COBALT (III) COMPLEXES OF CYSTEINE

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PREFACE

I want to thank my advisor, Dr. George Gorin, for his aid and advice in the preparation of this thesis and the carrying out of the experimental work on which the thesis is based. While at Oklahoma State University, I have gained a better idea of what research is and how a problem should be attacked. For much of this Doctor Gorin is responsible and I am appreciative of it.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The oxidation of mercapto groups to disulfides by atmospheric oxygen is a reaction of great biological interest. A better understanding of the mechanisms by which the process takes place is important.

A typical mercapto-disulfide system that has been extensively studied is that of cysteine and cystine. Cysteine is an amino acid with the formula: $HSCH_2CHNH_2COOH$. Commercially, it is usually available as the hydrochloride. Cystine, the product of mild oxidation, has the formula: $(SCH_2CHNH_2COOH)_2$.

Warburg and Sakuma[1] and Mathews and Walker[2] found that the oxidation of cysteine was catalyzed by traces of iron. Michaelis and his co-workers[3,4,5] worked on the problem of how iron catalyzed the oxidation. They found that traces of other metals also catalyzed the reaction. These metals had the common property of multiple valency, the ability of existing in more than one oxidation state. This suggested that the catalyzed reaction went through a cyclic path that involved the formation of a metal ion-cysteine complex, the oxidation of the complexed metal ion by atmospheric oxygen, and then the oxidation of cysteine by the oxidized metal ion. The reduced metal ion would then react with more cysteine and repeat the cycle. The identification and study of the intermediate compounds might be difficult because of their transitory nature.

Study of the complexes involving cobaltous and cobaltic ions has

advantages. The cobaltous complexes are readily formed and are stable as long as they are protected from air. The cobaltic complexes are very stable and can easily be prepared. Thus for the cobaltous-cobaltic system, metal-cysteine compounds with both the higher and lower valence states can be examined relatively easily. Another advantage is that cystine does not form cobalt complexes and, if formed, is an inert material.

Cysteine has one basic and two acidic groups. All three groups might take part in complex formation, although steric strain probably would prevent coordination to all three positions simultaneously. If two groups react with a single cobalt atom, a five or a six membered chelate ring will be formed, and such complexes would be quite stable.

Michaelis and Guzman-Barron[3] studied the cobaltous-cobaltic system without attempting to isolate products. They worked with phosphate buffers in the pH range 7.5-8.5. They found that (1) with excess cobalt present, the oxygen consumed was two-thirds of that required to oxidize cysteine to cystine, (2) with cysteine in excess the oxygen taken up depended on the cobalt concentration and was double that required to oxidize cobaltous ion to cobaltic ion and, (3) when cobalt and cysteine were mixed, the oxygen consumed was proportional to the amount of cobalt present up to a maximum cysteine-cobalt ratio of about 3-1. "At any event, it is decidedly greater than 2-1 and less than 4-1."[3] Thus they found that one atom of oxygen was taken up for every atom of cobalt and three molecules of cysteine.

Michaelis and Yamaguchi[4] investigated a brown cobaltic-cysteine complex in solution. On the basis of the oxygen consumed in the formation and of the amount of cobalt estimated to be present, they ascribed to this complex the formula: $Co(SCH_2CHNH_2COO)_3H_2$.

Michaelis[5] also concluded that chelation took place between the carboxyl and the sulfhydryl group, on the basis of the fact that similar reactions took place with thioglycolic acid, while thioacetic acid behaved differently. While the data reported by Michaelis has been confirmed by other workers, his conclusions have not been generally accepted.

Kendall and Holst[6] found that at a pH of 7.4 a cobaltic-cysteine complex could be formed from only two moles of cysteine and one atom of cobalt. The yield of complex varied with the oxidizing agent and experimental conditions, and ranged between 67-100%, based on cysteine. The cysteine that did not react with cobalt was oxidized to cystine; thus the oxygen consumption data were consistent with the findings of Michaelis and his co-workers. Kendall and Holst also believed that all three groups on the cysteine molecule coordinated with cobalt.

Kendall and Holst proposed an oxidation mechanism that involved an intermediate cobaltic tris-complex, which was decomposed to a cobaltic bis-complex and a molecule of cysteine:

(1) cobaltous cysteine = cobaltic triscysteine

(2) cobaltic triscysteine = cobaltic biscysteine + cysteine

(3) 2 cysteine = cystine

(4) cysteine + cobaltous ion = cobaltous cysteine.

They postulated that the rates of reactions (1) and (3) were variable and depended on the oxidant used, while velocities of reactions (2) and (4) were fixed for any given temperature and pH. Thus if reaction (1) was fast and much faster than reaction (2), reaction (1) would occur before reaction (3) could ever take place, and very little cystine would be formed. If reaction (1) was slow or the rates of reactions (1) and (2) were equal, reactions (1) and (3) would take place simultaneously,

and the amount of cobaltous cysteine formed would depend on the relative rates of reactions (3) and (4).

Schubert[7,8] was the first to prepare cobalt cysteine complexes in solid form and to determine their composition by analysis. He obtained three cobaltous-cysteine complexes and three cobaltic-cysteine complexes.

The cobaltous cysteine complexes were fount to have one, two, and three molecules of cysteine per atom of cobalt. The cobaltous monocysteine complex was reported to be a dimer with the formula $Co(OOCCHNH_2CHS)_2Co \cdot 4H_2O$. It was grass-green in color and very insoluble in water. However in dilute alkali and in the absence of oxygen, it deposited half its cobalt as cobaltous hydroxide and formed a red-violet cobaltous biscysteinate. The cobaltous monocysteinate was formed directly from cobaltous ion and cysteine at a pH of 7-8. At a pH of 11-12, by direct reaction, the cobaltous biscysteinate was formed. Schubert reported its formula to be $K_2(Co(SCH_2NH_2CHCOO)_2(H_2O)_2)$. Schubert thought it possible that the cobaltous monocysteinate might be the cobalt salt of the biscysteinate. In even more strongly alkaline solution, and with a cysteine-cobalt ratio of at least 3-1, a blue-violet cobaltous triscysteinate was obtained. Its formula was reported to be $K_4(Co(SCH_2CHNH_2COO)_3) \cdot 4H_2O$.

Schubert prepared three cobaltic cysteine complexes, both by direct reaction from cobaltous ion, cysteine, and alkali and by oxidation of cobaltous complexes. A brown cobaltic biscysteinate was formed by direct reaction at a pH of 7-8 and by air oxidation of the cobaltous monocysteinate or biscysteinate. The complex was precipitated as the free acid in an alcoholic acid solution. Schubert was not sure whether this material was a monomer with the formula: $H(Co(SCH_2CHNH_2COO)_2$

$(H_2O)_2) \cdot 3H_2O$, or a dimer with the formula: $H_4((SCH_2CHNH_2COO)_2Co\overset{OH}{\underset{OH}{}}Co(SCH_2CHNH_2COO)_2) \cdot 8\ H_2O$.

In the pH range 5-6 by direct reaction with cobaltous ion, air, and cysteine or by air oxidation of the cobaltous monocysteinate or biscysteinate in the presence of cysteine, a red cobaltic triscysteinate was formed, to which Schubert assigned the formula $H_3(Co(SCH_2CHNH_2COO)_3) \cdot 4\ H_2O$.

In the pH range 11-12 and in air with a cysteine-cobaltous ion ratio of 3-1 or greater, or by the air oxidation of the cobaltous triscysteinate, a green cobaltic triscysteinate was formed, for which Schubert[8] gave the formula $K_3(Co(SCH_2CHNH_2COO)_3) \cdot 3\ H_2O$. This material was stable only at pH's above 10. At lower pH's Schubert reported that it decomposed to the cobaltic biscysteinate and cysteine.

Schubert suggested that all chelation reactions took place between the carboxyl group and the sulfhydryl group. The amino group was not supposed to take any part in the reaction.

Neville and Gorin[9] found by cryoscopic measurements that the cobaltic biscysteinate was mononucleate. They examined the spectra of compounds structurally related to cysteine and they found that 2-aminoethanethiol, in which sulfhydryl-amino chelation was the only possibility, formed complexes with spectra very similar to the cysteine complexes. Mercapto compounds in which amino chelation was not possible either did not form complexes or gave ones with considerably different spectra. For both 2-aminoethanethiol and cysteine, a pair of complexes was formed and solutions of one were green and the other brown. This was considered good evidence that the cobaltic complexes were formed by sulfhydryl-amino chelation.

Neville[11] obtained further results in support of this belief. Neville[12] also studied the uptake of oxygen in cobalt (II)-cysteine solutions buffered with phosphate. In dilute solutions, with a cysteine-cobalt ratio greater than 3-1, one atom of cobalt was equivalent to 0.75 atoms of oxygen. With a cysteine-cobalt ratio of less than 3-1, one mole of cysteine was equivalent to 0.25 atoms of oxygen. In concentrated solutions, 0.5 atoms of oxygen were absorbed per atom of cobalt.

The work reported in this thesis deals with the cobalt (III)-cysteine complexes. The first section deals with the red triscysteinate complex. The conditions necessary for its preparation are better defined and an alternate and superior method of preparation is described. The spectrum of the complex in base is reported and compared with the spectrum of a tris-β-mercaptopropionate complex. The remarkable similarity of the spectra and of the properties of the two compounds are evidence that the red triscysteinate complex is formed by carboxyl-sulfhydryl chelation. The spectrum of the red triscysteinate in concentrated sulfuric acid is also reported. A trisodium salt has been prepared and described. The solubility of the red complex in buffers is used to learn about its strength as an acid.

The second section covers work with the green triscysteinate complex and the brown biscysteinate complex. The compounds are prepared directly from cobaltous ion, cysteine, and air. The green complex is also prepared from hexamminecobalt (III) chloride and cysteine. The spectra of various preparations are reported and compared with each other and with previous work.

New data on the stability of solutions of the green triscysteinate are given; concentration of the complex, pH, and cysteine concentration

are shown to be factors.

The behavior of solutions of the brown biscysteinate in alkali and in acid is described. Contrary to other reports,[7] the solutions do not readily react with cysteine to give the tris complex. Also the brown solution obtained by decomposing the green complex has a different spectrum from that of the biscysteinate prepared directly. The brown bis complex and preparations of the tris complex, decomposed under varying conditions, have been studied by ion-exchange chromotography. On the basis of the data presented, it is proposed that the biscysteinate complex prepared directly is a trans aquo isomer and that the triscysteinate decomposes to cysteine and other materials, which, in turn, break down to give diaquo biscysteinate isomers.

The third section covers the investigation of the reaction between cobaltous ion and cysteine in the presence of atmospheric oxygen. With an excess of cobalt, the reaction appears to be first order with respect to cysteine. With an excess of cysteine, no simple order could be ascribed to the reaction. It was found that varying stoichiometric quantities of cysteine were consumed per atom of cobalt present at different pH values. From this it was concluded that the reaction mechanism was complex.

CHAPTER II

PREPARATION AND PROPERTIES OF TRISCYSTEINATOCOBALTIC (III)-O, S ACID

Results and Discussion

A red cobaltic triscyteinate complex was first prepared by Schubert.[7] He made it by reacting cobaltous chloride, cysteine hydrochloride and potassium hydroxide in molar ratios of 1:1.91:4.32. The analysis was in accordance with the formula $H_3(Co(SCH_2CHNH_2COO)_3) \cdot 4H_2O$. On the basis of this formula and in accordance with modern nomenclature,[13] the name triscysteinatocobaltic (III)-O,S acid is proposed ; for brevity this compound will be referred to as TOS. Schubert also reported the absorption spectrum in the range 250-500 mu. Neville[14] repeated Schubert's preparation and reported the spectrum of the anion in the range 220-760 mu.

