REDUCTION OF THE DISULFIDE GROUP IN CYSTINE

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CHAPTER I

GENERAL INTRODUCTION

Cystine residues have an important role in the structure of proteins, especially the keratins, and the study of the reduction of cystine is of interest in connection with the chemistry of these substances. Several methods for the reduction of cystine have been tried and are described in the next chapter. The object of the research described in this thesis was to study the possible utility of sodium sulfide and sodium hyposulfite for this purpose. These reagents afford the additional advantage that excess of them might be removed easily by acidification after reduction is completed.

The ultimate purpose is to develop methods for the reduction of disulfide bonds in proteins, but the present study has been restricted to cystine, since it is better to investigate a substance of definitely known structure and purity. In the remainder of this introductory chapter, the role played by the disulfide group in the structure and activity of proteins and in keratolysis, is discussed. Some other relevant properties of cystine and cysteine are also given, knowledge of which is helpful in the investigation to follow.

The Importance of Disulfide Bonds in Proteins

Globular Proteins. Most globular proteins contain some cystine residues (17, 61). Some of the most important proteins and their

cystine contents are: insulin (12.5%), β-lactoglobulin (2.3%), ovalbumin (o.51%), pepsin (1.6%), casein (0.34%), ribonuclease (6.51%), human serum albumin (5.6%), lysozyme (6.8%) and ovomucoid (6.7%). If cysteinyl residues are absent, cystinyl residues may usually be detected by the nitroprusside test, after treatment with cyanide. Some proteins give the test only if denatured before treatment with cyanide, and some do not react at all.

The disulfide bond has profound structural significance in proteins. It may link two separate polypeptide chains or it may link two segments of a single chain; in the latter case a closed loop results. Disulfide bridges contribute to the folded nature of many proteins. Proteins with high cystine content are found to be highly resistant to all types of denaturation except that by alkali. This can be explained by the fact that the disulfide linkage forming the interchain bond in proteins is attacked by alkali.

Fibrous Proteins. The protective materials, hair, horn, wool and epidermis, consist largely of proteins together classified as keratins, that contain rather large quantities of cystine. The disulfide bonds link neighboring polypeptide chains together and the insolubility of the keratins is due partly to the cross-linking by these bonds. Reduction of the disulfide bonds solubilizes the keratin in acid or alkali (22). The characteristic mechanical and chemical stability of keratins is also due in large part to the disulfide cross-linking.

Insulin is an interesting case; although it has been classified as a globular protein, it can form fibers reversibly. Insulin retains its fundamental structure even in its fiber form (33), and apparently disulfide bonds are not involved in association to the fibrous form,

as the stability of insulin fibers is not affected by the creation or destruction of disulfide bonds.

Unhairing Action. Reduction of the disulfide bonds is important in unhairing. The mechanism of unhairing was formerly believed to involve reduction of the disulfide bridges and hydrolysis of the other linkages by alkali (66). According to the modern view, unhairing involves first the breaking of disulfide bridges by hydroxyl ions, while the depilatory substance (alkali sulfide, cyanide or sulfite) prevents the formation of new cross links between adjacent protein chains, which would lead to increased stability (24). Reduction of the disulfide linkages to sulfhydryl groups followed by re-oxidation to the disulfide state in a new orientation is the basis of the process for the "cold" permanent waving of hair (13).

Enzyme Activity. The liberation, measurement, and significance of the sulfhydryl and disulfide groups in enzymes has been treated in reviews by Neurath et al (42), by Anson (3), and, for the former only, by Barron (5). It was found by Vines (63) and by Mendel and Blood (41) that certain proteolytic enzymes could be activated by hydrocyanic acid, hydrogen sulfide, etc., and the reaction was said to be reversible. The active substance apparently contained the sulfhydryl group, while the inactive enzyme gave a negative nitroprusside test (48). On the other hand, the hormone insulin is active in its natural state and contains no sulfhydryl groups, but loses its activity when its disulfide linkages are reduced to sulfhydryl groups by thioglycolic acid, cysteine, etc., (29, 53). The product obtained by reoxidizing the sulfhydryl groups back to disulfide is completely inactive because of the low

probability that the original pairs of thiol groups would reunite. Disulfide groups are also necessary for the activity of enzymes, hormones, and crotoxin (45). Thus, drastic reducing conditions would tend to destroy the activity of most biological proteins that contain cystine (43).

Properties of Cystine and Cysteine

Cystine usually is prepared by hydrolysis of keratin from hair. Cysteine is prepared by reduction of cystine. Cystine is sparingly soluble in water, insoluble in alcohol or acetic acid, soluble in acids or alkalis. Cysteine is readily soluble in all these media. L-Cystine, which is the only isomer used in this work, has a high value of specific rotation, $[\alpha]_D = -214.4^\circ$ in 1 M hydrochloric acid at 25° (27). It is quite stable in acid but undergoes slow racemization in alkali. The specific rotation of L-Cysteine has a very low value, $[\alpha]_D = +7.6^\circ$ in 1 M hydrochloric acid (27). Cysteine in solution is oxidized by air; it is fairly stable in acid, but readily oxidized in alkali. Cysteine has a characteristic absorption peak at 232-240 mμ in strongly alkaline medium and very little absorption above 220 mμ in acid (7). Cystine has an absorption maximum at about 250 mμ both in acid and alkali (6).

CHAPTER II

REDUCTION OF THE DISULFIDE BOND IN CYSTINE AND PROTEINS--REVIEW OF THE LITERATURE

The Cystine-Cysteine System

As already stated cysteine is readily oxidized in solution, either by atmospheric oxygen or by other oxidizing agents. Cystine, on the other hand is relatively easily reduced to cysteine. Several attempts have been made during the last thirty years to determine the oxidation potential of the system,

$$\underset{\text{(Cysteine)}}{2\ RSH} \rightleftharpoons \underset{\text{(Cystine)}}{RSSR} + 2\ H^{+} + 2\ e^{-}$$

but greatly divergent results have been obtained (9), primarily because no suitable electrode system could be found which would respond reversibly to this mercaptan-disulfide system. The oxidation-reduction potential calculated from thermal and ionization-constant data was $E^{o} = +0.390$ v. at 25° and pH 7.0 (28). Ryklan and Schmidt (52) obtained a value of $E^{o} = +0.15$ v. (vs. N. H. E.) at pH 7.0; this was determined by potentiometric titration with iodine in the presence of 1 N hydrogen iodide. Kolthoff et al (32) obtained an average value of $E^{o} = +0.074$ v. (vs. N. H. E.) at the dropping mercury electrode in the pH range 4.98-9.2. This value is in good agreement with that obtained at a pretreated mercury macro-electrode (21), and that calculated from the equilibria in the iron cysteine-cystine system (60).

Eldjarn and Pihl (15) found that the oxidation-reduction potential of the cysteine system was higher than that of glutathione by 0.01 v. at pH 7.4 and 37°.

Methods of Reduction of Cystine

Several methods have been tried to reduce cystine; these are briefly described here.

Tin and Hydrochloric Acid. Harrison (25) reduced L-cystine by heating it with tin and 33% hydrochloric acid in the presence of a few drops of dilute platinum tetrachloride solution. Later Lavine (36) reduced 0.240 g. of cystine in 100 ml. of 1 N hydrochloric acid with tin dust. The dissolved tin was precipitated by hydrogen sulfide, and the hydrogen sulfide was blown out by carbon dioxide. The cysteine obtained was 101% of theoretical value.

Zinc and Hydrochloric Acid. This procedure is the one most commonly used for the reduction of cystine. Typical procedures used protein hydrolysate as the starting material. In one procedure, 10 ml. of the solution was taken, 0.5 ml. of concentrated hydrochloric acid and a small amount of powdered zinc were added, and the solution was gently warmed for a few minutes and allowed to stand for 1 hour at room temperature. The solution was then filtered and the precipitate washed (57).

In another procedure, sufficient protein to contain 2-5 mg. of cystine was boiled with about 50 times its weight of concentrated hydrochloric acid for 12 to 18 hours. Most of the hydrochloric acid was distilled off in vacuo and the residue dissolved in 10 ml. of

water. About 100 mg. of zinc dust was added and the cystine was reduced by heating under reflux for 1 hour (23). The reaction may be represented as

$$RSSR + 2\,H^{+} + Zn \longrightarrow 2\,RSH + Zn^{++}$$

Sodium Amalgam (58). Approximately 0.2% sodium amalgam was prepared by dilution of a 2% commercial preparation with mercury. Dilution rendered the amalgam liquid at room temperature and facilitated measurement of it by volumetric methods. Seven milliliters of cystine solution in 0.1 N hydrochloric or sulfuric acid was taken in a test tube and 1 ml. of the amalgam was added. It was allowed to stand for 1 hour with occasional shaking. The cystine was completely reduced.

Zinc Amalgam (36). Reduction to the extent of 99.8% was obtained when 1.20 g. of cystine in 50 ml. of 2 N hydrochloric acid solution was treated with an excess of amalgamated zinc for 17 hours.

Aluminum Amalgam (20). L-Cystine was dissolved in carbon dioxide-free water and 1-1.5 times its weight of aluminum amalgam was added. The mixture was heated for approximately one hour. Since evolution of hydrogen sulfide was noticed, the reaction proceeded to some extent to other products than cysteine. The material was filtered, the filtrate was acidified with hydrochloric acid and evaporated to dryness in a vacuum.

Electrolytic Reduction (26). Seventy-seven milligrams of cystine dissolved in 200 ml. of 2% hydrochloric acid was reduced electrolytically with a current of 40 milliamperes in a cell of 37 mm. diameter.

The cysteine formed was determined amperometrically. Reduction to the extent of 100% was claimed. The same theoretical amount of reduction was obtained when sulfuric, hydrochloric and sulfosalicylic acids (each 4%) were employed as media. The investigators claimed that not more than 90% reduction could be obtained at best using zinc.

Catalytic Hydrogenation. In general catalytic methods have not been used because of the difficulties of catalyst poisoning by sulfur and desulfurization, i.e., replacement of sulfur by hydrogen in the C-S bond; some attempts, however, have been made. Cystine (4.8 g.) in 60 ml. of 1 N hydrochloric acid in the presence of 1-2 g. of palladium sponge absorbed about 2 atoms of hydrogen in 6 hours and gave an almost quantitative yield of cysteine hydrochloride (8). Kavanagh (31) reduced cystine (2 g.) in 50 ml. 2 N hydrochloric acid quantitatively with a catalyst contaning 500 mg. polyvinyl alcohol, used as a protecting agent for the catalyst, and 100 mg. palladium in 50 ml. water; the reduction took 45 hours at room temperature and atmospheric pressure. Only partial reduction occurred with 10 mg. palladium.

Sodium Sulfite. In contrast to the methods of reduction so far mentioned, when sodium sulfite is added to cystine only one mole of cysteine is obtained per mole of cystine reacted. The reaction has been extensively studied. Clarke (12) represented the reaction as

$$R\text{-}S\text{-}S\text{-}R + Na_2SO_3 \rightleftharpoons R\text{-}S\text{-}Na + R\text{-}S\text{-}SO_3\text{-}Na$$

Stricks and Kolthoff (56), by means of polarographic measurements, showed that the reaction is reversible in alkaline medium (pH 8 to 13), the equilibrium being attained from both sides. The equilibrium constant

for the reaction was found to be 9×10^{-2} at pH 7.75 and 0.6×10^{-2} at pH 13.4. Cecil and McPhee (11) have studied the kinetics of the reaction. They concluded that the reaction above pH 9 is a simple reversible bimolecular reaction, but that the mechanism is complex below pH 9. They also found, in contradiction to some of the results reported earlier, that the disulfide bond of cystine reacts only with sulfite ions and not with bisulfite ions. Disulfides containing negatively charged groups were found to react much more slowly than those with no net charge (39). Thus there was an optimum pH for the reaction; below it, the sulfite concentration decreased, and above it the concentration of negatively charged disulfide ions increased.

