment 5). Maximal accumulation of radioactivity in hair occurred after 4 to 5 days, as compared with 1 to 2 days following injection of outer labeled thiosulfate or labeled cystine.

**DISCUSSION**

Since Abel and Geiling (29) first crystallized insulin, proteins have been observed to release cysteinyl sulfur on incubation in mild alkali (30), with formation of inorganic sulfide and lanthionine. However, this easy release of sulfur fails to occur generally with disulfides that are not in peptide linkage. Thus, the mechanism of release of sulfur from proteins must account for the failure of release from cystine under conditions that permit easy release from cysteinyl residues.

Detailed studies of simple disulfides have led to the proposal of various mechanisms for OH$^-$ attack. Danehy has recently reviewed these mechanisms (31), two of which will be considered in this discussion. Schöberl first postulated S-nucleophilic attack on the disulfide bond by OH$^-$ (32). The hypothetical sulfenic acid formed could decompose to yield H$_2$S. Tarbell and Harnish (33) first suggested that OH$^-$ acts instead as a base abstracting a proton from the sulfur $\beta$ to the sulfur atom. In the case of a peptide this would result in the formation of a cystinyl persulfide and a dehydroalanyl residue. The persulfide would decompose to yield free sulfur or, in the presence of a free sulphydryl group, inorganic sulfide.

\[
\text{RSS\textsubscript{CH}CH + OH}^- \rightarrow \text{RSS\textsubscript{CH}C}^- + \text{H}_2\text{O}
\]

The release of sulfur from insulin in alkaline solution occurs both by S-nucleophilic attack on disulfide bonds and by intermediate persulfide formation. The strong S-nucleophiles cyanide and sulfite cleave disulfide bonds with formation of organic thiocyanates (RSSCN) and organic thiosulfates (RSSO$_4^{2-}$), respectively. Alkaline incubation releases sulfur from these as SCN$^-$ or SS0$_3^{2-}$. Hydroxide itself, however, fails to behave as an efficient S-nucleophile in this system. Stapleton and Swan (34) observed the resistance to aqueous alkali of $\alpha,\alpha$-dimethyl-cystine, which contains no proton on either $\beta$ carbon, as compared with cystine. By contrast, both compounds have the same reactivity with the S-nucleophiles sulfite and cyanide, suggesting that OH$^-$ acts as a base and not as a nucleophile.

At low CN$^-$ concentration, increasing the concentration of OH$^-$ increased persulfide formation, with a resultant increased yield of SCN$^-$ from the reactive persulfide sulfur. An increase in CN$^-$ concentration resulted in competition for the disulfide, with formation of the less reactive thiocyanatoalanyl residues, thus decreasing the rate of SCN$^-$ formation. Metabolically, Catsimopoulos and Wood (35) have postulated that S-nucleophilic attack of CN$^-$ on cystine may be the major route by which SCN$^-$ is formed in vivo. By analogy, S-nucleophilic attack of SO$_4^{2-}$ may be a major route of SS0$_3^{2-}$ formation in vivo.

Incubation of GSSG in NaOH provides spectral evidence for the occurrence of persulfide intermediates. These data confirm the kinetic evidence showing persulfide formation from GSSG reported by Gawron and Odstrchel (36). Incubation of insulin provides similar evidence for persulfide intermediates. The sequential relationship of sulfide release following persulfide formation shows that the release of sulfur during alkaline incubation of protein is by the $\beta$-elimination mechanism of Tarbell and Harnish.

The ease of release of sulfur from interpeptide cystine as compared with free cystine upon alkaline incubation can be understood partly in terms of modification of the electron density about the $\beta$ carbon atom as this relates to proton abstraction by OH$^-$. Acetylation and esterification of cystine decrease electron density at the $\beta$ carbon atom in a fashion similar to the Schiff base formation with pyridoxal-5-P studied by Flavin (28), and permit significant persulfide formation. Peptide bond formation would be expected to have the same electronic effect as acetylation and esterification, and persulfide formation during alkaline incubation of GSSG was in fact observed.

In intact protein, additional considerations arise from the effects of secondary and tertiary structure. In insulin at least 1 of the carbon atoms $\beta$ to each of the interchain cystinyl residues must be positioned so that OH$^-$ attack with proton abstraction may take place. With albumin, in contrast, it appears that few of the cystinyl residues are so located as to permit persulfide formation, although others may be available for cyanide attack on the disulfide linkage, as shown by Catsimopoulos and Wood (37) and confirmed here. This effect may be a result of the S-nucleophilicity of CN$^-$ or it may reflect the ability of CN$^-$ to penetrate to hydrophobic regions of protein structure very readily, as it does in the rhodanese mechanism (38). Finally, the tertiary structure of proteins, by providing strategically positioned nucleophilic groups, may facilitate rapid breakdown of the intermedium persulfide or organic thiocyanate. Bohak (39) has shown the formation of N$\textsubscript{2}$-(or-2-amino-2-carboxyethyl)-L-lysine with disappearance of cystinyl and lysyl residues, in hydrolysates.
of several proteins incubated in 0.2 N NaOH at 40°C. He implicated the positional relationships of the lysine and cystine as determining the occurrence of the nucleophilic attack of the ε-amino group.