From the formula of the complex, it may be inferred that the equation for its formation would be:

(1) $$4Co^{2+} + 12H_3\ cys^{+} + 20\ OH^{-} + O_2 = 4\ TOS + 22H_2O$$

where cys represents $(SCH_2CHNH_2COO)^{=}$. Since the stoichimetric ratios of cobalt, cysteine, and base are 1:3:5, the proportions used by Schubert seemed puzzling. However, his results could be repeated, and it was found at first that if more nearly stoichimetric porportions of the reagents were used, TOS was not formed, or it was badly contaminated with a brown product. It was finally realized that it is of determining importance to maintain the pH on the acid side of 6. In Schubert's[7] preparation, two moles of base were added to a mole of cobalt and precipitated it as cobaltous hydroxide. The remaining 2.32 moles of base

were added to 1.91 moles of cysteine hydrochloride, and the reaction mixture was maintained at a pH of about 5.

The preparation was repeated with the stoichimetric ratio, one mole of cobalt to three moles of cysteine hydrochloride to five moles of base. Here four moles of base were added to the cysteine hydrochloride and one mole to the cobaltous chloride. TOS was obtained identical with that prepared by following Schubert's directions.

This suggested an alternate and preferable method of preparing TOS by the use of a suitable buffer system. TOS was obtained in 90-95% yields from a mixture of cobaltous chloride, cysteine hydrochloride, and sodium acetate in molar ratios of 1:3:15. The solution was buffered to a pH of about 5. In this preparation, as long as sufficient sodium acetate was present to buffer the system, the ratios of cobalt, cysteine, and acetate ion were not critical. This reaction may be written:

(2) $4Co^{2+} + 12H_3cys^{+} + O_2 + 20C_2H_3O_2^{-} = 4TOS + 2H_2O + 20\,HC_2H_3O_2$.

Thus sodium acetate absorbed the acid produced by the reaction and maintained the pH at around 5. The cobalt-cysteine ratio was not critical due to the fact that under these conditions, no other product was formed at a competitive rate. Cysteine is comparatively slowly oxidized by atmospheric oxygen but no other cobalt complex is formed.

After the reaction, the TOS was filtered off and the mother liquor was titrated with hydrochloric acid. A pH meter was used to determine the end point. The titration showed five moles of sodium acetate had been converted to acetic acid for every mole of TOS formed. This is in agreement with equation 2.

Other buffer systems were used to determine the pH range in which TOS could be obtained. A sodium formate buffer of pH 4.2 gave 90%

yields. About 10% yields were obtained in a sodium chloroacetate buffer with a measured pH of 2.6. As the pH was lowered, TOS was formed at a considerably slower rate. The reaction in chloroacetate buffer was very slow and the reported yield was obtained after the reactants had stood in air 36 hours. Because the reaction became increasingly slow, no sharp limit could be found on the lower end of the pH scale, below which the reaction would not occur. At pH's above 6, there is a competing reaction which forms the biscysteinate complex.

An investigation was carried out on the importance of several variables involved in the preparation of TOS. The preparation as described was carried out in very concentrated solutions and the yield of product was curtailed if the reaction mixture was diluted. This held both for Schubert's and the acetate preparation. This was illustrated by a series of experiments in which a stock solution, 2.00 M in sodium acetate and 0.600 M in cysteine hydrochloride was diluted with varying amounts of water and then sufficient cobaltous chloride was added to give a cysteine-cobalt ratio of 3-1. The weight of TOS obtained in each sample was determined. With the 0.600 M cysteine solution a 40% yield of TOS was obtained. The yield decreased linearly with decreasing cysteine concentration and no yield was obtained at a cysteine concentration of about 0.15 M. Figure 1 shows the results of two separate runs. If a solution too dilute to form TOS was evaporated to a suitable concentration, TOS was obtained.

Other experiments were run to determine the effect of a high concentration of a single component of the reaction mixture. A solution dilute with respect to cobaltous chloride and cysteine hydrochloride (H_3cysCl) but saturated with sodium acetate (NaAc) was prepared and

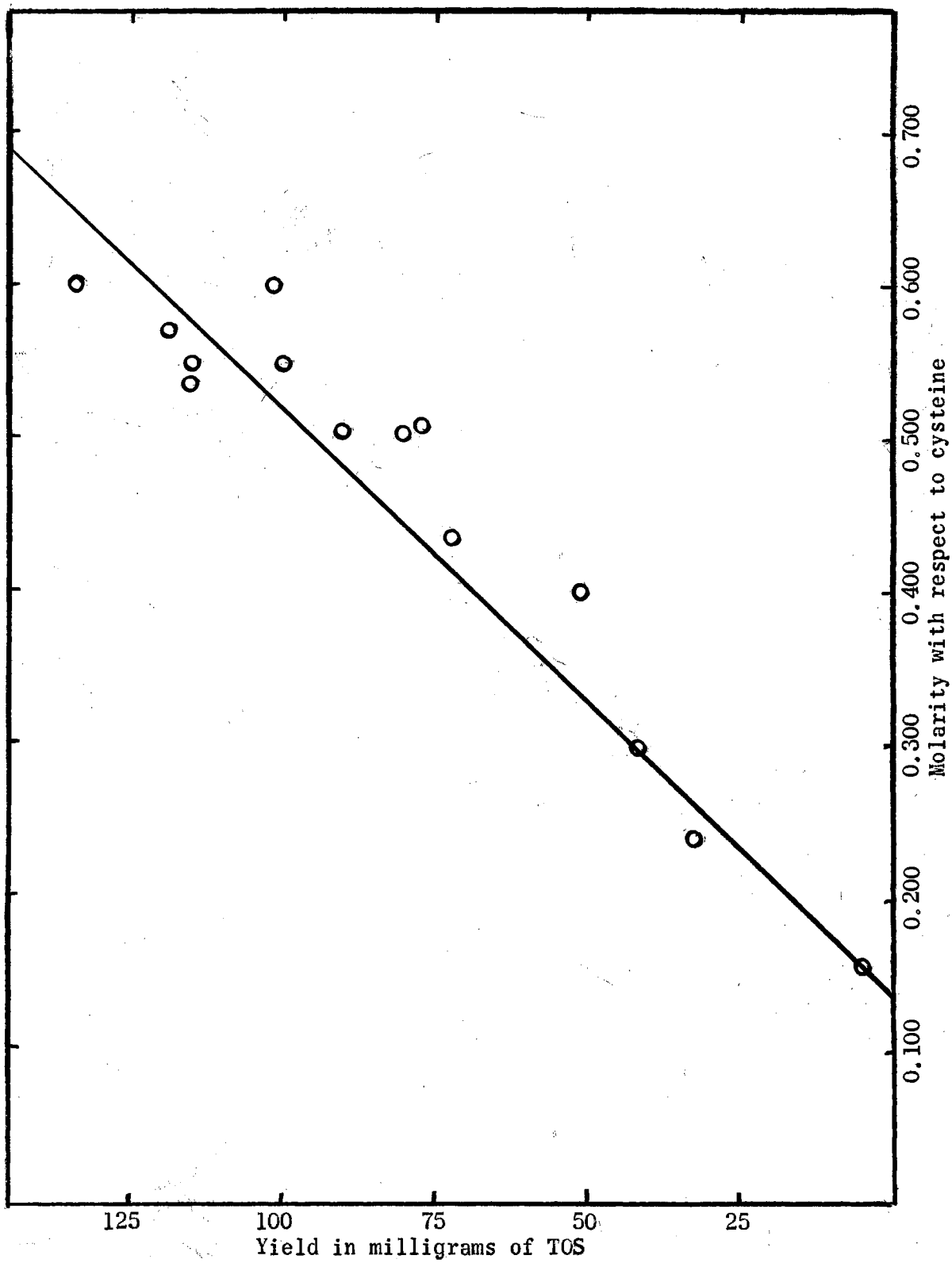


Figure 1. Yield of TOS in Acetate Buffer as a Function of Cysteine Concentration

aerated. No TOS was obtained. Thus a high ionic strength alone is not the determining factor in the formation of TOS.

Next investigated was a solution of 0.05 M in cysteine, 5 M in cobalt and 0.25 M in sodium acetate. No TOS was obtained. Finally a solution was prepared dilute with respect to cobalt and concentrated with respect to cysteine; TOS was obtained.

A possibility considered was that the reaction would depend upon the oxidation of cobalt by oxygen and that $Co(H_2O)_6^{2+}$ would be less likely oxidized than a less hydrated species. So, to have a minimum amount of water, cobaltous chloride heptahydrate was melted and, with heating, NaAc and H_3cys Cl were dissolved in the melt. No TOS was obtained, as the mixture had a low concentration of cysteine. Also investigated was whether TOS could be prepared from a dilute H_3cys Cl solution if the cobalt were present in the cobaltic state as a solution of hemaminecobalt(III) chloride in acetate buffer. No TOS was obtained.

Thus the TOS forming reaction depends upon a high cysteine concentration. TOS is insoluble at pH 5-6 and thus, once formed, it precipitates from solution. No other cobaltic cysteine complex is obtained under these conditions so TOS is probably formed by the oxidation of a cobaltous triscysteinate complex. This complex may be extensively dissociated and thus present in appreciable quantity only in the presence of a high cysteine concentration.

TOS when first formed was brownish-red in color though Schubert reported it to be dark red. When dissolved in base, a cherry red solution

was obtained. It is not easy to dissolve a TOS sample completely in base. The TOS had to be finely powdered and vigorous shaking was required. TOS was more soluble in potassium hydroxide than in sodium hydroxide so potassium hydroxide solutions were easier to work with. If the alkaline solution was acidified, a gelatinous dark-red precipitate was obtained. The dried product was insoluble in water and in organic solvents. The spectrum of TOS in base was measured in the range 210-760 mμ and showed the same peaks as those obtained by Neville. There are peaks at 225, 370, 417, and 510 mμ. As can be seen from Table I, the spectra of TOS prepared by Schubert's method or the acetate method are very similar. The peaks are at the same wave lengths and the molar absorbancies are close. The spectrum of TOS in alkaline solution was plotted in Figure 2. The spectra are the same whether one or more equivalents of base are present per mole of TOS. The spectrum is stable in 0.005 M base for at least a week and the TOS may be recovered by acidifying the solution.

TABLE I

MOLAR ABSORBANCIES OF ALKALINE TOS SOLUTIONS

Molar Absorbancies at Maxima			Molar Absorbancies at Minima		
Wave Length Mμ	Acetate	Schubert's	Wave Length Mμ	Acetate	Schubert's
225	8300	8500	245	7500	7600
270	8900	9600	365	4900	5300
417	8000	8500	450	7000	7400
510	24800	24100			

TOS also dissolves in concentrated sulfuric acid without decomposition. The peaks are shifted somewhat toward longer wave lengths and