Sodium Cyanide. Mauthner (38) first studied the action of cyanide on cystine. Pulewka and Winzer (47) later confirmed that the reaction occurs according to the equation

$$R\text{-}S\text{-}S\text{-}R + CN^- \rightleftharpoons R\text{-}S^- + R\text{-}SCN$$

According to Schöberl and Hamm (54) the cysteine and cysteine thiocyanate can be isolated in theoretical amounts, as the lead mercaptide and the copper salt respectively, if the reaction is stopped after 60 minutes. The thiocyanate was found to be surprisingly stable toward acids; it was not affected by refluxing 6 hours in 20% hydrochloric acid. With hydrocyanic acid, conversion of cystine to 0.49 equivalents of cysteine occurred when the mixture was heated to 95°C. for 1 hour; in no case did the yield of cysteine exceed 50%, showing that the reaction is similar to that with cyanide ion (18).

Sodium in Liquid Ammonia (62). Cystine in liquid ammonia was

reduced to cysteine by metallic sodium; 4 g.-atoms of sodium were needed to reduce 1 mole of cystine.

Irradiation (59). When a 0.5% solution of L-cystine in 0.1 N hydrochloric acid or sodium hydroxide was subjected to ultra-violet irradiation, a 5% yield of cysteine was obtained. Sulfur and hydrogen sulfide also formed.

Enzymatic Reduction (50). An enzyme, cystine reductase, was found in preparations from baker's yeast, Candida albicans, and pea seeds, which catalyzed the reduction of cystine to cysteine. Reduced diphosphopyridine nucleotide could serve as the source of hydrogen.

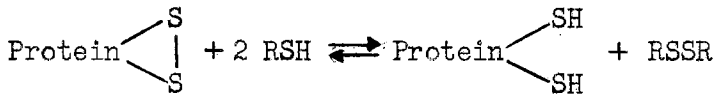
Reduction by a Mixture of Chromous and Chromic Chlorides and by Vanadous Chloride (46). These reagents were found to reduce cystine and the reaction rate was observed to be of the second order. The rate decreased with increasing acidity.

Borohydrides. Calvin (10) reported that there was considerable difficulty in reducing the disulfide bond by means of borohydride and suggested that the bond might be susceptible to attack only by free-radical-type reagents (e.g., zinc and acid).

Lithium Aluminum Hydride (4). The disulfide linkage in organic molecules was effectively reduced in ether-type solvents. The disulfide bond was broken and a lithium-aluminum complex was formed. Mercaptans were obtained by hydrolysis of the complex with dilute acid.

Methods for Reduction of Disulfide Bonds in Proteins

A considerable amount of work has been done on the reduction of disulfide linkages in proteins. It is generally brought about with an excess of a thiol compound like glutathione, cysteine, thioglycolic acid, monothioglycol or a low molecular weight mercaptan. Little is known about relative effectiveness but thioglycolate is claimed to be 50 times more effective in reducing the lactogenic hormone than cysteine (19). Studies have been made on insulin (65), crystalline urease (44), lens proteins (14) and papain (64). The reaction may be represented as



Although reductions have been carried out in solutions of extreme pH, neutral or slightly alkaline medium is generally used. Reducing agents like zinc or tin in acid, sodium amalgam, and sodium in liquid ammonia have been tried, but they are found to be most suitable only for proteins such as insulin which can withstand inactivation by the acid or other treatment. Cyanide and sulfite (or bisulfite) have been used and they have been found to act on the disulfide bond by addition.

Cyanides, sulfites, sulfides and thioglycolic acid have been tried as possible unhairing agents. Aliphatic amines, stannous chloride and mercaptans have also been proposed.

CHAPTER III

REACTION OF CYSTINE WITH SODIUM SULFIDE IN SODIUM HYDROXIDE SOLUTION

As has already been pointed out in the introductory chapter, cystine is a substance of considerable importance in biochemistry, primarily because of its participation in the composition of many proteins, and of the unique role which it plays in establishing and maintaining protein structure. Owing to the interest which attaches to this substance, many of its physical and chemical properties have been intensively studied, but these studies have not extended except in a cursory way, to the reaction with sodium sulfide (1, 2). The present investigation was undertaken to obtain fundamental information concerning this type of reaction. A considerable part of the material covered in this chapter is in course of publication (49).

EXPERIMENTAL

Reagents

All reagents were of analytical reagent grade, except as otherwise specified.

L-Cystine, "cfp" grade and L-Cysteine Hydrochloride Hydrate (purified grade) were obtained from the California Foundation for Biochemical Research, Los Angeles.

Nitrogen was of commercial grade and was purified by passing it

through a solution of vanadous ion (40).

Water used in the preparation of all solutions was distilled, deionized by passing through Amberlite MB-1 resin, deaerated by boiling and cooling with a stream of nitrogen bubbling through it, and stored out of contact with air.

Sodium Sulfide solutions were prepared by dissolving crystals of $Na_2S \cdot 9 H_2O$, which had been washed clean of yellowish spots of sulfur and polysulfide, in 0.2 M sodium hydroxide, except where otherwise stated.

Sulfur, "precipitated," was obtained from Baker and Adamson, New York, N. Y.

Polarimetric Measurements

These experiments were conducted to determine approximately the extent of reaction between cystine and sodium sulfide by means of a polarimeter. The method is based on the fact that L-cystine has a high value of levo-rotation whereas the rotation of cysteine is almost negligible, especially in acid medium.

Optical Activity of L-Cystine and L-Cysteine. Cystine, 1.00 g., was dissolved in 100 ml. of 1 M hydrochloric acid. The rotation of the solution at about 25° was found to be -4.17° in a 2-dm. tube, corresponding to $[\alpha]_D = -208.5°$. The molar rotation of cystine in 0.1 M sodium hydroxide was -92°. The rotation of cysteine in 1 M hydrochloric acid was found to be zero.

Oxidation of Cysteine. Some experiments were done to find a suitable oxidation procedure for cysteine. A 0.05 M cysteine solution was

prepared and adjusted to pH 8.0 by adding solid sodium hydroxide pellets. Ferrous sulfate was added to make the solution 1% in ferrous ions. Air was passed slowly through the solution for 1 hour, hydrochloric acid was added to 1 M concentration, and the optical rotation was determined. It was found that approximately 98% of the cysteine taken was oxidized to cystine by this procedure.

Standardization of Sodium Sulfide Solution. Approximately 1 M sodium sulfide solution was prepared in 1 M sodium hydroxide, and its titer determined exactly by titration. An aliquot of the sulfide solution was added with stirring to a known excess of standard 0.1 N iodine solution containing 1 M hydrochloric acid and 1 M potassium iodide. The excess iodine was titrated back with standard 0.1 N sodium thiosulfate solution; starch, the indicator, was added just before reaching the end point. The concentration of the sulfide solution used in these experiments was estimated to be 0.7935 M. The standardized solution was stored under nitrogen.

Reaction of Cystine with Sodium Sulfide. Cystine (0.03328 M) solution was made in 0.1 M sodium hydroxide. Nitrogen gas was bubbled through the cystine solution for 10-15 minutes. Fifty milliliters of the solution was pipetted into a 100 ml. conical flask and 4.2 ml. of the sodium sulfide solution was added. A yellow color developed gradually as the reaction proceeded. After two hours, 11.0 ml. of 6 N hydrochloric acid was added. Some sulfur precipitated on acidification and some remained in the colloidal state. The sulfur precipitate was filtered out, and the filtrate was centrifuged for half-hour. The optical activity of the solution (1 M in hydrochloric acid) was found

to be -1.08° using a 4-dm. tube, corresponding to $[\alpha]_D = -41°$. On the basis of this data, the reduction of cystine was calculated to be about 80% complete. In this experiment, two moles of sulfide were used for each mole of cystine. The experiment was repeated using 4 moles of sulfide per mole of cystine and about 85% reduction was obtained.

The procedure described under 'Oxidation of Cysteine' was employed to reoxidize the cysteine obtained by reduction of cystine. The acidified solution was neutralized with sodium hydroxide pellets to pH 8.0, ferrous sulfate was added, and air was passed in for 1 hour. The solution was acidified to 1 M acid concentration and the specific rotation was found to be -200°. This experiment showed that almost all the reduced cystine could be recovered and that the reduction product was cysteine.

Experiments with Rudolph Model 80 High Precision Polarimeter. More precise measurements were made by G. Gorin with the help of a Rudolph Model 80 high precision polarimeter, modified for photoelectric recording by Mr. Donald Sproul (Department of Biochemistry, University of California, Berkeley) (49). In a typical experiment, an 0.02 M solution of cystine in 0.2 M sodium hydroxide, having a rotation of -0.415° (1-dm. tube; $[\alpha]_D = -87°$) was treated with varying amounts of approximately 1 M sodium sulfide; in one case, 5.00 ml. was added, giving a concentration of 0.0166 M cystine and 0.188 M sulfide. The levo-rotation decreased rapidly, and finally levelled off at a value of -0.033°; this value varied little in the interval between 2000 and 6000 seconds. The reaction mixture was allowed to stand about 90 minutes, and then was made strongly acid. After removal of precipi-

tated sulfur and hydrogen sulfide, the optical rotation of the solution, then approximately 1 M in hydrochloric acid, was found to be -0.016°; this corresponds to less than 2% of the original amount of optically active cystine. An aliquot portion of this solution was made 1 M in potassium iodide, then treated with iodine solution drop by drop until a small excess had been added, and finally decolorized with a drop or two of sodium thiosulfate. Measurement of the optical activity then gave a value of -0.296°, which corresponded to regeneration of 95% of the original cystine activity (the solution having been diluted 3.3 times, but the activity now being measured in 1 M hydrochloric acid, in which $[\alpha]_D = -215°$).

Table I summarizes some data obtained with varying concentrations of sulfide.

TABLE I

POLARIMETRIC STUDY OF CYSTINE-SULFIDE MIXTURES

Concn. Cystine M	Concn. Sulfide M	"Half-Life" Secs.	Final Rotation 1-dm. Tube
0.0185	0.0689	760	-0.068°
0.0180	0.1030	503	-0.043°
0.0167	0.1550	250	-0.030°
0.0166	0.1880	-	-0.017°
0.0143	0.3100	-	-0.012°

The values called "half-life" actually measure the times required for the initial optical rotation to decrease to half the value, and do not truly correspond to half-lives of reaction, because the final rotations were not zero. However, these values were small enough to make little difference.

Effect of Alkali. Experiments were done to find out if any cystine was racemized or destroyed in the procedure used. It was found that about 3% of cystine disappeared during the experiment. The rotation of cystine solutions in 0.1 N sodium hydroxide solution was found to be almost constant over the period of time used for the experiment.

Spectrophotometric Measurements

General. Spectra were scanned with a Beckman DK-1 Spectrophotometer; some optical density measurements at a fixed wave length were done with a Beckman DU Spectrophotometer. Sodium sulfide solutions were prepared shortly before measurement. In all these experiments, except where specifically indicated otherwise, both the cystine and the sulfide solutions were made up in 0.2 M sodium hydroxide, which in turn was prepared in carefully deaerated water, and stored under nitrogen. The solutions were mixed and transferred to the spectrophotometer cell, taking care to minimize exposure to air; the cell had a ground glass stopper and was filled completely with liquid, i. e., no air space was left above the solution.

Ultraviolet Spectra of the Reactants and the Products. The spectra of cystine, cysteine, and sodium sulfide solutions are shown in Figures 1 and 2. A small quantity of sulfur was added to 0.2 M sodium hydroxide and spectrum measured against sodium hydroxide; no absorption was found between 280 and 360 mμ.