It is likely that all of the foregoing mechanisms are operative in the release of cystinyl sulfur from proteins in alkaline cyanide solution. Moreover, the nonenzymatic mechanism for β-elimination of sulfur from cystinyl residues is also in accord with biochemical mechanisms postulated from the investigations of Cavallini et al. (16, 40), and of Flavin (28) for the action of cystathionase upon cystine. The possibility for an enzyme system acting directly upon cystinyl residues to form persulfides has not been examined, but it seems probable that cystine sulfur does become available metabolically at the persulfide level.

Following formation of persulfide from cystine or cystinyl residues, rhodanese may catalyze the transfer of the persulfide sulfur to sulfite to form thiosulfate (8). In more general terms, cystathionase upon cystine. The possibility for an enzyme system catalyzing the transfer of the persulfide sulfur may contribute to a pool of metabolically active inner sulfur atoms of polythionates and, possibly, elemental sulfur itself. In this state, sulfur is at a metabolic branch point. Oxidation to sulfate and excretion may occur, or the sulfur may be retained. Szczepkowski, Skarzynski, and Weber (41) have demonstrated retention of the outer sulfur atom of SSO₃⁻ under conditions leading to oxidation of the inner sulfur atom to sulfate. Furthermore, Koj et al. (9) have been unable to account for all of the outer sulfur atom as sulfate in their mitochondrial system after complete metabolism of the thiosulfate, again suggesting a retention or recycling of this sulfur.

Previously we had demonstrated the incorporation of the outer sulfur atom of thiosulfate into cysteine without participation of sulfide as an intermediate by a rat liver mitochondrial preparation (13). Now similar incorporation has been demonstrated to occur in vivo. As in the system in vitro, a large excess of unlabeled sulfide failed to decrease the level of cysteine synthesis from ³⁵SSO₃⁻. Huovinen and Gustafsson (12) have demonstrated incorporation of ³²S⁻ into cysteine in germ-free rats. If ³²S⁻ were an intermediate between ³⁵SSO₃⁻ and ³⁵S-cystine, addition of unlabeled sulfide would be expected to dilute the relative radioactivity and, thus, the specific activity of the protein. It is unlikely that the metabolic pool of sulfide as such is large since small doses of sulfide are fatal. This is in contrast to the demonstrated large metabolic pool in which the outer sulfur atom of thiosulfate participates. Thus, either sulfide is incorporated into cystine by a pathway separate from that of the outer sulfur atom of thiosulfate or both may contribute to a common pool which is very large relative to the administered concentration of either.

The increased cystine biosynthesis from ³⁵SSO₃⁻ when intestinal bacteria were suppressed confirmed that this was mammalian, not bacterial, biosynthesis of cysteine. The failure of the inner sulfur atom of thiosulfate to be incorporated indicates that sulfur in vivo is irreversibly lost when highly oxidized.

Only a small percentage of the label from injected ³⁵SSO₃⁻ was incorporated into the cystine of hair immediately. Labeled thiosulfate was only 3% as effective an immediate source of radioactivity in hair as was ³⁵S-cystine itself, indicating a limited rate of synthesis of cystine utilizing the outer sulfur atom of thiosulfate. Furthermore, a diet with limited methionine for 2 to 7 days before injection of thiosulfate failed to increase the effective immediate yield of ³⁵S-cystine. Nevertheless, a significant synthesis of ³⁵S-cystine continued for a substantial period of time. The slower rate in decline of ³⁵S-cystine appearing in hair after injection of ³⁵SSO₃⁻ as compared with the pulse label of ³⁸S-cystine suggested the existence of a pool of slowly metabolized sulfur.

Elemental sulfur and polythionate sulfur would be possible members of this pool of slowly metabolized sulfur. Both appear to be immediate products of thiosulfate metabolism. Furthermore, both were incorporated into cystine in vivo much as was the outer sulfur atom of thiosulfate. Meister, Fraser, and Tice (42) have demonstrated the formation of elemental sulfur from β-mercaptoptiyruvate by several rat tissue preparations in vitro; β-mercaptoptiyruvate is a possible intermediate product of cystine degradation. The extent to which elemental sulfur occurs in vivo associated with protein could not be determined readily. It was not possible to distinguish such sulfur from the cystinyl sulfur of protein on the basis of reactivity with alkali and cyanide, for example. Release of this sulfur occurred under the same conditions that caused spontaneous decomposition of cystinyl residues via persulfide intermediates as well as the conditions leading to direct nucleophilic attack on intact disulfide bonds.

During the 1930's, injection of "colloidal" elemental sulfur was used in the treatment of rheumatoid arthritis. A review by Comroe (43), however, revealed a lack of convincing proof that this treatment was therapeutically effective, and the use of elemental sulfur was discontinued. Although the postulated role of the injected elemental sulfur was to replenish depleted sulfur stores, especially cystine, the metabolic fate of the injected sulfur was never established. It is noteworthy, however, that elemental sulfur associated with intracellular protein would provide a form of retained sulfur having metabolic activity that could account for the prolonged synthesis of cystine from the injected outer sulfur atom of thiosulfate.

Moreover, although it has not been feasible to measure quantitatively the various forms contributing to the pool of metabolically active retained sulfur, the tracer experiments with thiosulfate showed the effective total pool to be of substantial size. Thus, the outer sulfur of thiosulfate may contribute to an important metabolic function. Although this sulfur can be oxidized to sulfate and excreted in the urine, with diminution of the sulfur stores of the body, a portion of it can also be transformed into a retained metabolic intermediate. Elemental sulfur associated with protein appears to be an appropriate form for this intermediate, being slowly metabolized to provide sulfur for cysteine biosynthesis over a long period of time.

REFERENCES
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