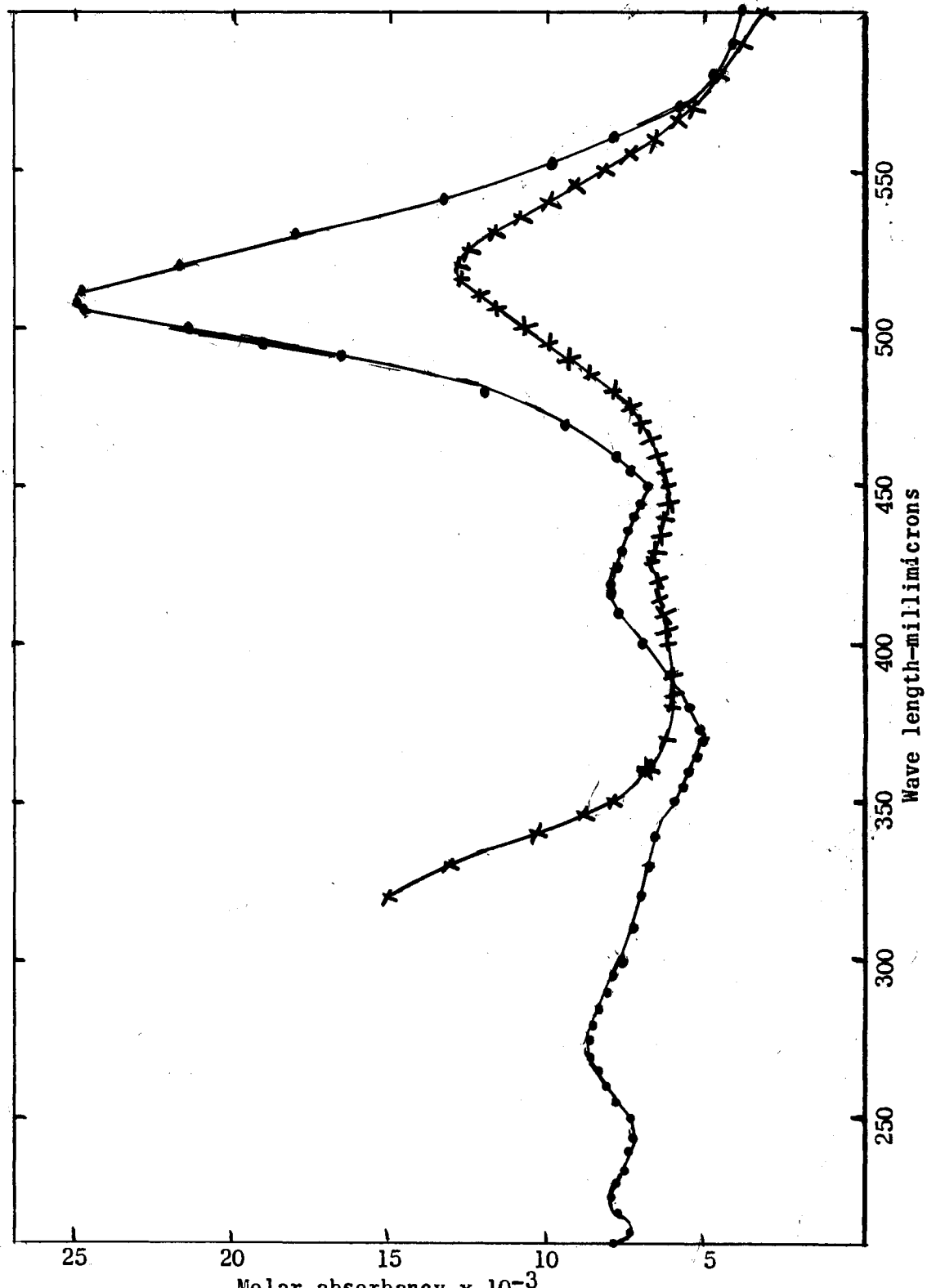


Figure 2. Molar Absorbancy of TOS in Concentrated Sulfuric Acid (XX) and in 0.05 M Sodium Hydroxide (●—●)

the molar absorbancies are changed. The complex can be recovered by diluting the acid with water. Table II shows the absorbancies at the maxima for sulfuric acid solutions of TOS. Figure 2 is a plot representing the absorption spectrum of the solution.

TABLE II

SULFURIC ACID SOLUTIONS OF TOS

Wave Length Mu	Molar Absorbancies
265	8000
423	5300
520	12300

The next question to be considered is what groups are coordinated to the cobalt (III) ion. Schubert deemed it likely that the sulfhydryl and carboxyl groups were involved. Neville[14] prepared a complex from N-formylcysteine which had a spectrum very similar to that of TOS; from this he suggested that chelation involved the sulfhydryl and carboxyl groups. If this is so, then 3-mercaptopropionic acid would form a cobalt (III) complex much like TOS.

This expectation was fulfilled. The 3-mercaptopropionic acid complex, like TOS, could be prepared in acetate buffer. Both complexes were brick-red when freshly prepared and when reprecipitated from basic solution by acidification gave gelatinous red precipitates. When the precipitates were dried, a shiny green material was obtained in both cases. The spectra of the alkaline solutions were very similar. Peaks occurred at the same wave lengths as can be seen from Figures 2 and 3. Table III shows the molar absorbancies of the 3-mercaptopropionic acid

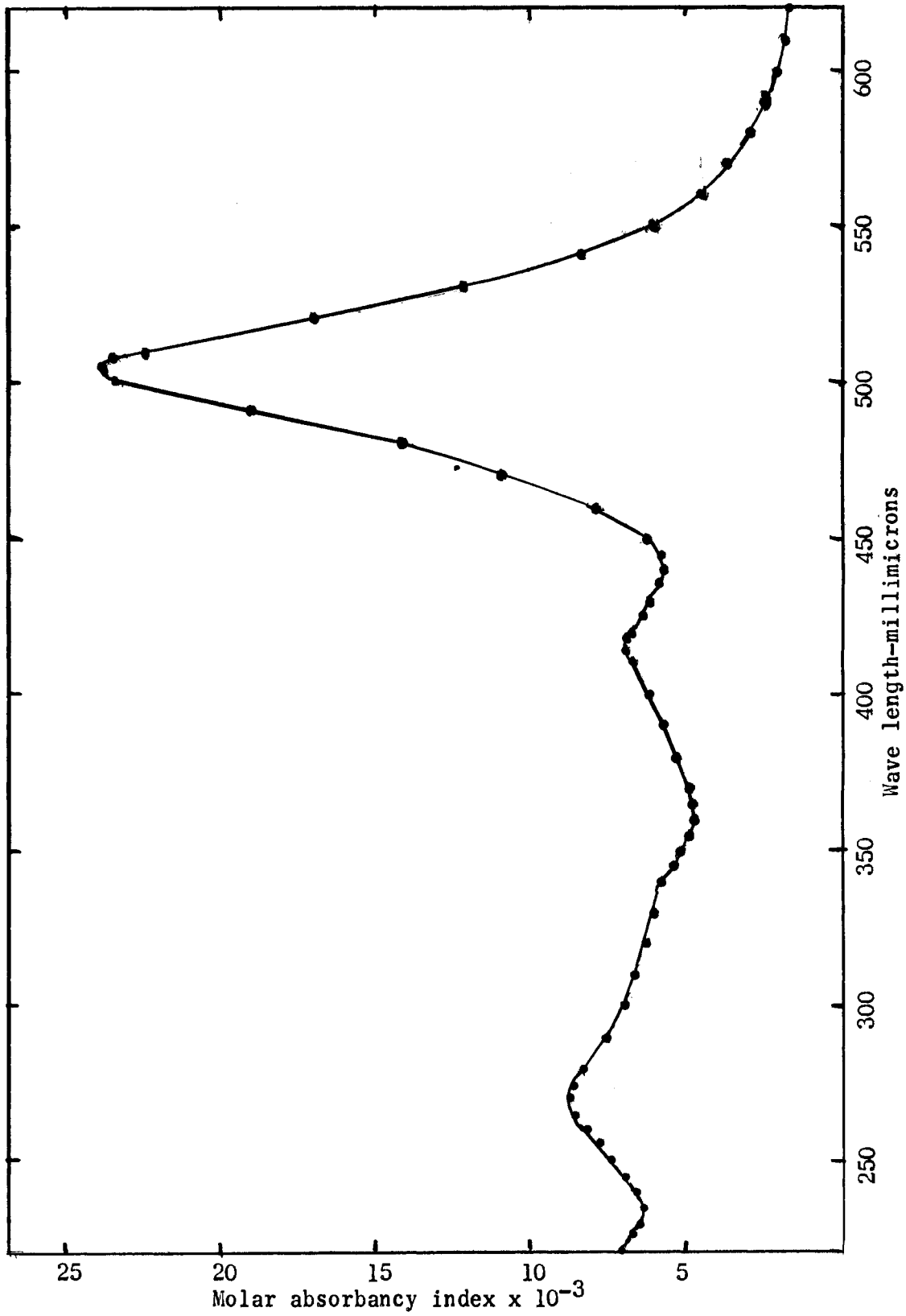


Figure 3. Molar Absorbancy Index of Tris(3-Mercaptoproprionato)cobaltate (III) Complex in 0.05 M Sodium Hydroxide Solution

complex are of the same order as that of TOS. The only difference in the spectra is that TOS has a peak at 225 mu.

TABLE III

MOLAR ABSORBANCIES OF THE 3-MERCAPTOPROPIONIC ACID COMPLEX

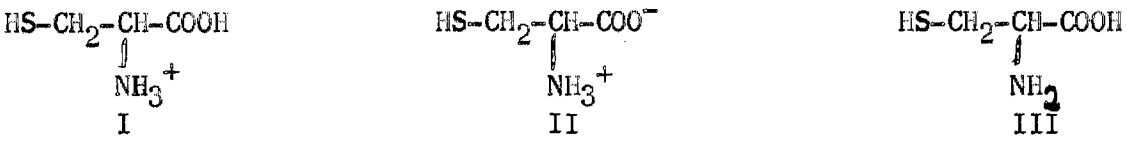
Wave Length Mu	Molar Absorbancies at Maxima	Wave Length Mu	Molar Absorbancies at Minima
270	8200	240	6000
415	6400	365	4300
510	22600	445	5400

A trisodium salt of TOS was prepared by dissolving TOS in an excess of sodium hydroxide and adding alcohol. The salt precipitated. It was red when freshly precipitated; when dry, like the acid, it was green. Unlike the acid, the salt was water soluble and it dissolved completely and readily. An analysis for sodium on a flame photometer gave excellent agreement with the formula $Na_3(Co(SCH_2CHNH_2COO)_3) \cdot 6H_2O$. Drying in an oven 4 hours at 110^0 produced a weight loss corresponding to the removal of 5½ molecules of water.

Efforts were made to prepare a methionine ($CH_3SCH_2CH_2CHNH_2COOH$) complex. In this case the sulfur atom is substituted and it was hoped to obtain a complex with carboxyl-amino chelation, having different properties from those of TOS. However no reaction or evidence of complex formation was observed in acetate buffer or in alkaline solution even after heating to 80^0 and allowing the mixture of alkali, cobalt, and methionine to stand three days.

The evidence is clear that sulfhydryl-carboxyl chelation occurs

in the reaction of cobaltous ion, cysteine, and air in the pH range 2.5-6, while, as will be seen, sulfhydryl-amino chelation occurs at higher pH values, resulting in a brown bis or a green tris complex, depending on the conditions. This may be explained by the fact that in the pH range 2.5-6,[15] cysteine exists as the positively charged ion (I) and the neutral molecule. The neutral molecule exists almost entirely in the dipolar form (II) rather than the unchanged form (III). Owing to the positive charge on the amino group, sulfhydryl-carboxyl is favored.



Attempts to prepare TOS in a formate buffer in methanol were not successful. The product formed instead was, presumably, the brown bis-cysteinate complex. In less polar solvents form III was more prevalent than form II.[15] So the complex obtained was dependent upon what cysteine species was available for chelation rather than the acidity of the solution.

TOS is weakly acidic. A dough determination of K_3, the third ionization constant, was made by dissolving a weighed amount of the trisodium salt in a known volume of water and measuring the pH of the solution. From this, the hydrolysis constant can be determined and an approximate value of K_3 can be calculated. Thus for a 0.0142 M solution, the pH was 10.45. Neglecting activities, the hydrolysis constant was 1.0×10^{-5} and K_3 was 1.0×10^{-9} or $pK_3 = 9.0$.

This is obtained by assuming the hydrolysis reaction to be:

$$(3) \quad TOS^{\equiv} + H_2O = TOS + OH^-$$

(TOS with the appropriate charge is used as a symbol for any of the anionic forms). The hydrolysis constant, K_h, is given by

$$(4) \qquad K_h = \frac{(TOS^{=})\ (OH^{-})}{(TOS^{\equiv})}$$

where the terms in parentheses stand for the concentrations of the various materials. (OH^-) is determined from the pH of the solution. $(TOS^{=})$ is equal to (OH^-) and $(TOS^{\equiv})$ is equal to the calculated concentration, based on the quantity of trisodium salt used, less (OH^-). Both sides of (4) are multiplied by K_w or $(H^+)\ (OH^-)$ to give

$$(5) \qquad K_h = \frac{K_w\ (TOS^{=})\ \ (OH^{-})}{(TOS^{\equiv})\ \ (H^{+})\ \ (OH^{-})}$$

Since

$$(6) \qquad K_3 = \frac{(H^{+})\ \ (TOS^{\equiv})}{(TOS^{\equiv})}$$

$$(7) \qquad K_h = \frac{K_w}{K_3}$$

Experiments were done to determine the solubility of TOS as a function of pH. It was hoped to determine from the slope of a log (TOS) vs log (OH^-) plot, in what form TOS dissolved in alkali; i.e. dit it dissolve as (TOS^{-}) or $(TOS^{\equiv})$.