When cystine and sodium sulfide solutions were mixed, rapid spectral changes took place. The resulting spectra are shown in Figures 3, 4, 5 and 6; except in some cases, as noted, the spectrum was allowed to develop fully before it was determined. The composition

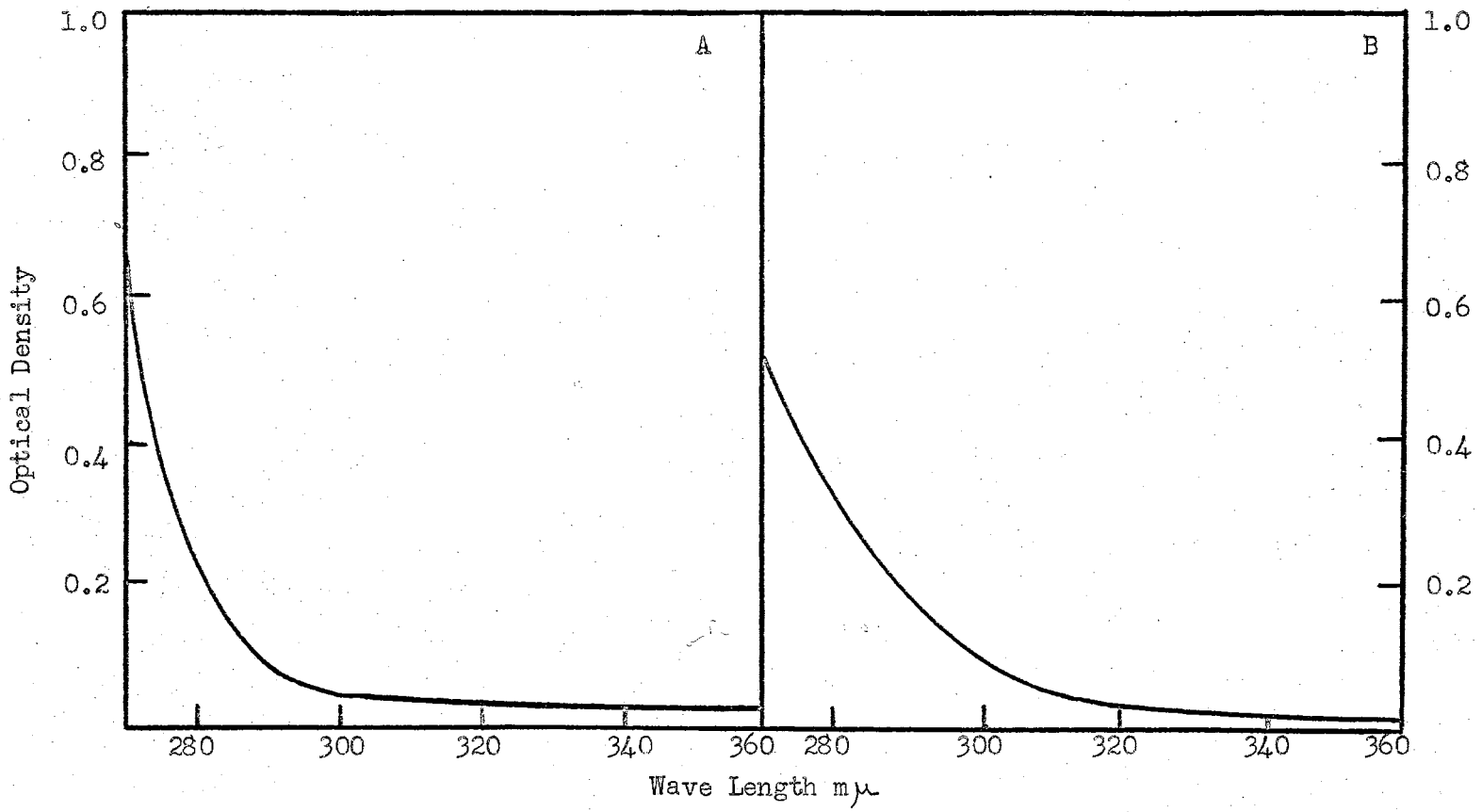


Figure 1

A: 0.004 M Cysteine in 0.2 M NaOH.

B: 0.002 M Cystine in 0.2 M NaOH.
0.2 M NaOH used as reference in both cases.

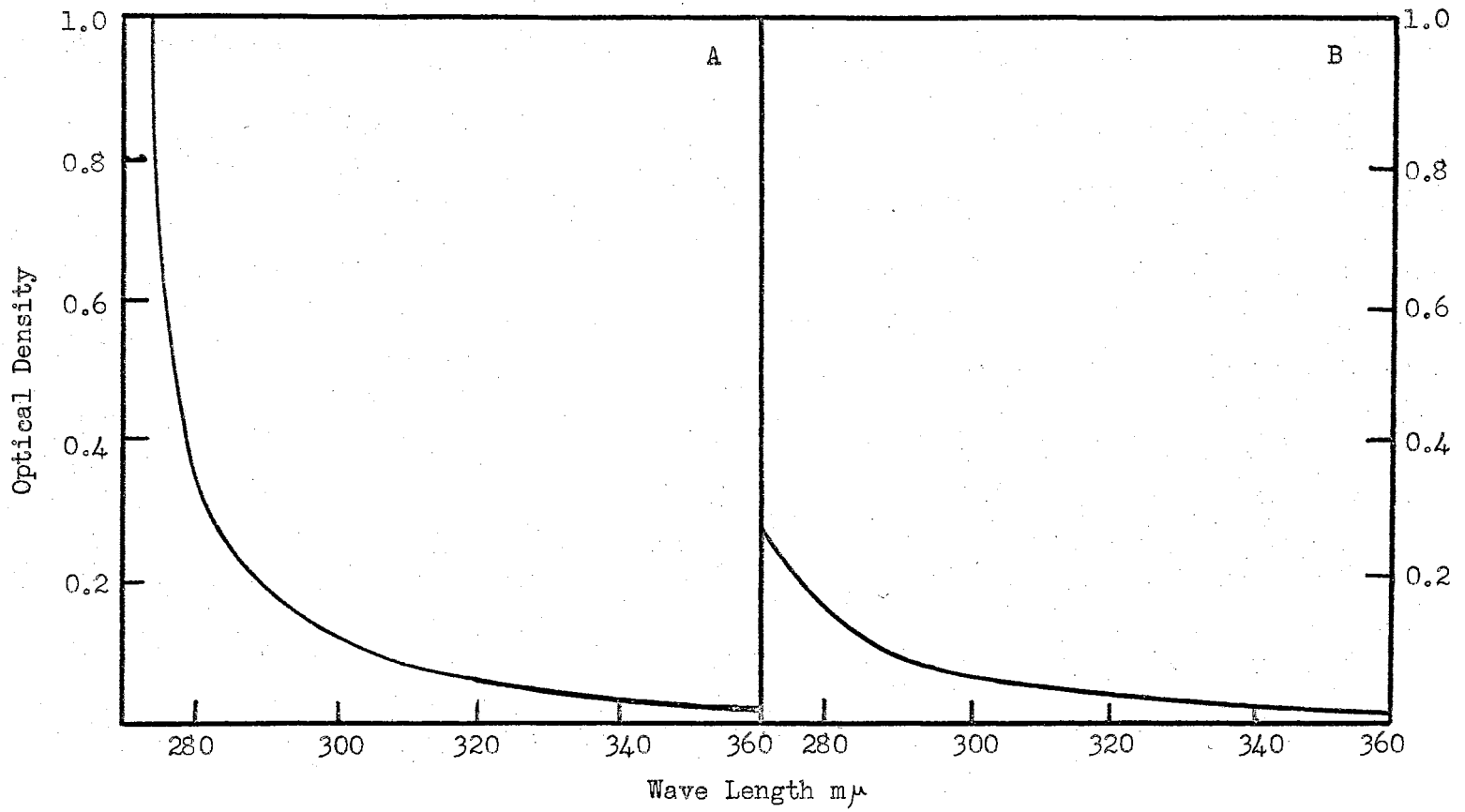


Figure 2

A: 0.1 M Na_2S made in 0.2 M NaOH vs. 0.2 M NaOH.
B: 0.004 M Cysteine in 1 M Na_2S vs. 1 M Na_2S.
0.2 M NaOH used as solvent medium.

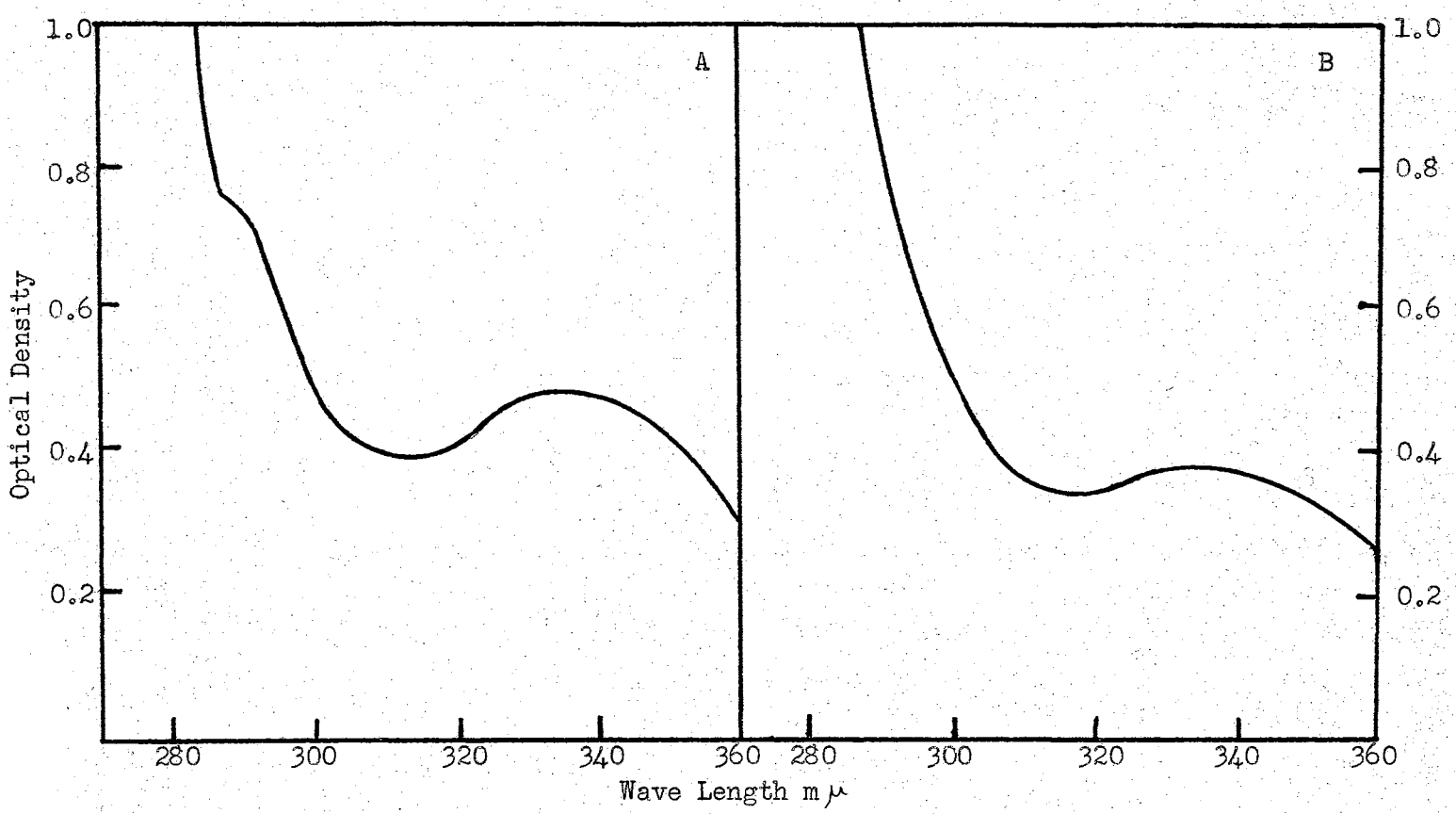


Figure 3

A: 25 ml. 0.04 M Cystine + 1 ml. 0.052 M Na_2S, 1 hr. after mixing, vs. 0.0385 M Cystine.
B: 25 ml. 0.04 M Cystine + 4 ml. 0.52 M Na_2S, 28 mins. after mixing and diluted 25 times with 0.2 M NaOH vs. 0.2 M NaOH.