The reaction would be:

$$(8) \qquad n\ OH^- + TOS = TOS^{n-} + nH_2O.$$

As TOS is a solid, its activity may be taken as 1 so the equilibrium constant can be written as

$$(9) \qquad K_{eq} = \frac{(TOS^{n-})}{(OH^{-})^n} \qquad \text{or}$$

$$(10) \qquad \log K_{eq} = d \log (TOS^{n-}) - n\ d \log (OH^{-})$$

$$(11) \qquad d \log K_{eq} = d \log (TOS^{n-}) - n\ d \log (OH^{-}) = 0$$

hence

$$(12) \qquad d \log (TOS^{n-}) = n\ d \log (OH^{-})$$

$$(13) \qquad \frac{d \log (TOS^{n-})}{d \log (OH^{-})} = n = \text{slope}$$

So by dissolving TOS in a series of buffers and measuring the pH of the buffers and the concentration of TOS in the solutions, it was expected

that the value of n might be found. The results were non-reproducible and inconclusive.

This is believed due to the fact that TOS dissolved very slowly and that equilibrium was not reached even if the solutions were continuously shaken or let stand for several days. Also many of the buffers were sodium salts and the sodium salt of TOS is sparingly soluble. It was found that less than one equivalent of sodium hydroxide per mole of TOS dissolved some TOS. From this it seems likely that TOS can go into solution as a monosodium salt.

TOS started to dissolve in significant (10^{-5} M) quantities at a pH range of 8.5-9.0 and it can be estimated from this that K_1 is not much larger than K_3. The estimation of K_1 is done as follows: At a pH of 8.5, the TOS concentration is about 10^{-5} M. The ionization constant is measured by:

$$(14) \qquad K_1 = \frac{(H^+)\ (TOS^-)}{(TOS)}$$

where (TOS) is the concentration of unionized TOS in solution. The solubility vs pH plots showed that the TOS concentration at a pH of 7 (or in pure water) is of the order of 10^{-7} M. Then K_1 is about 3×10^{-7} or 30 times K_3. All three protons are weakly acidic and of near equal strength.

The addition of silver ion to an alkaline solution of TOS caused the color of the solution to change from dark red to a reddish-purple color. There was a single absorption peak in the visible at 540 mμ. The molar absorbancy at 540 mu was 23,800, which was about the same as that of TOS at its 510 mμ peak. The general effect was that absorbancies were shifted toward longer wave lengths.

The formula of this species was determined by the method of continual

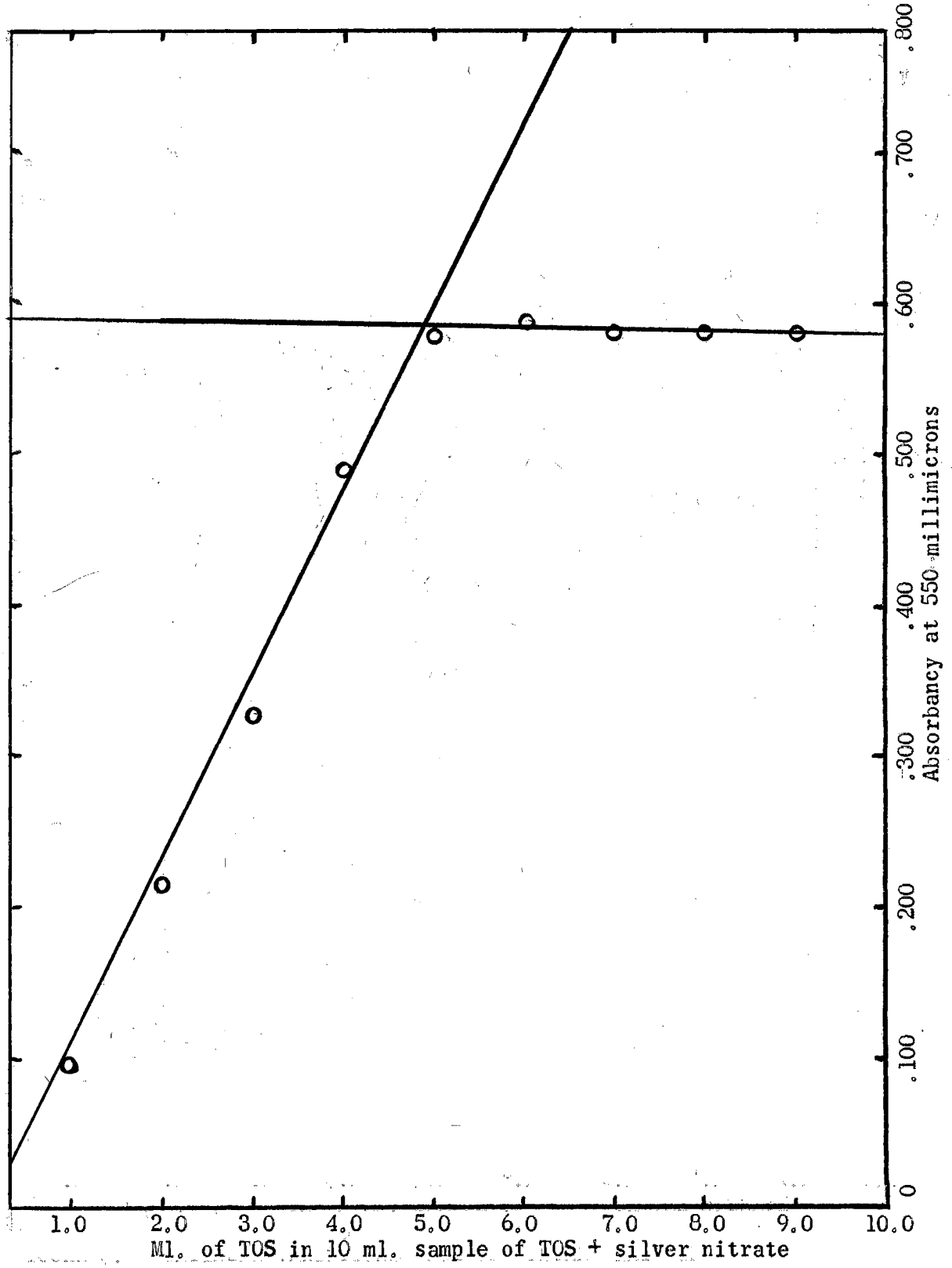


Figure 4. Continual Variations Plot of Silver Salt of TOS

variations[16] and it was found that one equivalent of silver ion reacted with one mole of TOS. Figure 4 shows the continual variation plot. The intersection of the two lines was where the silver ion concentration was equal to the concentration of TOS.

Experimental

1. Chemicals: Cysteine hydrochloride hydrate was obtained from the California Corporation for Biochemical Research, Los Angeles, California and from the Schwarz Laboratories, Mount Vernon, New York. The California Corporation cysteine hydrochloride was their "B Grade". 3-mercaptopropionic acid was obtained from the Aldrich Chemical Company, Milwaukee, Wisconsin and purified by distillation, the middle third being retained for use. All other chemicals were of commercial reagent quality.

2. Preparation of TOS by Schubert's Method: Potassium hydroxide, 7.5 molar, was added to 1 M cobaltous chloride in quantity just sufficient to precipitate the cobalt as cobaltous hydroxide, to this solution was added 10 M cysteine hydrochloride which had been partially neutralized with 1.4 equivalents of 7.5 M potassium hydroxide per mole of cysteine. Air was passed through the solution one hour and the mixture was then allowed to stand 24 hours. A brownish-red precipitate was obtained. It was separated by filtering and then dissolved in 0.05 M potassium hydroxide. Acidification with concentrated hydrochloric acid precipitated a red gelatinous product which was filtered. Preliminary centrifuging appreciably reduces the filtering time as the filtering process is slow. The precipitate was washed with water until free of chloride ion and then dried in an oven at 110^{0}. Green lumps or sometimes thin

shiny-green sheets were obtained. This product was suspended in water 30 minutes and tested for absorbed chloride. Rinsing was repeated until the decanted wash water gave no precipitate with silver nitrate. Yields were 90-95% based on the amount of cobalt used.

3. Preparation of TOS in Acetate Buffer: To cysteine hydrochloride and sodium acetate in a molar ratio of one to five, enough water was added to make a thick paste. Then 0.33 moles of cobaltous chloride per mole of cysteine were added as a 1 M solution. The suspension was stirred thirty minutes and allowed to stand twelve hours in air. More water was added, if necessary, to maintain a thick paste. After standing, the suspension was treated with water and filtered. The precipitate was dissolved in base and reprecipitated in the manner described in section 2. Yields were 90-95% based on the amount of cobalt used. Calculated for $(Co(SCH_2CH(NH_3)COO)_3) \cdot 3H_2O$, Co 12.94% found 12.68%, loss of weight on drying 7.97%, found 8.05%, nitrogen 9.23% found 9.20%, carbon 23.74% found 23.37% and hydrogen 4.87%, found 4.86%.

4. Titration of the Sodium Acetate Mother Liquor: The washings from section 3 were titrated with 1.000 M hydrochloric acid using a Beckman model G pH meter as an indicator.

The end point was determined by the differential method where the change in pH divided by the change in mililiters of acid $(\frac{\Delta pH}{\Delta mc})$ was plotted against the total milliliters of acid. A cusp-shaped curve was obtained with the end point being at the peak of the cusp. The initial amount of sodium acetate was determined by weight. The amount unconverted to acetic acid was determined by titration. The difference was the amount of sodium acetate that reacted in the formation of TOS. The amount of TOS obtained was determined by weight. End point calculated

for five moles of sodium acetate being converted to acetic acid for every mole of TOS formed: 20.43 ml., 21.93 ml.; found 20.0 ml., 20.5 ml.

5. Preparation of TOS in Formate Buffer: To a solution 3 M with respect to cysteine and .1 M in cobaltous chloride was added enough solid sodium formate to provide 5 moles of it for every mole of cysteine present. The mixture was stirred 30 minutes and then let sit 48 hours in air. The precipitate was then treated in the manner described in section 3. The yield was 85% based on the amount of cobalt used.

6. Preparation of TOS in Chloroacetate Buffer: The same procedure was used as described in section 5 except sodium chloracetate was used instead of sodium formate. The yield was 10%, based on the amount of cobalt used.

7. Gradual Dilution Experiment: A stock solution was made up 2.00 M in sodium acetate and 0.60 M in cysteine hydrochloride. The solution was diluted with water as required and then 0.33 moles of cobaltous nitrate per mole of cysteine were added. The solutions after mixing stood in air 24 hours and then the precipitates were washed into Gooch crucibles. The precipitates were washed with water, alcohol, and ether, and then air dried and weighed.

8. Ionic Strength Experiment: A solution 0.05 M in cobaltous chloride, 0.15 M in cysteine hydrochloride and saturated with sodium acetate was stirred 30 minutes and then let stand 24 hours. Filtration showed that no TOS had formed.

9. High Cobalt Concentration Experiment: A solution 0.15 M in cysteine hydrochloride and 0.75 M in sodium acetate was saturated with solid cobaltous nitrate hexahydrate, stirred and treated as described in section 7. No TOS was obtained.

10. High Cysteine Concentration Experiment: A 5 M cysteine hydrochloride solution was saturated with sodium acetate with solid sodium acetate being present. Enough 1 M cobaltous nitrate solution was added to make a solution 0.05 M with respect to cobalt. The solution was treated as described in section 7. Some TOS was obtained.