0.2 M NaOH used as solvent medium in all cases.

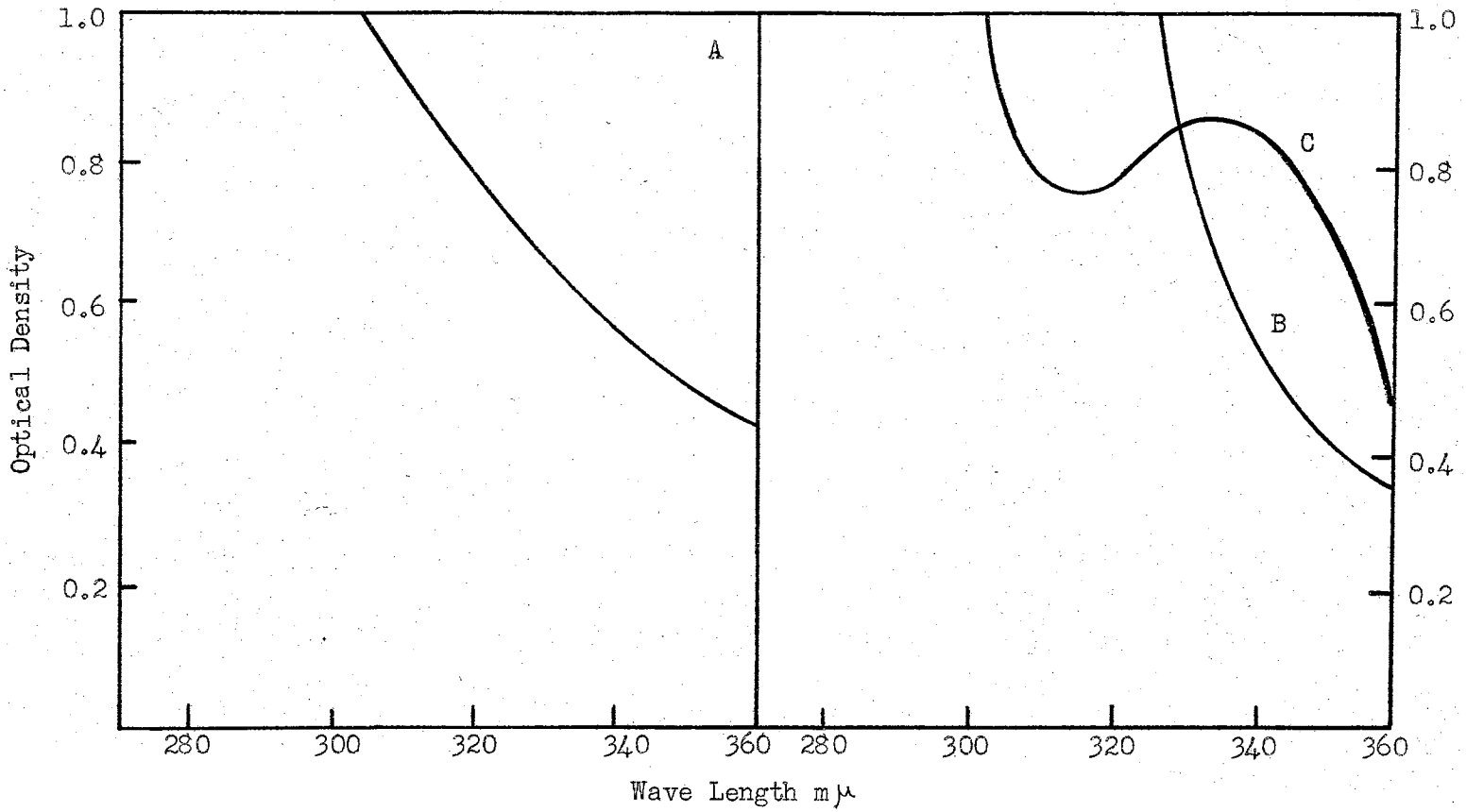


Figure 4

A: 3 ml. 0.008 M Cystine + 2 ml. 0.516 M Na_2S + 7 ml. 0.2 M NaOH, 25 mins. after mixing, vs. 0.086 M Na_2S.

B: 0.05 M disulfide prepared by dissolving 0.054 g. Sulfur in 30 ml. of 1.5 M Na_2S, diluted 25 times with 0.2 M NaOH vs. 0.06 M Na_2S.

C: 0.002 M disulfide + 0.36 g. Cysteine vs. 0.06 M Na_2S + 0.36 g. Cysteine.

0.2 M NaOH used as solvent medium in all cases.

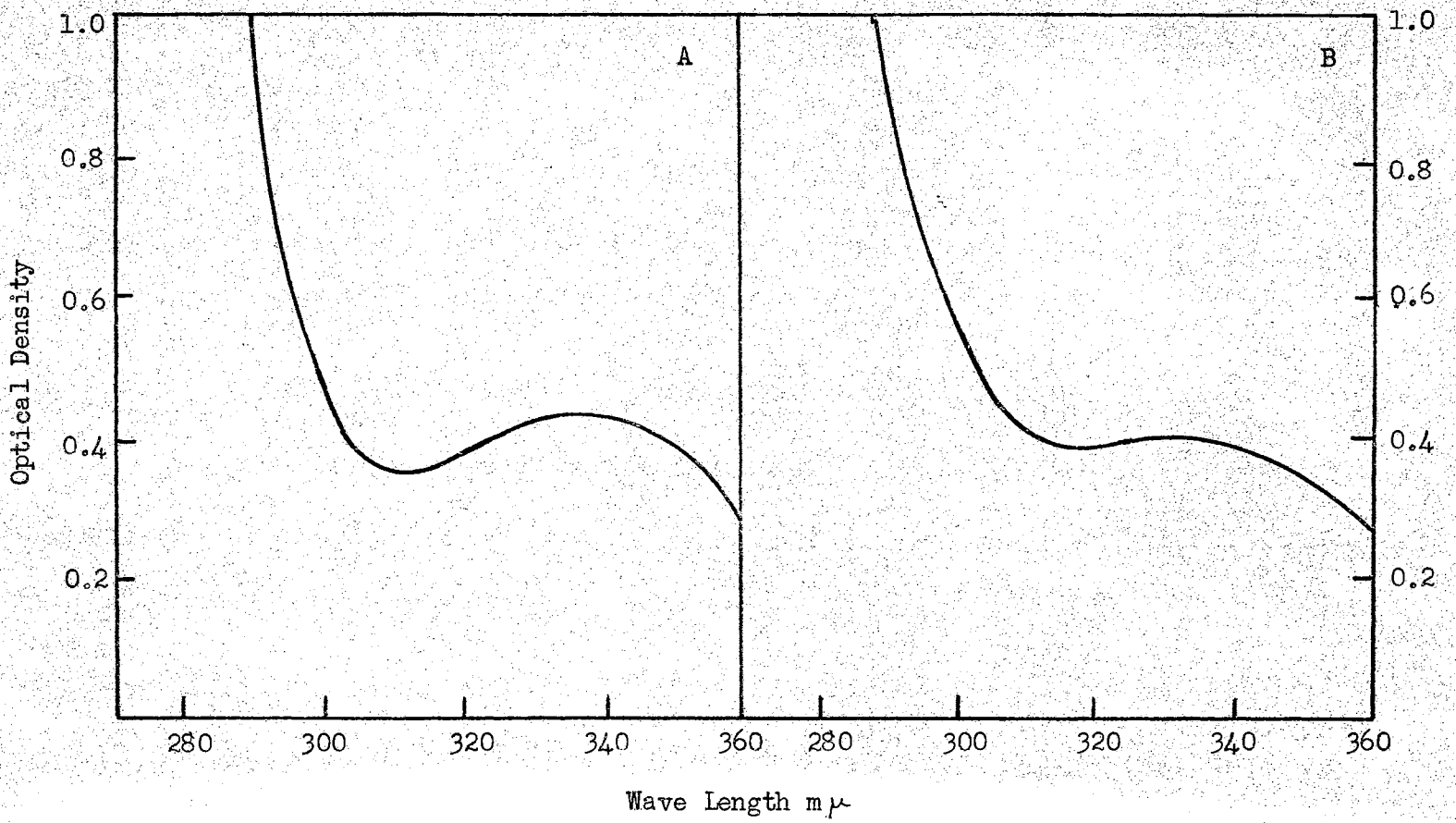


Figure 5

A: 25 ml. of solution B Fig. 3 + 0.002 g. Cysteine vs. 0.2 M NaOH + 0.002 g. Cysteine.
B: 25 ml. 0.04 M Cystine + 6 ml. 0.52 M Na_2S, 30 mins. after mixing and diluted 25 times with 0.2 M NaOH vs. 0.2 M NaOH.
0.2 M NaOH used as solvent medium in all cases.

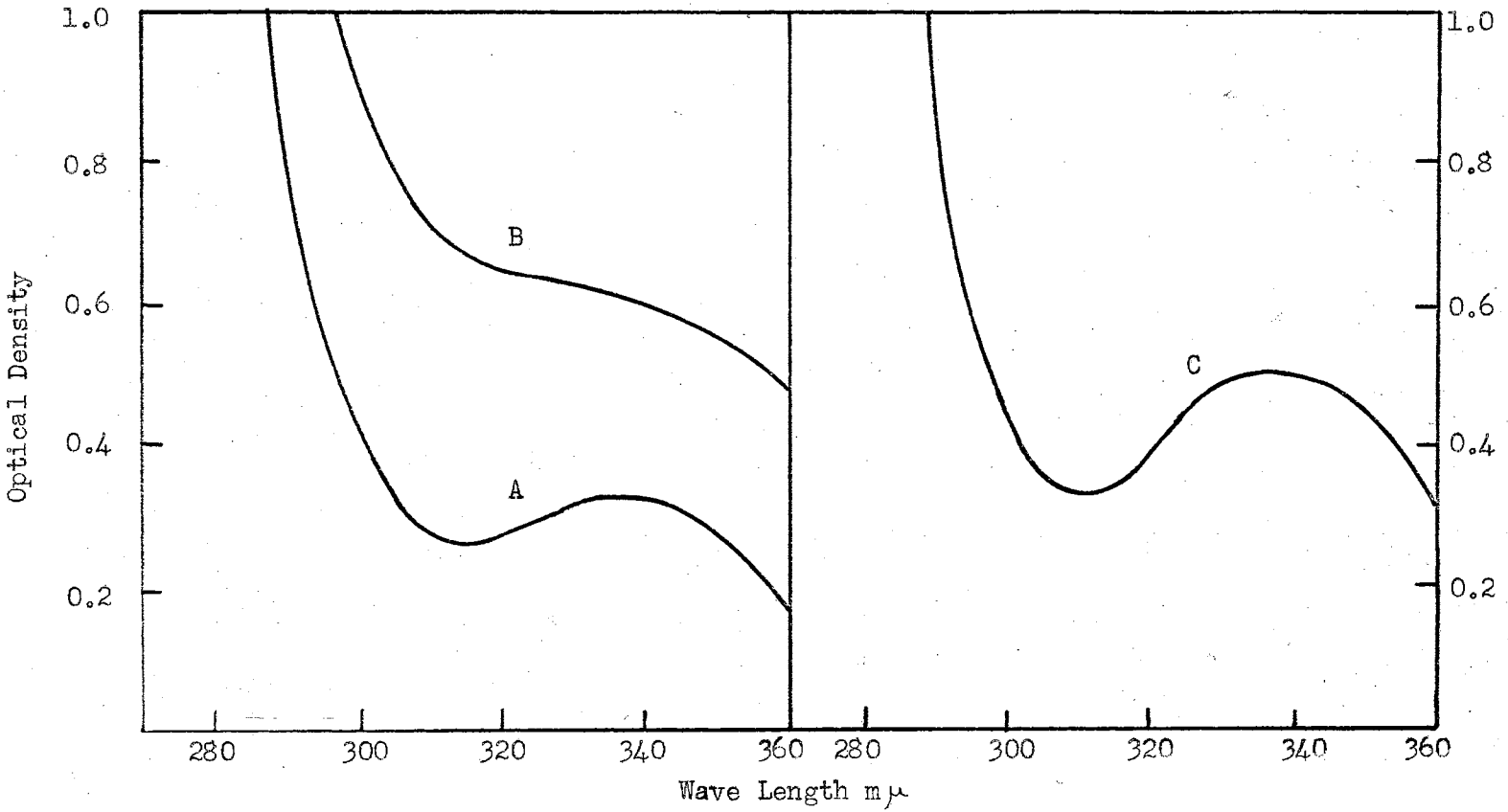


Figure 6

A: 3 ml. 0.008 M Cystine + 2 ml. 0.516 M Na_2S + 7 ml. 0.2 M NaOH, 7 mins. after mixing, vs. 0.086 M Na_2S.
B: Solution A, 15 mins. after mixing.
C: 0.02 M Cysteine in 0.2 M NaOH + a small quantity of sulfur kept for 7 mins. after stirring vs. the Cysteine solution.
0.2 M NaOH used as solvent medium in all cases.

of each mixture is fully detailed below each Figure, but the list below gives a brief description of the experiments done. The Beckman DK-1 spectrophotometer was used to scan these curves.

(1) A large excess of cystine was mixed with sodium sulfide; no yellow color was visually perceptible-Fig. 3, Curve A.

(2) Two equivalents of sodium sulfide were added to one equivalent of cystine-Fig. 3, Curve B.

(3) A large excess of sulfide was added to a small quantity of cystine, and the spectrum measured after about 7, 15 and 25 minutes; a bright yellow color developed. An absorption maximum developed at about 270 mμ (not shown) on keeping the solution for some time-Fig. 6, Curves A and B, and Fig. 4, Curve A, respectively.

(4) A small quantity of cysteine was added to solution (2)-Fig. 5, Curve A.

(5) One more equivalent of sodium sulfide was added to solution (2)-Fig. 5, Curve B.

(6) Sodium disulfide was prepared by dissolving 0.055 g. of sulfur in 50 ml. of 1.5 M sodium sulfide; the solution developed a lemon-yellow color. An absorption maximum (not shown) was obtained at about 270 mμ -Fig. 4, Curve B.

(7) Cysteine was added to the disulfide solution (6) until its concentration was approximately equal to that of sulfide-Fig. 4, Curve C. The slope of the disulfide curve gradually flattened in the range 300-340 mμ as cysteine was added, but the absorption maximum at 335 mμ did not clearly develop until the cysteine concentration was brought to the level stated.

(8) A small quantity of sulfur was added to cysteine dissolved in 0.2

M sodium hydroxide, stirred and quickly measured-Fig. 6, Curve C. On keeping the solution in contact with sulfur, within about an hour's time the solution turned yellow and the maximum at 335 mμ disappeared. The curve then assumed the shape of Fig. 4, Curve A.

Rate and Extent of Reaction. These experiments were done to establish the effect of sulfide concentration on the rate and the extent of reaction of cystine with sodium sulfide. Table II shows the change in optical density measured at 335 mμ with time, at constant cystine concentration with varying equivalents of sulfide. In the case where one equivalent of sodium sulfide was used, 25 ml. of 0.04 M cystine, 2 ml. of 0.5 M sodium sulfide and 4 ml. of 0.2 M sodium hydroxide were mixed together. In all other cases, 25 ml. of cystine solution was mixed with varying volumes of sodium sulfide and sodium hydroxide and the total volume was kept at 31 ml. The solutions after mixing were immediately transferred to the spectrophotometer cell and the optical density was measured at various intervals of time. Nine-millimeter spacers were used in both the cells. Sodium hydroxide (0.2 M) was used as blank, as the absorption due to cystine or sodium sulfide was not significant in these experiments.

In Table III, the maximum optical density reached at 335 mμ, and the time required to reach the maximum, as the sulfide concentration was changed, are shown. The cystine concentration was kept constant at 0.03226 M in the first set of data and at 0.0182 M in the second.

TABLE II

CHANGE IN OPTICAL DENSITY OF CYSTINE - SULFIDE MIXTURES WITH TIME

0.03226 M Cystine in 0.2 M NaOH vs. 0.2 M NaOH

Time in Minutes	Optical Density at 335 mμ, 1 mm. Optical Path: No. of Equivalents of Sodium Sulfide per Equivalent of Cystine				
	0.5	1.0	1.5	2.0	3.0
5	0.051	0.111	0.222	0.250	0.410
10	0.065	0.180	0.390	0.450	0.700
15		0.248	0.531	0.623	0.920
20	0.084	0.310	0.650	0.760	1.050
25		0.369	0.750	0.880	1.100
30	0.101	0.419	0.810	0.960	1.135
35		0.480	0.870	1.035	1.148
40	0.115	0.534	0.920	1.052	1.152
45		0.590	0.940	1.075	1.152
50	0.129	0.630	0.970	1.075	1.152
60		0.700	1.005	1.075	
70	0.160	0.735	1.020		
90		0.772	1.020		
115	0.213	0.800			
125	0.215	0.800			
145	0.225				
155	0.225				

TABLE III

MAXIMUM OPTICAL DENSITY AND TIME TAKEN TO REACH THE MAXIMUM IN CYSTINE-SULFIDE MIXTURES

Moles Sodium Sulfide per Mole Cystine	Maximum Optical Density, 1 mm. Optical Path	Time Taken to reach the Maximum, Minutes
0.03226 M Cystine in 0.2 M Sodium Hydroxide		
0.5	0.225	145
1.0	0.800	115
1.5	1.020	70
2.0	1.075	45
3.0	1.152	36
0.0182 M Cystine in 0.2 M Sodium Hydroxide		
1.5	0.498	157
1.8	0.518	110
2.0	0.535	89
3.0	0.543	50

Analytical Determination of the Extent of Reaction

Procedure for the Determination of Cysteine. The iodimetric procedure for the estimation of cysteine was standardized. Iodine solution was prepared just before use by mixing about 3 ml. each of 6 N potassium iodide and 6 N hydrochloric acid, and 10 ml. of standard potassium iodate, the final solution being approximately 1 M in iodide and acid. Cysteine (0.2-0.3 g.) was weighed into the iodine solution, allowed to react for 3-5 minutes while shaking the solution, and the excess iodine was titrated back with standard thiosulfate solution. The results obtained are shown in Table IV. By this method the cysteine used was found to be about 97 to 98% pure, which is in agreement with the value obtained by the amperometric procedure (30).

TABLE IV

IODIMETRIC DETERMINATION OF CYSTEINE IN A COMMERCIAL SAMPLE

Amount of Cysteine taken g.	% Cysteine Found	Deviation From Mean %
0.2029	97.7	0.3
0.2372	97.5	0.1
0.2448	97.6	0.2
0.2452	97.1	0.3
0.2466	97.7	0.3
0.2572	99.1	1.7
0.2615	95.9	1.5
0.2954	98.3	0.9
	Mean 97.4	Average Deviation 0.65

Reaction of Cystine with Sodium Sulfide in 0.2 M Sodium Hydroxide. Cystine (about 0.123 g.) was accurately weighed into a 50-ml. round-bottom flask, and sufficient 0.2 M sodium hydroxide was added so the final volume, after addition of 2.4 M sulfide, would be 20 ml. The solution was stirred with a magnetic stirrer until the cystine had dissolved, the sodium sulfide solution was added, the air above the solution was displaced with nitrogen, the flask was closed, and the solution stirred for the length of time required to give constant cysteine titer (1 hour for the smallest concentration of sulfide to 15 minutes for the largest). The temperature was maintained at 30°C. Then, approximately 5 ml. of ice-cold 6 N hydrochloric acid was added,

and the hydrogen sulfide was expelled. In one set of measurements, this was accomplished by stirring and bubbling nitrogen through the solution for 1.5 hours, and in the other by boiling the solution under vacuum with gentle heating and vigorous stirring for 10-15 minutes, both procedures giving nearly the same results. The solution freed from hydrogen sulfide was cooled and added to about 25 ml. of standard iodine solution, prepared from standard potassium iodate and sufficient potassium iodide and hydrochloric acid to give a final concentration of approximately 1 M in iodide and hydrogen ions; after standing a few minutes, the excess iodine was titrated with standard sodium thiosulfate. The data obtained with different amounts of sodium sulfide are given in Table V.

TABLE V

CONVERSION OF CYSTINE TO CYSTEINE

Cystine - 0.0256 M

H_2S distilled off under vacuum		H_2S expelled by passing Nitrogen	
Moles Sulfide/ Mole Cystine	% Conversion Based on Cystine	Moles Sulfide/ Mole Cystine	% Conversion Based on Cystine
0.5	38.4		
1.0	70.1	1.5	88.0
2.0	89.7	2.3	90.0
3.9	95.2	2.9	93.5
7.8	95.8	9.5	95.0
10.1	96.0	14.3	95.8

Blank experiments were run, first on solutions containing a representative amount of sodium sulfide, by which it was established that the procedure employed expelled all but a small amount of hydrogen

sulfide; and, secondly, on mixtures of cysteine (0.2 to 0.3 g. of cysteine hydrochloride hydrate) and sodium sulfide (1 to 10 equivalents) in which about 97% of the cysteine originally taken could be recovered; the two corrections, i.e., for residual hydrogen sulfide and for loss of cysteine during manipulation nearly cancelled one another, so no correction was applied to the results obtained by titration on the cystine-sulfide samples. The accuracy of the determination is believed to be about $\pm$ 2%.

Some experiments were done in which excess of cystine was allowed to react with sodium sulfide and the cysteine formed was determined. The general procedure was essentially the same. In this set of experiments the sodium sulfide concentration was kept constant at about 0.019 M and the concentration of cystine varied; 0.6 M sodium hydroxide was used to dissolve the sodium sulfide and cystine. The data obtained are given in Table VI. The data from Tables V and VI are summarized and shown in terms of moles in Table VII.

TABLE VI

CONVERSION OF CYSTINE TO CYSTEINE

Sulfide - 0.019 M

Moles Cystine/ Mole Sulfide	% Conversion Based on Sodium Sulfide
1.0	71.0
2.0	81.6
7.0	94.8

TABLE VII

CONVERSION OF CYSTINE TO CYSTEINE

10^{-4} Moles in 20-ml. Samples		
Cystine	Na_2S	Cysteine
5.0	2.5	3.8
5.0	5.0	7.0
5.0	7.5	8.8
5.0	10.0	9.0
5.0	15.0	9.4
5.0	50.0	9.6
3.8	3.8	5.4
7.6	3.8	6.2
26.6	3.8	7.2

Slow Acidification of High-pH Reaction Mixture. Cystine, 1.214 g., was dissolved in 25 ml. of 0.6 M sodium hydroxide, 3 ml. of 2 M sodium sulfide was added, and the air in the flask displaced by nitrogen. The solution was stirred for 2.5 hours. Carbon dioxide freed from oxygen was bubbled through the solution for 2 hours to expel the hydrogen sulfide. There was bulky white precipitate which was filtered out. The pH of the filtrate was found to be 7.0. The precipitate was washed with a small quantity of water, dried and the weight found to be 1.164 g. (96% of the original). The precipitate was identified as cystine by means of the optical rotation.

DISCUSSION

Preliminary Considerations

Before entering upon a discussion of the experimental results, it is necessary to make reference to two problems, which are pertinent to

that discussion, namely, the acid-base properties of sulfide ion, and the nature and absorption of polysulfide ions.