11. Attempted Preparation of TOS from hexamminecobalt(III) chloride: To a solution, 0.10 M in cysteine hydrochloride and 0.50 M in sodium acetate was added sufficient hexamminecobalt(III) chloride to make the solution 0.05 M with respect to it. The flask was stoppered and allowed to stand 24 hours. No TOS was obtained.

12. Preparations of TOS by Concentrating a Dilute Solution: A solution 0.15 M in cysteine hydrochloride, 0.75 M in sodium acetate and 0.05 M in cobaltous nitrate after standing 12 hours had produced no TOS. Upon evaporation of the solution, some TOS was obtained.

13. Spectral Measurements: All spectral work was done using a Beckman Model DU Spectrophotometer. The solutions were made up by dissolving weighed amounts of the materials studied in known volumes of solvent. The concentrations were chosen to give absorbancies between 0.200 and 1.100. The molar absorbancy is equal to the absorbancy divided by the molarity of the solution. The solvent was the reference solution in all cases.

14. Preparation of Tris(3-Mercaptopropionato)cobaltic(III) Acid: 3-Mercaptopropionic acid was immediately added to an acetate buffer to mask the odor. One mole of acid was added for each five moles of sodium acetate used. Then enough 1 M cobaltous chloride was added to the solution to give a cobalt-acid ratio of one to three. The procedure was identical with that described in section 3 as the purpose of the experi-

ment was to see if the complex could be prepared under the same conditions as TOS. The yield was 95% based on the amount of cobalt used. Calculated for $(H_3(Co(SCH_2CHCOO)_3)$, cobalt 15.75%, found 15.88%, loss of weight on drying none, found none, carbon 28.88%, found 29.05% and hydrogen 4.04%, found 4.24%.

15. Attempts to Prepare a Cobalt (III) Methionine Complex: Methionine was dissolved in 1 M sodium hydroxide and cobaltous chloride added to give a cobalt-methionine ratio of one to three. A precipitate of cobaltous hydroxide was obtained but even after standing a day, no evidence of any other reaction was observed. A similar mixture was heated to 80^0 and then allowed to stand three days. No evidence of complex formation was observed. Methionine was also suspended in sodium acetate solution in the presence of cobaltous chloride. There was no apparent reaction.

16. Preparation and Analysis of the Trisodium Salt of TOS: TOS, 0.7 grams, was dissolved in 500 ml. of 0.10 M sodium hydroxide. The solution was well shaken, allowed to stand 14 hours and then filtered. Alcohol was added to the filtrate. A precipitate was obtained. The solution was filtered, washed with alcohol, then ether, and dried in air. A sample of the salt was dissolved in a solution which was 500 parts per million in lithium hydroxide. Standard sodium samples were made up by dissolving sodium chloride in a solution 500 ppm in lithium hydroxide. The standard solutions were measured on a Perkin-Elmer flame photometer and used to calibrate the instrument. The trisodium salt solution was run and compared with the curve obtained from the standards. Calculated for $Na_3(Co(Cys)_3) \cdot 6H_2O$, 50.3 ppm sodium in the solution, found 51.0 ppm.

Another sample was heated four hours at 110^0 in an oven to drive

off water. The weight loss was 20.7%. Calculated for complete loss of water from $Na_3(Co(cys_3) \cdot 6H_2O$, 22.1 %, for $Na_3(Co(cys)_3) \cdot 5H_2O$, 18.4%.

17. Reaction of Silver Ion with TOS: When silver nitrate solution was added to an alkaline solution of TOS, the color changed from cherry-red to a reddish-purple. The reaction product was stable enough that an ammonical solution of silver nitrate could be used. This was convenient as it prevented the precipitation of silver hydroxide from a basic solution if an excess of silver in the amount required to react with TOS were present. That the reaction took place at the expense of the silver diammine complex gave an idea of the stability of the product. This result showed that this product was more than a simple salt and was probably some form of silver-TOS complex.

The formula of the silver-TOS compound was determined by using the method of Continual Variations.[16]

One solution was prepared of 1.77×10^{-4} M TOS in sodium hydroxide. Another solution was prepared 1.69×10^{-4} M in silver nitrate and 10^{-3} in ammonia. A series of solutions were prepared using 10 ml of the TOS solution, 9 of TOS and 1 silver nitrate, 8 of TOS and 3 of silver nitrate, et cetera. The absorbancies of each of these solutions were measured at 550 mu. (Eight milliliters of water were added to every sampleto get more convenient absorbancy values). On the ordinate was plotted the ratio of complex to silver of the solutions and on the abscissa was plotted the absorbancy at 550 mu. Two straight lines resulted. Their intersection corresponded to a value on the ordinate of a one to one ratio. This showed that the compound was formed by the reaction of one mole of silver and one mole of TOS.

18. Solubility Experiments: Weighed amounts of TOS were added to

20 ml. volumes of sodium hydroxide or sodium carbonate-sodium bicarbonate buffer solutions. The pH of the solutions was determined with a Beckman Model G pH meter. Some solutions were continually mechanically shaken for up to 3 days. Others were let stand with intermittent manual shaking for up to three days. Samples were kept in a constant temperature bath at $36 \pm 1^{\circ}$ C. The TOS concentration of the solutions was determined by pipetting out a one ml. portion of the solution and determining the absorbancy, quantitatively diluting the samples as necessary to obtain more convenient absorbancy values. Despite careful fine grinding of the TOS, results were not reproducible. Data from the runs was listed for examination.

Run 1 was made by suspending 5×10^{-5} moles (.0227 g.) of TOS in 20 ml. of various buffer solutions prepared by using 0.100 M sodium carbonate and 0.100 M sodium bicarbonate and allowing the solution to sit with occasional shaking for three days.

TABLE IV

TOS SOLUTIONS IN CARBONATE-BICARBONATE BUFFERS - I

pH of the Solution	TOS Conc.-Molar $\times 10^{-5}$
9.23	.45
9.31	1.60
9.44	1.50
9.58	2.40
9.55	9.35
9.73	4.35
9.73	14.2
9.79	16.8

Complete solution of TOS would have resulted in a TOS concentration of 2.50×10^{-3} M.

Run 2 was made by suspending 5×10^{-5} moles of TOS in buffer solutions made from 0.0123 M sodium carbonate and 0.0171 M sodium bicarbonate. The pH of the solutions was measured before adding the TOS. Then TOS was added and the suspensions were mechanically shaken three days. The pH of the solution was then redetermined and the concentration of TOS obtained from absorbancy measurements. For all pH measurements, the pH meter was standardized against commercial pH 7 and pH 10 buffers.

TABLE V

TOS SOLUTIONS IN CARBONATE-BICARBONATE BUFFERS - II

pH of Buffer With No TOS	pH of Buffer With TOS	Conc. of TOS-Molar
8.30	7.70	4.3×10^{-6}
8.53	8.17	2.5×10^{-6}
8.69	8.50	4.7×10^{-6}
8.95	8.48	9.5×10^{-6}
9.09	8.53	9.6×10^{-6}
9.21	9.02	1.34×10^{-5}
9.31	8.70	3.88×10^{-5}
9.38	8.90	1.02×10^{-4}
9.48	9.16	1.34×10^{-4}
9.64	9.40	2.46×10^{-4}
9.67	9.33	2.63×10^{-4}
9.71	9.38	3.09×10^{-4}
9.75	9.45	5.55×10^{-4}
9.83	9.47	7.78×10^{-4}
9.91	9.57	7.97×10^{-4}
9.99	9.90	9.34×10^{-4}
10.10	9.98	1.18×10^{-3}
10.26	9.91	1.31×10^{-3}
10.40	10.08	1.40×10^{-3}
10.60	10.22	2.04×10^{-3}
10.63	10.29	2.24×10^{-3}

If the entire sample dissolved, the TOS concentration would have been 2.50×10^{-3} M.

Run No. 3 was made to determine whether TOS went into solution as a mono, bi, or tri-sodium salt. To 10^{-4} moles (0.0455 g) of TOS was added sodium hydroxide solution to give varying alkali to TOS ratios. The samples were shaken 48 hours and the TOS concentration was then determined spectrophotometrically. The spectra of TOS solutions were not affected by the base/TOS ratios. The results of Run 3 are shown in Table VI.

TABLE VI

TOS SOLUTIONS IN SODIUM HYDROXIDE - I

Base/TOS Ratio	pH	TOS Conc.-Molar x 10^{-3}
.100	7.07	.09
.200	7.00	.12
.300	7.73	.21
.400	8.52	.40
.500	9.13	1.41
.600	9.40	2.44
.750	Not measured	3.89
.750	Not measured	3.69
.850	Not measured	3.84
.900	Not measured	1.41
.950	Not measured	4.34
1.00	Not measured	3.94
1.05	Not measured	4.44
1.10	Not measured	4.39
1.20	Not measured	4.75
1.30	Not measured	3.49
1.50	Not measured	4.29
2.00	9.85	4.44
2.50	10.43	4.34
3.00	10.58	4.14
3.50	10.66	4.44
4.00	10.72	3.79

Complete solution of the TOS would have resulted in a TOS concentration of 5.00×10^{-3} M.

Run 4 was made by adding six moles of sodium hydroxide per mole of TOS, shaking the samples manually at ten minute intervals for two hours and then back titrating with hydrochloric acid to give a desired base to TOS ratio. The samples were then shaken and allowed to stand 24 hours with intermittent shaking. The TOS concentration and pH's of the solutions were then determined. The results of Run 4 are shown in Table VII. 4.48×10^{-4} moles (.2036 g) of TOS were used in each sample.

TABLE VII

TOS SOLUTIONS IN SODIUM HYDROXIDE - II

Base/TOS Ratio	pH	TOS Conc. M x 10^{-3}
.10	6.88	--
.20	7.22	--
.30	7.41	--
.40	8.22	.005
.50	9.20	.31
.60	9.40	2.31
.70	9.52	2.50
.80	9.78	2.56
.90	9.87	2.76
1.00	9.95	2.76

Complete solution of TOS would have resulted in a TOS concentration of 22.4×10^{-3} molar.

19. Analyses: Cobalt analyses were made by decomposing the sample with hot concentrated sulfuric acid on an electrically heated Kjeldahl rack until all organic matter was decomposed. The acid solution was then diluted, cooled, and made alkaline with ammonia. Cobalt was then determined by electrodeposition as the metal.[17] Sodium was determined with the use of a flame photometer as described in section 16.[18] Nitrogen, carbon, and hydrogen were determined commercially by the Micro-Tech Laboratories, 8000 Lincoln Avenue, Skokie, Illinois.

CHAPTER III

PREPARATION AND PROPERTIES OF COBALT (III) CYSTEINE COMPLEXES FORMED BY SULFHYDRYL-AMINO CHELATION

Results and Discussion

In the pH range 7-9, a brown solution of a cobaltic biscysteinate is formed. Schubert[7] first prepared the complex and established that the formula was $H(Co(SCH_2CHNH_2COO)_2(H_2O)_2) \cdot 3H_2O$. He believed that the complex was a dimer, but on the basis of cryoscopic evidence, Neville and Gorin[9] found that the monomeric formula was correct. The suggested name is biscysteinatodiaquocobaltic (III) acid-N,S trihydrate or BNS for short.

Experiments by Neville and Gorin[10] and Wessler[19] established that chelation involved the sulfhydryl and amino groups of cysteine and that the carboxyl group was not directly connected to the cobalt atom.

BNS is obtained from cobalt chloride, cysteine, and oxygen in the pH range 7-9 regardless of the cysteine-cobalt ratio, and at pH values above 10 and cysteine-cobalt ratios of 3-1 or more a triscysteinate complex is obtained. BNS is formed as the anion but it is precipitated from solution at a pH of about 2. Thus BNS is an insoluble acid. Titration of BNS[14] showed an end point around pH 7 and this indicates that the acid was extensively dissociated in solution as the end point for a slightly dissociated acid would be above pH 7. Unlike TOS, BNS dissolved rapidly in base with no special grinding or stirring being required. The solid acid was black or brown and the alkaline solutions were brown.