The second ionization constant of hydrogen sulfide is so small that sulfide is almost completely hydrolyzed in aqueous solution; unfortunately, there is lack of agreement in the values reported for the ionization constant (34, 55), so that the relative concentrations of sulfide and hydrosulfide cannot be calculated accurately. In most of the experiments done, 0.2 M sodium hydroxide was used as the medium; this would serve to reduce the hydrolysis, and maintain a nearly constant, although undetermined, ratio between the sulfide and hydrosulfide ions. Even in this alkaline medium, considerable hydrosulfide ion is present (37), and it should be kept in mind in that the reactions and equations ascribed to sulfide ion might involve hydrosulfide ion instead, or both ions.

With reference to the nature and properties of polysulfide ions, it should first be admitted that they are not well understood. However, it is known that polysulfide ions form when sulfur is dissolved in sodium sulfide solution, and that they exist in mobile equilibrium with each other (16); these facts are of importance in the work to be discussed. Comparatively small amounts of sulfur were dissolved in an excess of sulfide, and disulfide was, accordingly, the predominant species, but higher polysulfide ions were probably present to an appreciable extent. This should be understood, even though disulfide will be the only species specifically named throughout. Disulfide has a characteristic lemon-yellow color, and absorbs fairly strongly in the near ultraviolet. Attempts to determine its spectrum by dissolving known amounts of sulfur in excess sodium sulfide resulted in an absorption that changed gradually with time, probably because of

the gradual establishment of the polysulfide equilibria previously alluded to, and of the occurrence of side reactions, such as interaction with oxygen. The solutions were protected from air as well as was conveniently possible, but oxidation could not be completely prevented. Qualitatively, the spectrum exhibits a broad band with a maximum about 270 mμ, and a gradually decreasing absorption toward higher wave lengths; for the purposes of comparisons to be made in the subsequent discussion, it should be noted that the molar absorption coefficient (calculated from the amount of sulfur) at 335 mμ is about 310, and the absorption at 310 mμ is four times greater.

Polarimetric Experiments

The preliminary experiments using the polarimeter show that sodium sulfide is a reducing agent for cystine. In a solution containing 0.0307 M cystine and 0.1230 M sulfide, as well as about 0.2 M hydroxide, the levo-rotation due to cystine was reduced by 85%, and could be almost completely restored by oxidation. The extent of reaction evidenced by the data is probably less than actually occurred, because the reaction mixture was centrifuged and kept for long time, and some cysteine was probably reoxidized by air. The solution after acidification and centrifuging was still not fully transparent, and the optical rotation could be read only with some difficulty. However, the results were in fair agreement with the more precise ones obtained by means of the Rudolph 80 Photoelectric Polarimeter.

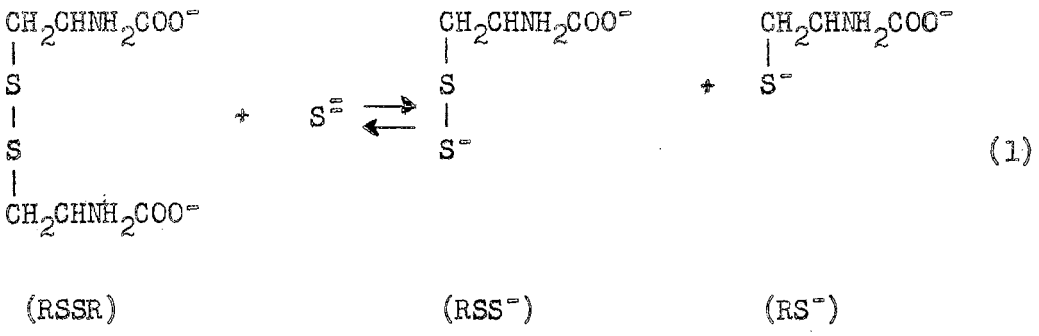
About 96% of the original cystine could be recovered on re-oxidation of product, by bubbling air through a mildly alkaline solution in the

presence of traces of ferrous ions. This confirms that cysteine is the product of the reaction.

Spectrophotometric Experiments

The spectra of the various reactants and products are shown in Figures 1-6. Cystine, cysteine and sodium sulfide have rather high absorption below 280 mμ, but little or none, at the concentrations involved here, above 300 mμ. However, significant absorption develops in this region when cystine and sulfide react.

Figures 3-6 represent some typical spectra obtained in the reaction of cystine and sodium sulfide. In all cases, development of the absorption was fairly rapid, although slow alterations of the spectrum would generally be observed over a long period of time; the curves represent, in general, the spectra developed when the first rapid reaction had reached essential completion. Curve A (Fig. 3) represents the spectrum developed from a large excess of cystine and sodium sulfide, and a clearly defined absorption is seen at 335 mμ. This maximum cannot be due to disulfide, or to cysteinate ion (7), and it is postulated that it is due to the species RSS^-, formed according to equation (1)



(1)

The assumption is supported by Curve C (Fig. 6), obtained when sulfur was dissolved in alkaline cysteinate solution. This also shows the absorption maximum at 335 mμ. RSS^- would be formed in this case by addition of the sulfur to RS^-.

As the proportion of sulfide was increased, the absorption at 310 mμ increased, both in absolute value and especially with respect to that at 335 mμ. In the experiment represented by Curve B (Fig. 3), sodium sulfide and cystine were mixed in 2:1 molar ratio, and the maximum at 335 mμ is still visible, but the relative absorption at 310 mμ is a little higher than in Curve A (Fig. 3). With 3:1 molar ratio, represented by Curve B (Fig. 5), the maximum at 335 mμ has diminished more, and the absorption at 310 mμ is of the same order of magnitude as that at 335 mμ. The concentrations of reaction product in experiments A and B (Fig. 3) are not known exactly, but are not the same, and therefore the intensities of absorption should not be compared directly. In the experiment represented by Curve A (Fig. 4) sodium sulfide was used in large excess, and it is seen that the absorption at 310 mμ is now much higher than at 335 mμ. It is postulated that, in excess sulfide, disulfide is formed, according to equation (2),

$$RSS^- + S^= \rightleftharpoons RS^- + S_2^= \qquad (2)$$

and that its absorption obliterates the minimum at 310 mμ, observed in the other three cases. Unfortunately, the absorption of disulfide is not very distinctive, and its quantitative features are somewhat uncertain, as has already been stated; furthermore, the other product of the reaction, cysteinate ion, absorbs fairly strongly in the region of the disulfide maximum. For these reasons, a quantitative interpretation of the curves has not been attempted. However, it can be

deduced from the appearance of the Curve B (Fig. 3) that only a little disulfide could have been formed in that experiment, even though sufficient sulfide had been added to react with RSS^- completely according to equation (2); it follows that the equilibrium constant of reaction (2) is small.

This conclusion is supported by Curves B and C (Fig. 4). Curve B represents the spectrum of a disulfide solution prepared by dissolving 0.002 M sulfur in excess sodium sulfide, and Curve C the spectrum developed when cysteine was added to this in a concentration comparable to that of sulfide. It is seen that the absorption typical of RSS^- is developed, by reversal of reaction (2). Similarly, when a small quantity of cysteine was added to the solution of Curve B (Fig. 3), the maximum at 335 mμ became more pronounced, indicating an increase in the concentration of RSS^-.

Curves A and B (Fig. 6) show that reaction (1) predominates at the beginning and that reaction (2) becomes important only when sufficient concentration of RSS^- is built up.

The effect of increasing the sulfide concentration on the rate and extent of reaction was shown in Tables II and III. When the sulfide concentration was increased from 0.5 to 3.0 equivalents per equivalent of cystine the maximum optical density at 335 mμ increased, indicating that the reaction gradually goes toward completion, as the sulfide concentration is increased. The time taken to reach the maximum absorption decreased from 145 minutes to 40 minutes, as might be expected. These values serve to indicate the approximate speed of the reaction, which is fairly great.

When solutions showing the absorption maximum at 335 mμ were treated with strong hydrochloric acid, the maximum disappeared quickly, and sulfur precipitated. The possible reaction taking place is discussed in the next section.

Analytical Determinations

More concentrated solutions were next investigated. In this series of experiments, the reaction between cystine and sulfide was allowed to come to substantial completion, the reaction mixture was made strongly acid, excess hydrogen sulfide was expelled, and the remaining solution was titrated with iodine, for the determination of cysteine. The results have been shown in Tables V, VI, and VII. At sulfide-cystine ratios smaller than 2:1, the amount of cysteine formed was greater than the amount of sulfide; this shows that the reaction cannot be represented by an equation such as (3), according

$$RSSR + 2\,S^{=} \longrightarrow 2\,RS^{-} + S_2^{=} \qquad (3)$$

to which the amount of cysteine and sulfide should, at most, be equivalent. The data can be explained, however, if it is further postulated that the species RSS^{-} is unstable in acid solution, and decomposes according to equation (4). Such a postulate is reasonable, in

$$RSS^{-} + H^{+} \longrightarrow (RSSH) \longrightarrow RSH + S\downarrow \qquad (4)$$

view of the similar behavior of inorganic disulfides toward acidification. The decomposition is probably not instantaneous. In many cases, it was observed that the deposition of sulfur, although largely complete upon the first addition of acid, continued for some time

afterwards. This may be due to the temporary existence of RSSH. In the experiments described, where acidification was followed by expulsion of hydrogen sulfide and long standing, the separation of sulfur appeared to be complete before other determinations were undertaken, and it has been assumed that no RSSH remained.

Reaction (4) occurs only when strong acid in sufficient amount is used to decompose the RSS^-.

In the experiment where cystine and sulfide in 1:1 molar ratio were allowed to react and the reaction mixture was acidified slowly to pH 7.0 by passing carbon dioxide, more than 96% of the cystine was recovered as a precipitate. Slow acidification probably permits the occurrence of reaction (5)

$$RSSH + RSH \longrightarrow RSSR + H_2S\uparrow \qquad (5)$$

The results of Tables V, and VI show quantitatively that the reactions do not go to completion. However, there is a gradual shift toward completion as the excess of either reagent is increased, in accordance with the Law of Mass Action. It cannot be deduced from the data to what extent reactions (1) and (2) contribute to the total reaction; indeed, the results might be explained in terms of reactions (1) and (4) alone. However, the occurrence of reaction (2) and the formation of disulfide ion is clearly indicated by the spectrophotometric experiments, and by the strong yellow coloration observed in the more concentrated solutions containing excess disulfide.

An approximate equilibrium constant for reaction (1)

$$RSSR + S^{=} \rightleftharpoons RS^- + RSS^-$$

was calculated using the expression

$$K = \frac{(RS^-)\ (RSS^-)}{(RSSR)\ (S^=)}$$

from the data obtained with sodium sulfide not in excess, assuming that (1) was the only reaction taking place. The values calculated were in the range of 5 to 10. While this value cannot be accurate, in view of the assumption involved, it gives an indication of the order of magnitude of the constant.

Disulfide Reduction in Proteins

To evaluate from these results the possible utility of sodium sulfide for reducing disulfide bonds in proteins, one must keep in mind that the free energy of disulfide bonds in proteins is not the same as that of the bond in cystine. Accordingly the values of the equilibrium constants may be different. To the extent that protein disulfide bonds would show the same reactivity as those in cystine, it can be inferred that sodium sulfide in moderate excess would reduce disulfide bonds almost completely to give -S^- and -SS^- residues, and that, in a large excess of sulfide, reduction to two -S^- residues could be expected. Acidification of the solution would convert both types of residues to -SH groups in any case; some time may be required for reaction. During and after acidification, excess reagent, now in the form of hydrogen sulfide, can be removed easily.

CHAPTER IV

THE EFFECT OF pH ON THE REACTION BETWEEN CYSTINE AND SODIUM SULFIDE

In the previous chapter the reaction between cystine and sodium sulfide was studied in 0.2 M sodium hydroxide. Since the high alkalinity has a deleterious effect on cystine, and would be even more harmful to proteins, it was desirable to study the reaction at somewhat lower alkalinity. The reaction was studied spectrophotometrically and the extent of reduction determined by iodimetry, as in the case of experiments done in 0.2 M sodium hydroxide.

EXPERIMENTAL

Reagents

The reagents used were of the same purity as those mentioned in the previous chapter.

Trimethylolaminomethane ("Tris") was obtained from the Matheson Co., Inc., East Rutherford, N. J. Tris buffer was prepared in all the cases by dissolving the proper amount of the substance in deionized and deaerated water and neutralizing it to the required pH with hydrochloric acid.

Spectrophotometric Measurements

About 0.5 g. of cystine was stirred in 60 ml. of 1 M tris buffer (pH 8.3) for 1 hour under nitrogen. The undissolved cystine was

filtered out. The optical density of the filtrate at 250 mμ was measured with the tris buffer as reference, to determine the cystine concentration in the solution (ε taken as 380). To 20 ml. of the filtrate (1.25×10^{-3} M) was added 1.2 ml. of 0.154 M sodium sulfide and the flask shaken for 2-3 minutes. The optical density of the solution was measured at 335 mμ in the Beckman DU spectrophotometer at different time intervals, using the buffer mixed with sulfide but without cystine as reference. In a separate flask, another portion of the original cystine solution in tris buffer was brought to pH 12.2 by adding sodium hydroxide pellets and the reaction of cystine with sulfide was followed in the Beckman DU spectrophotometer; the reference was also brought to pH 12.2. These experiments were all done at room temperature (about 30°C). The results obtained are given in Table VIII.

TABLE VIII

REACTION OF CYSTINE (1.18×10^{-3} M) WITH SODIUM SULFIDE (8.72×10^{-3} M)

At pH 8.3		At pH 12.2	
Time of Reaction, Mins.	O.D. at 335 mμ	Time of Reaction, Mins.	O.D. at 335 mμ
4	0.060	4	0.143
6	0.085	6	0.162
8	0.097	10	0.203
10	0.101	18	0.275
18	0.103	31	0.365
35	0.103	54	0.510

A comparative rate study was also done with the reactions at pH 9.2 and 12.2, following the same procedure except in that cystine was

dissolved in tris buffer of pH 9.2 instead of pH 8.3. Sodium sulfide (1.2 ml. of the 0.154 M solution) was added to 20 ml. of 6.4×10^{-3} M cystine solution in 1 M tris buffer and mixed well. The optical density at 335 mμ was measured at different time intervals as in the previous case. The results obtained are shown in Table IX.

TABLE IX

REACTION OF CYSTINE (6.04×10^{-3} M) WITH SODIUM SULFIDE (8.72×10^{-3} M)

At pH 9.2		At pH 12.2	
Time of Reaction, Mins.	O.D. at 335 mμ	Time of Reaction, Mins.	O.D. at 335 mμ
5	0.48	6	0.11
6	0.56	12	0.20
8	0.66	19	0.31
11	0.75	44	0.66
17	0.82	60	0.81
24	0.85	84	1.04
37	0.86	98	1.14
55	0.86	172	1.43

Some spectra scanned with the Beckman DK-1 spectrophotometer are shown in Figure 7. Curve A represents the spectrum of the reaction mixture at pH 9.2 about 28 minutes after mixing and Curve B represents the spectrum of the same on being brought quickly to a pH of about 12.

Analytical Determinations

Reaction of Cystine with Sodium Sulfide at pH 8.0, 8.4 and 8.9 and Titration of Cysteine. The procedure followed here is essentially the same as used for experiments done in 0.2 M sodium hydroxide. For

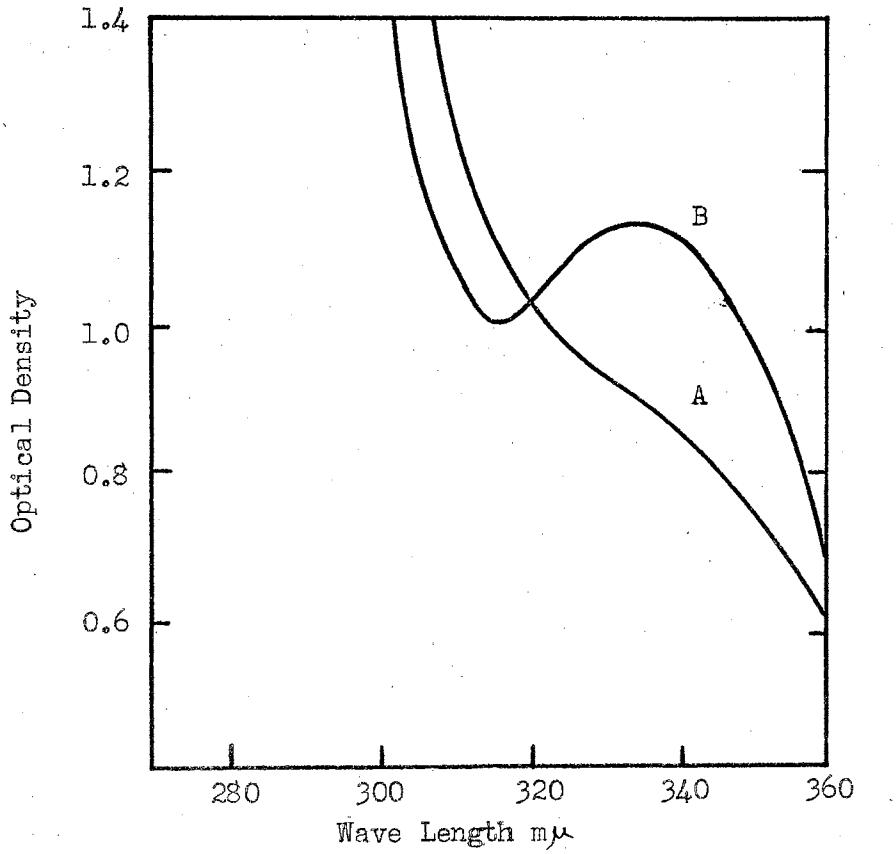


Figure 7.

A: 20 ml. 6.04 x 10^{-3} M Cystine (in 1 M tris buffer) + 1.2 ml. 0.154 M Na_2S, 28 mins. after mixing (pH 9.2) vs. 20 ml. 1 M tris + 1.2 ml. 0.154 M Na_2S.

B: Solution A (pH 9.2) brought to pH 12 by adding NaOH pellets vs. blank also brought to pH 12.

the data given in Table X, cystine was weighed into a 100-ml. round-bottom flask, 18 ml. of tris buffer of pH 7.8 (2 M) and then 2 ml. of 2.4 M sodium sulfide were added. The pH of the reaction mixture was 8.0. The air above the solution was displaced by passing in nitrogen for 3 minutes, the flask was closed, and the solution stirred for 1.5 hours at 30°C. Then the solution was acidified with ice-cold 6 N hydrochloric acid, and the hydrogen sulfide was expelled by distilling under reduced pressure. The cysteine obtained was determined by iodimetry as described earlier.

TABLE X

REDUCTION OF CYSTINE WITH SODIUM SULFIDE AT pH 8.0 IN TRIS BUFFER

Amount of Cystine g.	Moles Na_2S/ Mole Cystine	Cystine Reacted, g.	Moles Cystine Reacted/Mole Na_2S added
0.586	2.0	0.146	0.139
0.401	2.9	0.135	0.128
0.342	3.4	0.128	0.121
0.211	5.4	0.121	0.115
0.208	5.5	0.118	0.112
0.110	10.6	0.090	0.085
0.110	10.6	0.089	0.084

Table XI represents the data obtained when different amounts of solid cystine were added to 20 ml. of a 0.315 M sodium sulfide solution at pH 8.4, prepared by neutralizing a concentrated sodium sulfide solution with hydrochloric acid. In this set of experiments no other buffer was added, and the sodium sulfide solution acted as its own buffer. The rest of the procedure was the same as described above.

TABLE XI

REDUCTION AT pH 8.4 WITHOUT TRIS BUFFER

Amount of Cystine g.	Moles Na_2S/ Mole Cystine	Cystine Reacted, g.	Moles Cystine Reacted/Mole Na_2S added
0.604	2.5	0.190	0.124
0.400	4.0	0.172	0.112
0.382	4.1	0.172	0.112
0.220	7.3	0.165	0.107
0.207	7.6	0.151	0.098

Table XII shows the data obtained when different amounts of solid cystine were added to a mixture of 17 ml. of tris buffer (2 M) and 3 ml. of 2.2 M sodium sulfide, when the pH of the solution was 8.9.

TABLE XII

REDUCTION AT pH 8.9 IN TRIS BUFFER

Amount of Cystine g.	Moles Na_2S/ Mole Cystine	Cystine Reacted, g.	Moles Cystine Reacted/Mole Na_2S added
0.697	2.3	0.240	0.152
0.489	3.3	0.230	0.145
0.462	3.5	0.228	0.144
0.335	4.8	0.201	0.127
0.206	7.8	0.161	0.102
0.206	7.8	0.157	0.099

Blank experiments done with cysteine and sodium sulfide in tris buffer showed that in the conditions employed all but a small amount of hydrogen sulfide could be expelled, and that cysteine was oxidized only to

a small extent; the two corrections, i.e., for residual hydrogen sulfide and for loss of cysteine during manipulation, nearly cancelled one another.

DISCUSSION

Before proceeding with a discussion concerning the reaction proper, it is necessary to consider the state of sulfide at pH 8-9. It should be clear from what has been said in the previous chapter, that sulfide ion cannot exist to any appreciable extent at pH 8-9; when, as in the experiments described, sodium sulfide is added to a buffer of adequate strength to maintain the pH at these levels, the sulfide ion would be converted to hydrosulfide and hydrogen sulfide. The formation of the latter presents a difficulty, since it is not very soluble in water, and it may be lost from the condensed phase. In the experiments, the sulfide titer was determined after the buffered solution had been mixed, and it was ascertained that in the conditions employed the loss of hydrogen sulfide was not great, though it was appreciable (about 5% of sulfide taken). In the discussion to follow, the term sulfide is intended to comprise hydrosulfide and hydrogen sulfide.

Spectrophotometric Measurements

In the reaction between cystine and sulfide at pH 8-9 no absorption peak develops at 335 mμ, although there is an increase in absorption at and around this value, as is shown by Figure 7, Curve A. Curve B represents the spectrum obtained when the reaction mixture represented by Curve A is brought to a pH of about 12 by addition of sodium hydroxide pellets. The characteristic absorption maximum at 335 mμ

develops at full intensity when the reaction mixture is brought to pH 12, and again disappears when the solution is brought down to pH 9 with acid.

It is postulated that the reaction at pH 8-9 proceeds similar to that at pH 12, except that the product is now RSSH instead of RSS^-. The reaction may be represented as

$$RSSR + HS^- \longrightarrow RS^- + RSSH \qquad (6)$$

It is believed that the maximum at 335 mμ would develop only when the RSSH is ionized to RSS^-.

On the assumption, which is only approximately correct, that no reaction other than ionization takes place in the conversion from Curve A to Curve B, it is seen from Figure 7 that the specific absorbancy of the product at pH 12 is 1.3-1.4 times that of the product at pH 9.2, at 335 mμ. Then the optical density of 0.86 at pH 9.2 corresponds to about 1.15 at about pH 12, which is about 80% of 1.43. On this basis, it may be inferred that the extent of reaction taking place at pH 9.2 is roughly 80% of that at pH 12.

The rates of development of absorption at different pH values can be compared from the data given in Tables VIII and IX. For a reaction mixture containing 1.18×10^{-3} M cystine and 8.72×10^{-3} M sodium sulfide the time of half-reaction at pH 8.3 is less than 4 minutes, while at 12.2 it is about 15 minutes. For a different solution containing 6.04×10^{-3} M cystine and 8.72×10^{-3} M sodium sulfide the time of reaction at pH 9.2 is less than 5 minutes, while at pH 12.2 it is about 50 minutes. Thus the reaction at lower pH appears to be much faster than that at higher pH, for a given concentration of cystine and sulfide.