BNS also dissolved in 0.1 M hydrochloric acid. The acid solutions were brown and had the same absorption spectrum as the basic solutions. The wave lengths of the peaks and the peak molar absorbancies in the range 210-760 mμ are shown in Table VIII.

TABLE VIII

PEAK MOLAR ABSORBANCIES OF BNS

Wave Length of Peak - mμ	Molar Absorbancy $\times 10^{-3}$
280	7.10
350	5.80
442	1.82

The proton in BNS is probably loosely bound to one of the two carboxyl groups and in sufficiently acid solutions, the second carboxyl group probably also accepts a proton. Thus in 0.1 M hydrochloric acid, BNS would dissolve as $(Co(SCH_2CHNH_2COO)_2\ (H_2O)_2)^+$.

When a solution of BNS in 1 M sodium hydroxide was boiled 45 minutes, a precipitate resulted that gave a qualitative test for cobalt and that probably was cobalto-cobaltic oxide (Co_3O_4). The absorption spectrum of the resulting solution showed a single peak at 280 mμ. Thus the composition of the solution was changed.

If the alkaline solution of BNS was allowed to stand a week at room temperature, a brown cobalt-containing precipitate resulted that was probably cobalto-cobaltic oxide. The absorption spectrum of the solution showed peaks at 280 and 350 mμ.

At pH values above 10 and with a cysteine-cobalt ratio of 3-1 or

greater, green solutions of a cobaltic triscysteinate complex are obtained. Schubert[8] first prepared the triscysteinate as the potassium salt and determined the formula to be $K_3(Co(SCH_2CHNH_2COO)_3) \cdot 3H_2O$. In accordance with modern nomenclature,[13] the name of this compound is potassium triscysteinatocobaltate (III) - N,S trihydrate or TNS for short. The complex was prepared by Schubert by reacting cysteine hydrochloride and cobalt chloride in 4 M potassium hydroxide in the presence of air. The salt was precipitated with alcohol. Schubert reported that the dry salt was green and stable. However the writer's preparations, following Schubert's directions, were gray-blue and they decomposed in air or when protected from moisture in a desiccator to give a brown powder.

TNS was also prepared from sodium hydroxide, cysteine hydrochloride, and hexamminecobalt (III) chloride. Sufficient alkali to give a pH above ten, and sufficient cysteine to give a cysteine-cobalt ratio of 3-1 were combined and added to an aqueous solution of hexamminecobalt (III) chloride. At room temperature about three hours were required for complete reaction.

TNS was also prepared from BNS, alkali, and cysteine hydrochloride. It was necessary to heat the reactants to about 70° for 30 minutes to form TNS. The reaction did not proceed to any measurable extent in 12 hours at room temperature.

As BNS did not react with cysteine and base at room temperature, it was decided to try to replace the two molecules of water with ethylenediamine. The absorption spectrum was not changed after 12 hours' standing at room temperature. So apparently BNS has no marked affinity for a third molecule of ligand.

The absorption spectrum of TNS was the same regardless of the

method of preparation. The absorbancy vs. wave length plot of a TNS solution is shown in Figure 5. Table IX shows the peak molar absorbancies in the wave length range 210-760 mμ.

TABLE IX

PEAK MOLAR ABSORBANCIES OF TNS SOLUTIONS

Wave Length mμ	Molar Absorbancy
584	269
444	570
280	1.64×10^4

The spectrum was independent of the cysteine-cobalt ratio, as long as it was 3-1 or higher.

TNS is not stable at below pH 10. Schubert[8] found that cystine and a brown solution resulted and he supposed that the solution was one of BNS. However, an examination of the absorption spectrum of this solution (Figure 6) in the range 220-760 mu shows that the spectrum was decidedly different from that of BNS. The products obtained were dependent on the conditions in which TNS was decomposed.

Neville[10] reported that TNS was susceptible to oxidation even above pH 10, BNS and cystine being the products. However Schubert[8] prepared TNS in good yields (85%) by bubbling air through a solution five hours. When the writer repeated the preparation, TNS was precipitated with alcohol and the supernatent liquid had very little brown color. Under these conditions BNS is soluble so it should have been present in the liquid phase. The precipitate dissolved giving a clear green solution free from any brown coloration. Thus after five hours of bubbling air

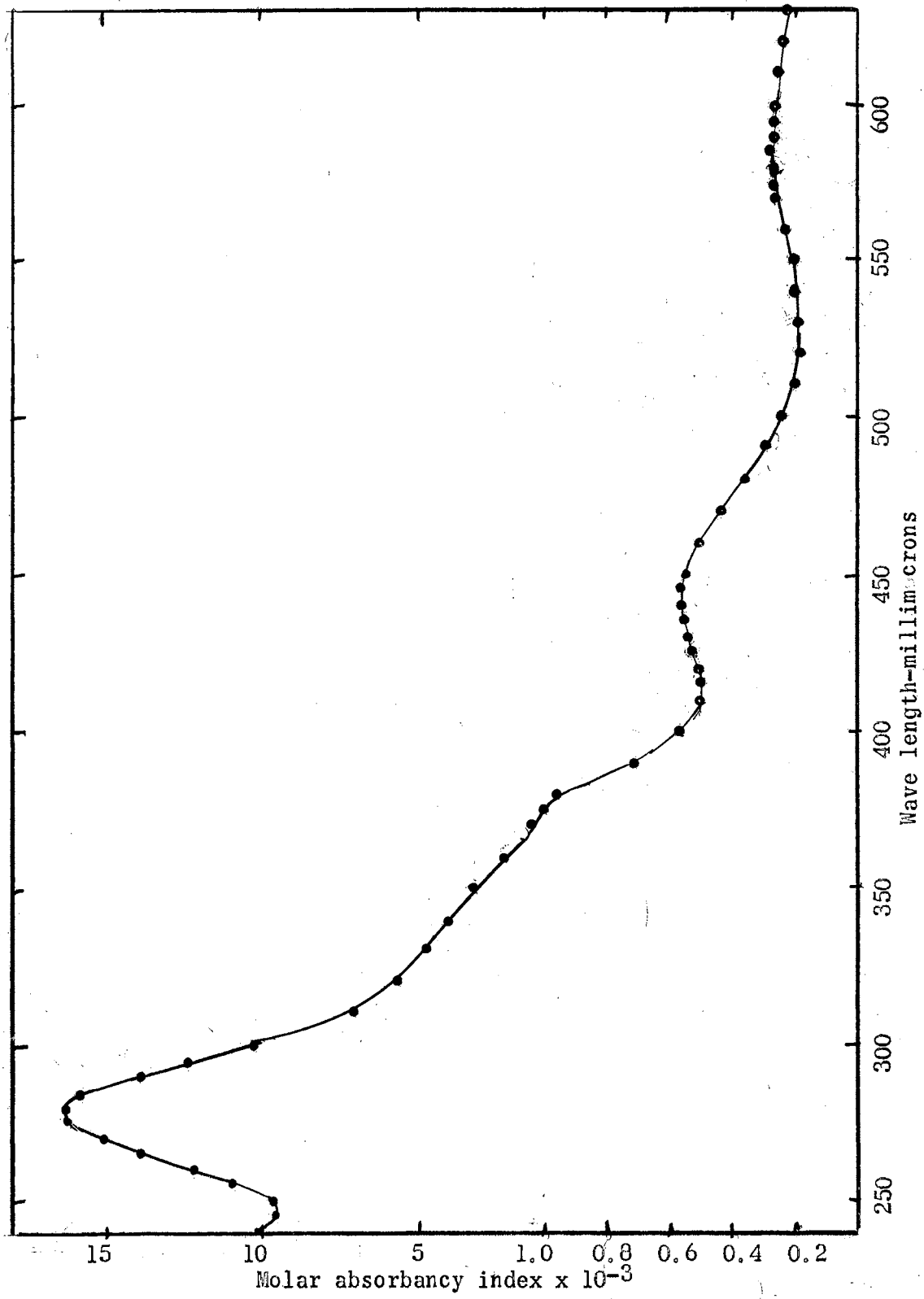


Figure 5. Molar Absorbancy Index of TNS in 0.1 M Sodium Hydroxide Solution

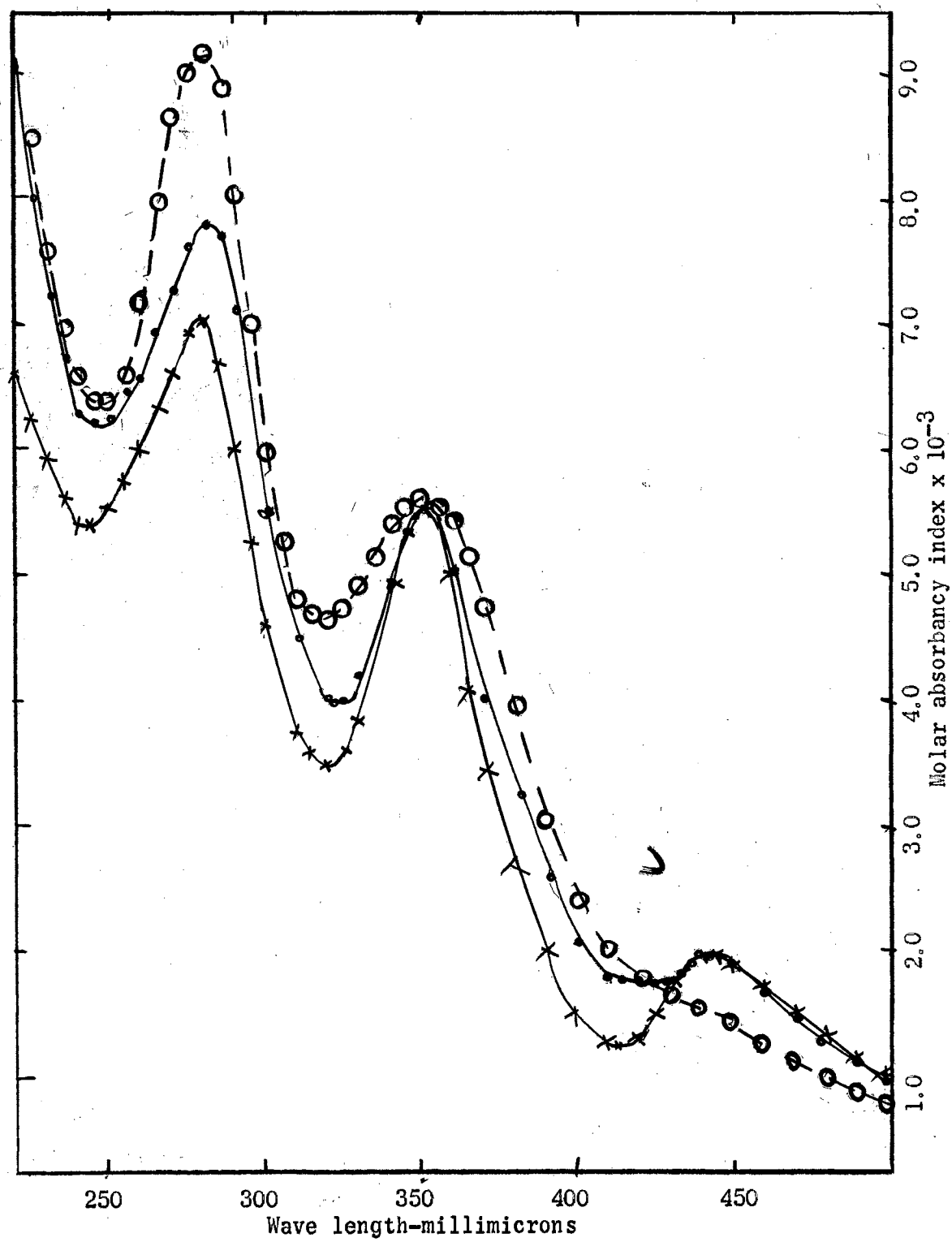


Figure 6. Molar Absorbancy Index of BNS (XXX), Acid Decomposed TNS (____) and pH 8 Decomposed TNS (-o-o-o-o-) all in 0.1 M Sodium Hydroxide Solution

through the system and an additional twelve hours of letting the mixture stand in air, very little oxidation occurred.