Analytical Results

In the experiments reported in Tables X, XI and XII the reaction between cystine and sodium sulfide was allowed to proceed at pH values 8-9, acidified, the hydrogen sulfide expelled, and the cysteine formed determined by iodimetric titration. A heterogeneous system was involved, because the solubility of cystine is very low at these pH values. In no case did the cystine react completely, as some solid was always left undissolved, even when the sulfide-cystine ratio was large.

It should therefore be possible to regard the activity of cystine as constant, and the cysteine-sulfide ratio should also be constant at equilibrium. This expectation was not realized experimentally. The ratio of number of moles of cystine converted per mole of sodium sulfide used increased as the amount of solid cystine was increased, keeping the sulfide added constant, as can be seen from Tables X, XI and XII. The cysteine-sulfide ratio increases even more as the amount of solid cystine taken is increased. This suggests that a true equilibrium had not been reached; but it was shown that in any one case, no more solid cystine would go into solution by lengthening the time of reaction as much as two- or three-fold. Experiments done at pH 8.4 without any buffer showed same type of results, so that the presence of buffer could not be held responsible for the anomalous results obtained. The nature of the solid phase was checked by separating it on a filter, washing with water, and dissolving in hydrochloric acid; no sulfur was found.

Extent and Rate of Reaction

While no satisfactory explanation can be given for the analytical data obtained, one thing appears to be clear, i.e., at pH 8-9, not more than 0.1-0.15 mole of cystine reacted per mole of sodium sulfide used. Thus the equilibrium constant of the reaction at pH 8-9 appears to be considerably lower than that in the medium of 0.2 M sodium hydroxide, described in the previous chapter. The low yields obtained at pH 8-9 may be due to the fact that a heterogeneous system is involved, since the spectrophotometric experiments mentioned earlier indicated that the reaction at pH 9.2 takes place to an extent 80% as great as that at pH 12. In the spectrophotometric experiments, the reaction took place in a homogeneous system.

It is also clear from Tables VIII and IX that hydrosulfide is the effective reagent at pH 8-9 and that the reaction goes considerably faster at or around this pH than at pH 12.

While the reduction at pH 8-9 is found to be not as effective as that at higher pH, sulfide can be used to reduce cystine and disulfide bonds in proteins in this pH range, but with low conversion of disulfide to mercapto groups.

CHAPTER V

REACTION OF CYSTINE WITH SODIUM HYPOSULFITE (DITHIONITE) IN SODIUM HYDROXIDE SOLUTION

Sodium hyposulfite is a very strong reducing agent in alkaline solution, and study of its reaction with cystine was therefore undertaken. The oxidation potentials of hyposulfite in base and acid are (35)

$$S_2O_4^{=} + 4\ OH^- \longrightarrow 2\ SO_3^{=} + 2\ H_2O + 2e^- \qquad E^\circ = 1.12$$

$$HS_2O_4^- + 2\ H_2O \longrightarrow 2\ H_2SO_3 + H^+ + 2e^- \qquad E^\circ = 0.08$$

Accordingly, the reducing action of hyposulfite is powerful in alkaline media, but much less so in acid. It is unstable with respect to the decomposition

$$2\ S_2O_4^{=} + H_2O \longrightarrow S_2O_3^{=} + 2\ HSO_3^- \qquad \Delta F = -32.5\ \text{K cal.}$$

and the rate of decomposition is increased in acid solution, and retarded in alkali.

EXPERIMENTAL

Materials

All the materials used here are again of the same purity as described in chapter III.

Sodium Hyposulfite, Practical grade, was obtained from Eastman Kodak Co., Rochester, N. Y.

Formaldehyde, Reagent, 36-38% HCHO, Merck and Co. Inc., Rahway, N. J.

Assay of the Sodium Hyposulfite

The pH of 0.1 N hyposulfite solution in deionized and deaerated water was found to be about 5.0 and after standing for 30 minutes it decreased to 3.5. Titration curves were run on a freshly prepared 0.1 N hyposulfite solution with 0.1 N sodium hydroxide under an atmosphere of nitrogen. The curve showed a buffer region with the midpoint at about pH 7.

When a 0.1 N solution of hyposulfite was prepared in aqueous formaldehyde (prepared by mixing equal volumes of formaldehyde and water), it had a pH of about 9 which remained unchanged for at least 1 hour. Titration curves were run on this solution using 0.1 N sulfuric acid as titrant. A buffer region was then found at pH 3-4.

The sodium hyposulfite was assayed by the iodimetric method using the formaldehyde procedure (51). About 1 g. of the hyposulfite was accurately weighed in a glass-stoppered flask and 10 ml. of formaldehyde and 10 ml. of water were added in that order, thus avoiding the decomposition that might occur if water were added first. The solution was allowed to stand for 30 minutes with frequent shaking. The solution was transferred to a 250-ml. volumetric flask, and 150 ml. of water and 2 drops of methyl orange were added. The solution was titrated with 1 N sulfuric acid to a slight acid reaction, and made up to 250 ml. with water (neutralization with acid to the methyl orange

end-point converts any sodium sulfite present to sodium bisulfite. This, as well as any bisulfite originally present and that to be formed in the titration with iodine combine with the formaldehyde to give sodium formaldehyde bisulfite, which does not react with iodine). Fifty milliliters of the solution was taken and titrated with 0.1 N sodium hydroxide to a slight alkalinity using phenolphthalein as indicator. This solution was then titrated with 0.1 N iodine, using starch indicator. The hyposulfite content was found to be 88%.

Reaction of Cystine with Sodium Hyposulfite and Titration of Cysteine

The procedure followed was essentially the same as the one described for the reaction with sodium sulfide. The proper amounts of cystine and hyposulfite were each time weighed into a 100-ml. round-bottom flask, 20 ml. of 0.2 M sodium hydroxide was added, air was displaced by nitrogen and the flask was closed. The solution was stirred for 1.5 hours at 30°C and acidified with ice-cold 6 N hydrochloric acid, nitrogen was bubbled through the solution for 15 minutes to expel sulfur dioxide. The remaining sulfur dioxide was removed by distillation under reduced pressure. The cysteine formed was estimated by iodimetry. The results obtained are shown in Table XIII.

Experiments were done to determine how much of sulfur dioxide was still left in the solution; the amount was found to be negligible. Blank experiments were done with cysteine and hyposulfite, and it was found that there was some loss of cysteine depending on the amount of hyposulfite used. The actual results obtained were corrected for this loss and the corrected results are included in Table XIII.

TABLE XIII

REDUCTION OF CYSTINE WITH SODIUM HYPOSULFITE

Equivalents of Hyposulfite/Equivalent of Cystine	Amount of Cystine Taken, g.	Amount of Hyposulfite Taken, g.	% Conversion Based on Cystine	% Conversion Corrected for the Loss in Cysteine
0.6	0.259	0.131	9.2	11.0
1.2	0.259	0.266	18.5	22.2
1.8	0.258	0.398	24.1	28.5
2.4	0.260	0.531	27.9	34.1
2.5	0.528	1.115	32.6	39.0
5.1	0.260	1.105	37.0	49.8

DISCUSSION

Sodium hyposulfite is a rather unstable substance, as is witnessed by its behavior in aqueous solution, in which it is apparently converted quickly to sulfite. In alkaline solution the substance is more stable. The commercial product used in this work contained about 88% hyposulfite, and further purification was not attempted, since the product could not be expected to remain pure, and since the impurities present, namely sulfite and bisulfite, would not affect the main reaction adversely.

Blank experiments were first performed, in which cysteine and hyposulfite were mixed, acidified, and the sulfur dioxide removed; considerable loss of cysteine occurred, the loss being greater the greater the amount of hyposulfite. The loss was not due to direct reaction between cysteine and hyposulfite, because the loss occurred even when the hyposulfite was acidified and decomposed prior to adding to the cysteine. Also, when blanks were done using sodium sulfite and sulfur in the place of sodium hyposulfite no loss in cysteine occurred. It is

presumed that the loss was due to oxidation of the cysteine by the colloidal sulfur formed on acidifying hyposulfite, probably according to equation (7)

$$2\,RSH + S \longrightarrow RSSR + H_2S\uparrow \qquad (7)$$

Even when considerable excess of hyposulfite was used not more than half of the cystine was converted into cysteine. It may be postulated that the reaction takes place according to equation (8)

$$RSSR + S_2O_4^{=} \rightleftharpoons RS^- + RS\text{-}S_2O_4^- \qquad (8)$$

This would be analogous to the reactions with sulfite or cyanide, described in the second chapter.

In view of the low conversion yields, the hyposulfite is not considered a suitable reducing agent for cystine or for the disulfide bonds of proteins.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The reaction of cystine with sodium sulfide in 0.2 M sodium hydroxide solution produces a product with an absorption maximum at 335 mμ; it is postulated that this absorption is due to ($^-SSCH_2CHNH_2COO^-$). In the presence of excess sulfide this species reacts further, and, overall, there are produced two cysteinate ions and disulfide or polysulfide ions. The mechanism of the reaction may be represented by

$$\underset{\text{(Cystine)}}{RSSR} + S^{=} \rightleftharpoons \underset{\text{(Cysteine)}}{RS^-} + RSS^- \qquad (1)$$

$$RSS^- + S^{=} \rightleftharpoons RS^- + S_2^{=} \qquad (2)$$

That the absorption maximum at 335 mμ is due to the species RSS^- is supported by the observation that the maximum also develops when sulfur is dissolved in an alkaline cysteinate solution. That the reaction (2) takes place is supported by the fact that the spectrum obtained when excess sulfide is added to cystine resembles closely that of sodium disulfide prepared by dissolving sulfur in sodium sulfide of comparable concentration. Reaction (2) can be reversed by adding some cysteine to a solution of sodium disulfide. Reactions (1) and (2) are consecutive reactions, and reaction (2) becomes important only when excess of sulfide is used. When the reaction mixture is made

strongly acid, the absorption maximum at 335 mμ disappears, sulfur precipitates, and cysteine is found; with 5-10 equivalents of sodium sulfide the reaction is more than 95% complete. It is postulated that the species RSS^- is unstable in acid solution, and decomposes according to the equation

$$RSS^- + H^+ \longrightarrow (RSSH) \longrightarrow RSH + S\downarrow$$

Such a postulate is reasonable, in view of the similar behavior of inorganic disulfides toward acidification.

The reaction of cystine with sodium sulfide was also studied at pH 8-9. It is found, on the basis of spectrophotometric data, that the reaction goes faster at pH 8-9 than at pH 12. The reaction product does not exhibit the maximum at 335 mμ, but does so when brought to a pH of 12. It is postulated that the product at pH 8-9 is RSSH, which does not exhibit the absorption maximum at 335 mμ, unless ionized to RSS^-. The extent of reduction of cystine is found to be considerably less at pH 8-9 than in 0.2 M sodium hydroxide.

The reaction of cystine with sodium hyposulfite in 0.2 M sodium hydroxide was investigated briefly. Excess hyposulfite can be destroyed by acidifying the solution and sulfur dioxide can be removed by distillation under reduced pressure. However, in blank experiments conducted with cysteine and hyposulfite, it is found that considerable loss of cysteine occurs, possibly due to oxidation by colloidal sulfur. Correction is made for this cysteine loss. Even when excess of hyposulfite is used, not more than 1 mole of cysteine can be obtained from each mole of cystine reacted. The reaction taking place is postulated as

$$RSSR + S_2O_4^{=} \rightleftharpoons RS^{-} + RS\text{-}S_2O_4^{-}$$

The product formed appears to be quite stable in strong acids.

Thus, sodium sulfide is found to be a good reducing agent at high pH but not as good a reducing agent at pH 8-9. Sodium hyposulfite is not found to be a suitable reducing agent even at high pH because half of the cysteine is combined with the hyposulfite and thus not more than 50% yield of cysteine can be obtained.

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