The solution from which TNS was precipitated was about 0.35 M with respect to that material. On the other hand for spectrophotometric work in the visible range, a 1.00×10^{-3} M solution was required. Solutions of this composition turned brown fairly quickly. By making the solution with boiled, deaerated water and carefully protecting the solutions from air, the solutions were stable enough for spectrophotometric work for about six hours. Solutions, 2.00×10^{-5} M, were required for ultraviolet spectrophotometric work. Even when boiled water and an excess of cysteine were used, the solutions were stable for only 45 minutes to an hour. So the concentration of a TNS solution definitely affected its stability.

This was more fully investigated by preparing solutions of TNS of differing concentrations and passing air through them. The extent of decomposition was followed by measuring the absorbancy of the solutions at 442 mu. The molar absorbancy of a fresh TNS solution was 570_{μ} while that of a decomposed solution was about double that.

First, TNS was dissolved in water to give solutions that were 10^{-4} M, 10^{-3} M, and 10^{-2}M. All solutions were decomposed with the more dilute solutions reacting faster. As the measured pH of these solutions was 6.0, 8.0, and 9.5 in order of increasing TNS concentration, this factor alone would cause decomposition.

In another set of experiments, three more solutions were prepared with 1 M sodium hydroxide as solvent instead of water. The solutions still decomposed but the rate of decomposition was slowed, particularly for the more dilute solutions. Aliquot portions of the solutions, which

had not been subjected to air, after 4.67 hours had decomposed to some extent but not as much as the aerated solutions.

Next investigated was the effect of the presence of an excess of cysteine. TNS was dissolved in a solution 1 M in sodium hydroxide and 0.11 M in cysteine. The initial absorbancy of the 1.11×10^{-4} M solution was abnormally low, but when it reached a normal value, the solution was fairly stable for 1.5 hours. Cysteine stabilized all solutions. However it had the least effect on the more concentrated solutions. Data and experimental details of these runs are given in the experimental section.

From the information obtained from these experiments, it seems reasonable that TNS decomposed to give cysteine and other materials and that an excess of cysteine shifts the equilibrium in favor of TNS. The extent of decomposition seems to be greater in dilute solutions than in more concentrated ones.

If TNS or the brown product resulting from its air decomposition was acidified to a pH of about three, a brown precipitate resulted. When this material was dissolved in base, the absorption spectrum obtained was very similar to that of BNS. The peaks were at the same wavelengths, 280, 350, and 442 mμ, and the peak molar absorbancies agreed within 5%. The only difference was that the 413 mu minimum was shifted to 423 mu and the absorbancy at the 442 mμ peak was only 12% higher than the absorbancy at 423 mμ. For BNS, the 442 mμ absorbancy was about 30% higher than the 413 mμ absorbancy. The absorption spectrum of an alkaline solution of this material is shown in Figure 6. Assuming this product to be an isomer of BNS, only about a 60% yield was obtained, based on the amount of TNS initially present.

If TNS was decomposed in air, alkali or in pH 8 solution and the product was dissolved in base, a brown solution resulted that had absorption peaks at 280 and 350 mμ but no peak at 442 mμ. The molar absorbancy at 280 and 250 mμ was higher than that of a solution of BNS or of the product resulting from the acid decomposition of TNS.

It was now clear that, regardless of experimental conditions, BNS was not produced by the decomposition of TNS and that more than one product could result from the decomposition of TNS. So it was decided to investigate both BNS and the decomposition products of TNS by ion-exchange chromotography.

The details of the construction and operation of the ion-exchange column may be found in the experimental section.

The samples were dissolved in a pH 8 medium, either by dissolving it directly in the eluent or by dissolving the sample in a small volume of hydrochloric acid and then making the solutions alkaline by the dropwise addition of concentrated ammonia. The difference was that in the latter case the sample had been momentarily acid while in the former case, the pH had not been allowed to go below 8. The eluent solutions were a series of ammonium chloride-ammonia solutions of varying concentrations with the ammonium ion-ammonia ratio being 20-3. These solutions had a pH of 8-8.5. The column was filled with the eluent solution. Then the sample solution was placed on the column and followed by more of the eluent. The components of the sample passed through the column and came out at the bottom. The concentrations of the solutions were measured by determining the absorbancy and when a plot of absorbancy or concentration vs ml. of eluent was made, the separate components showed characteristic peaks.

When BNS was eluted, three products were obtained. The spectra of all three were identical. The first material made up 1-2% of the total sample and it was completely eluted with 0.100 M ammonium chloride-0.015 M ammonia. The 0.200 M ammonium chloride-0.030 M ammonia eluent produced a partial separation of two materials in an estimated 2-1 ratio. The concentration (absorbancy) vs. ml. of eluent plot is shown in Figure 7.

When TNS was decomposed and the brown solution was eluted, five products were obtained. The first was eluted with the 0.100 M ammonium chloride eluent and by the absorption spectrum and the volume of eluent required, it was found to be the same material that was the minor constituent of BNS. Elution with 0.20 M ammonium chloride eluent produced a partial separation of two materials with identical spectra. The absorption spectra were the same as that of an acid-decomposed TNS solution. As can be seen from the concentration (absorbancy) vs. ml. of eluent plots in Figures 8 and 9, a peak was produced by 0.300 M ammonium chloride eluent but this was due to the change in eluent concentration from 0.200 to 0.300 M. This would increase the concentration of material in the eluent about 50%.[20] The increase was of that order.

Two more materials were obtained. Their spectra had peaks at 280 and 350 mμ and their spectra were the same as that obtained by dissolving an air-decomposed sample of TNS in water. One product was eluted with 0.500 M ammonium chloride eluent and the other with 1.000 M ammonium chloride eluent. The latter material absorbed more strongly in the ultraviolet than did the former material.

It was found that the proportions of the various products obtained from the elution of decomposed TNS were very much dependent upon the

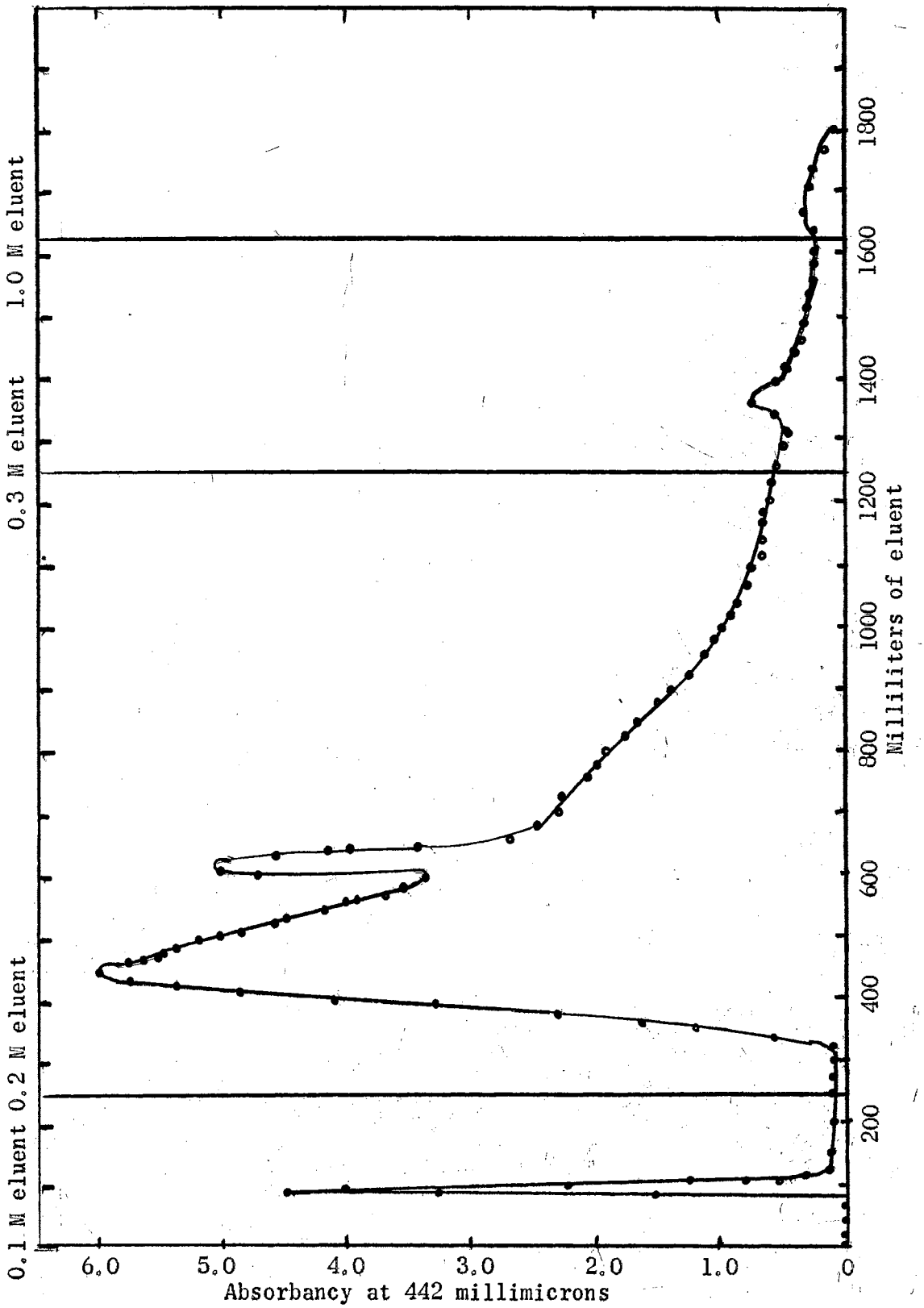


Figure 7. Elution of BNS

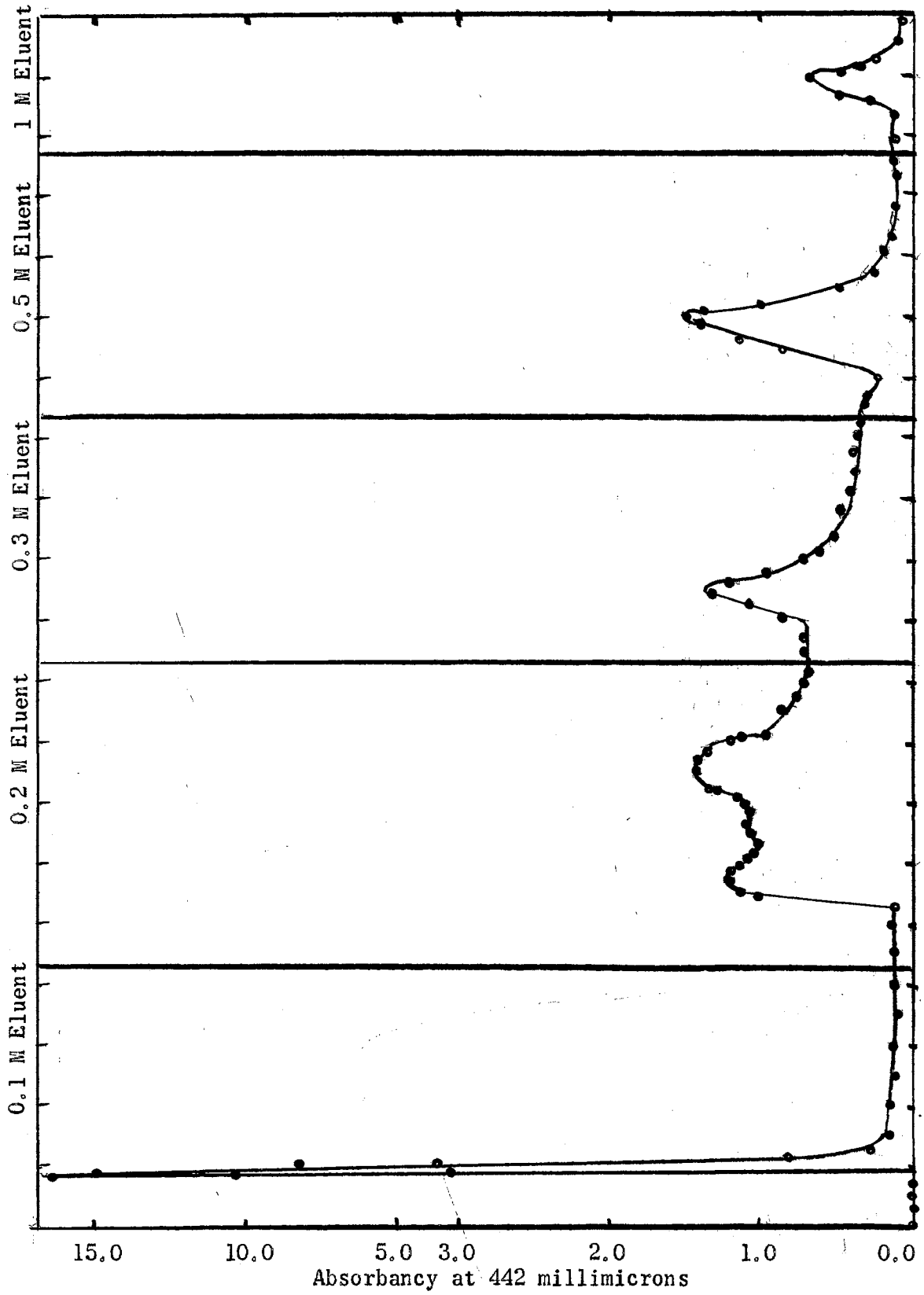


Figure 8. Elution of Acid Decomposed TNS

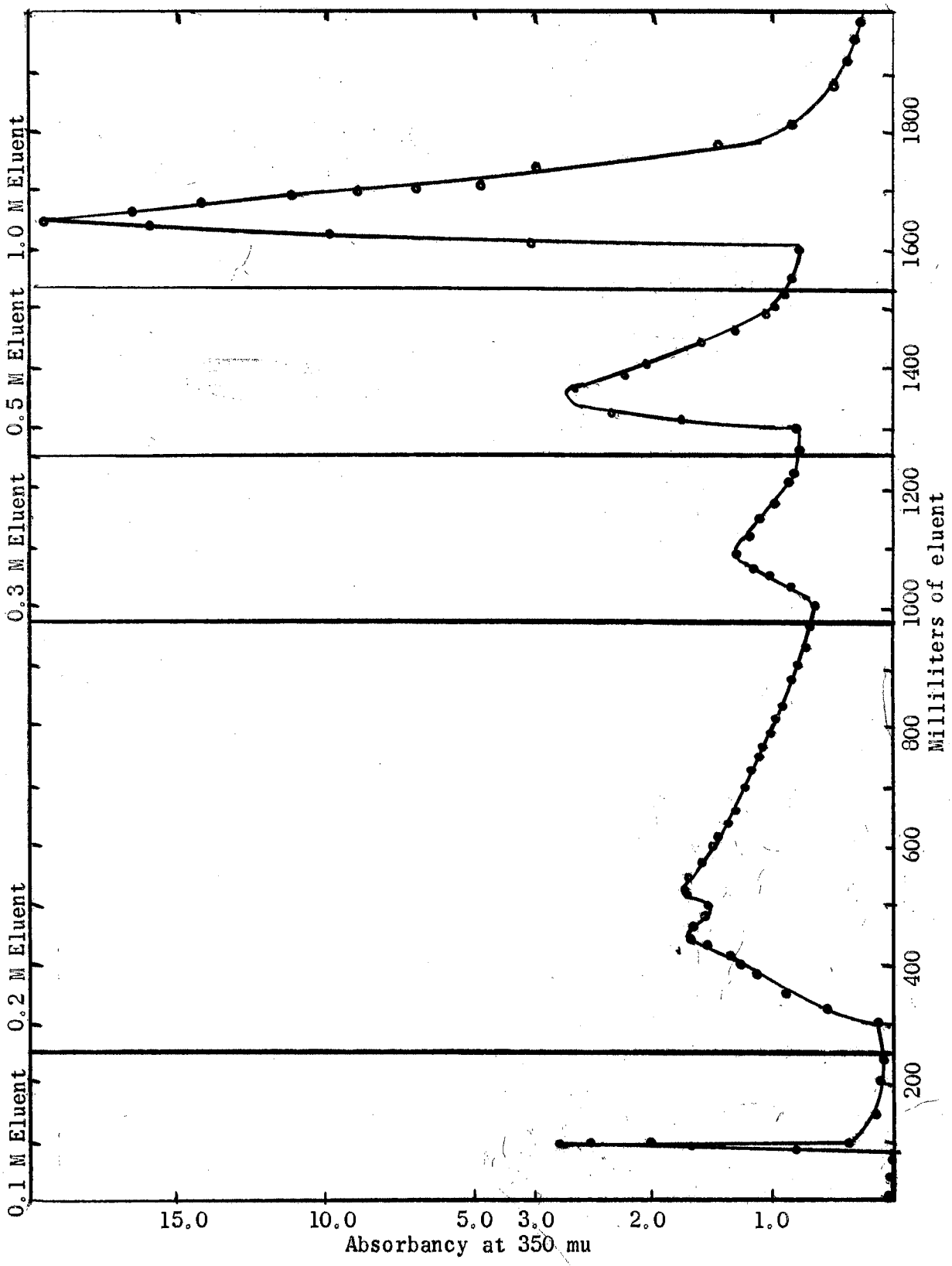


Figure 9. Elution of TNS Decomposed at pH 8

conditions of decomposition. Table X shows the proportions of products obtained from the elution of two TNS samples. One sample was dissolved in acid and then immediately made alkaline with ammonia. So the solution was momentarily acid. (The absorbancy vs. ml. of eluent plot for this sample is shown in Figure 8.) The other sample was dissolved in pH 8 buffer and allowed to stand until it turned brown. (The absorbancy vs. ml. of eluent plot for this sample is shown in Figure 9.

TABLE X

RELATIVE PROPORTIONS OF PRODUCTS OBTAINED FROM THE ELUTION OF TNS SAMPLES

Product	Acidified	Decomposed at pH 8
1	28%	1%
2	10%	10%
3	27%	18%
4	20%	17%
5	15%	54%

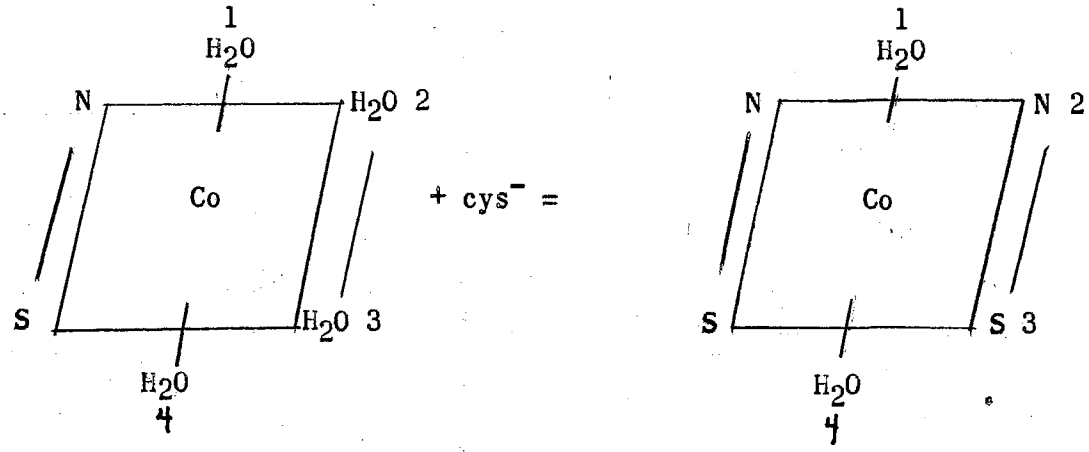
When solutions of products 1, 2, and 3 were acidified and let stand 10 minutes, the spectra were unchanged. When solutions of products 4 and 5 were similarly treated, the solutions now had a peak at 442 mμ and the absorbancy at the peak was about 20-25% higher than the absorbancy at the 415 mμ minimum. This behavior was what would be expected from a mixture of products 1, 2, and 3.

Each of these five materials was reacted with cysteine and base at room temperature. All reacted to give green solutions of TNS. The first

product reacted in about an hour, the second and third in about three hours and the fourth and fifth in about half-an-hour.

Since the fourth and fifth products react most readily with cysteine to give TNS and since the first three products can be obtained from their decomposition in acid solution, it appears that TNS initially decomposes to give cysteine and these materials and that the fourth and fifth products further break down at rates that are pH dependent to give the first three products.

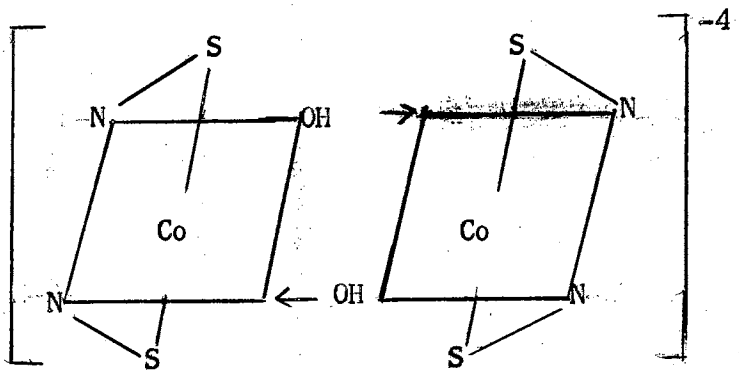
The first three products are very similar in appearance, spectra, and solubility to BNS but they definitely are not the same material. BNS also shows a reluctance to add a third molecule. From this it seems reasonable that BNS formed directly by Schubert's[7] method is a trans diaquo isomer. Thus as can be seen from Figure 10, the non-chelated positions are at opposite ends of the molecule and a third group can not be added without rearrangements of the molecule. Apparently when one molecule of cysteine is coordinated, the neighboring positions are relatively inert and hence the second cysteine molecule has only one place to go. As can be seen from the diagram when one molecule of cysteine is coordinated, positions 1 and 4 are cis to both the nitrogen and sulfur atoms and positions 2 and 3 are cis to only one filled position. So for the



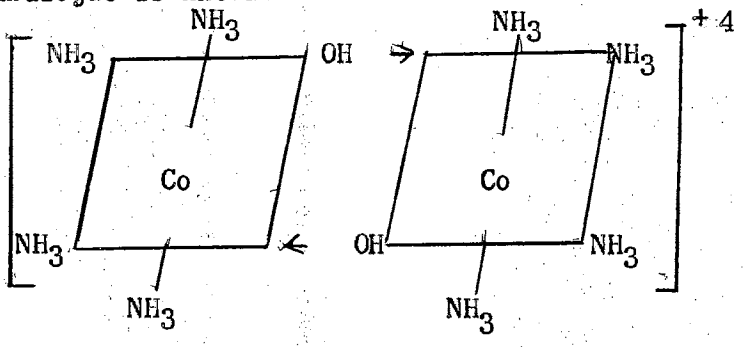
main part (98-99%), only the trans aquo isomer is formed by direct reaction. There are two possible trans diaquo isomers (I and II). The third material, present in small amounts in BNS, is believed to be a cis diaquo isomer. The reluctance of BNS to add a third chelating group and the fact that BNS is composed mainly of two materials is good evidence in support of this argument.

If a bis complex is formed by the removal of a molecule of cysteine from TNS, a cis diaquo isomer is expected. Due to the similar spectra, appearance, and properties of the first three materials to be eluted from decomposed TNS, it is believed they are the cis diaquo isomers.

The last two materials to be eluted from decomposed TNS are believed to be some type of polymeric material that breaks down to give cis diaquo isomers. This material must also be anionic. One possibility would be:



The ammonia analogue is known:[21]



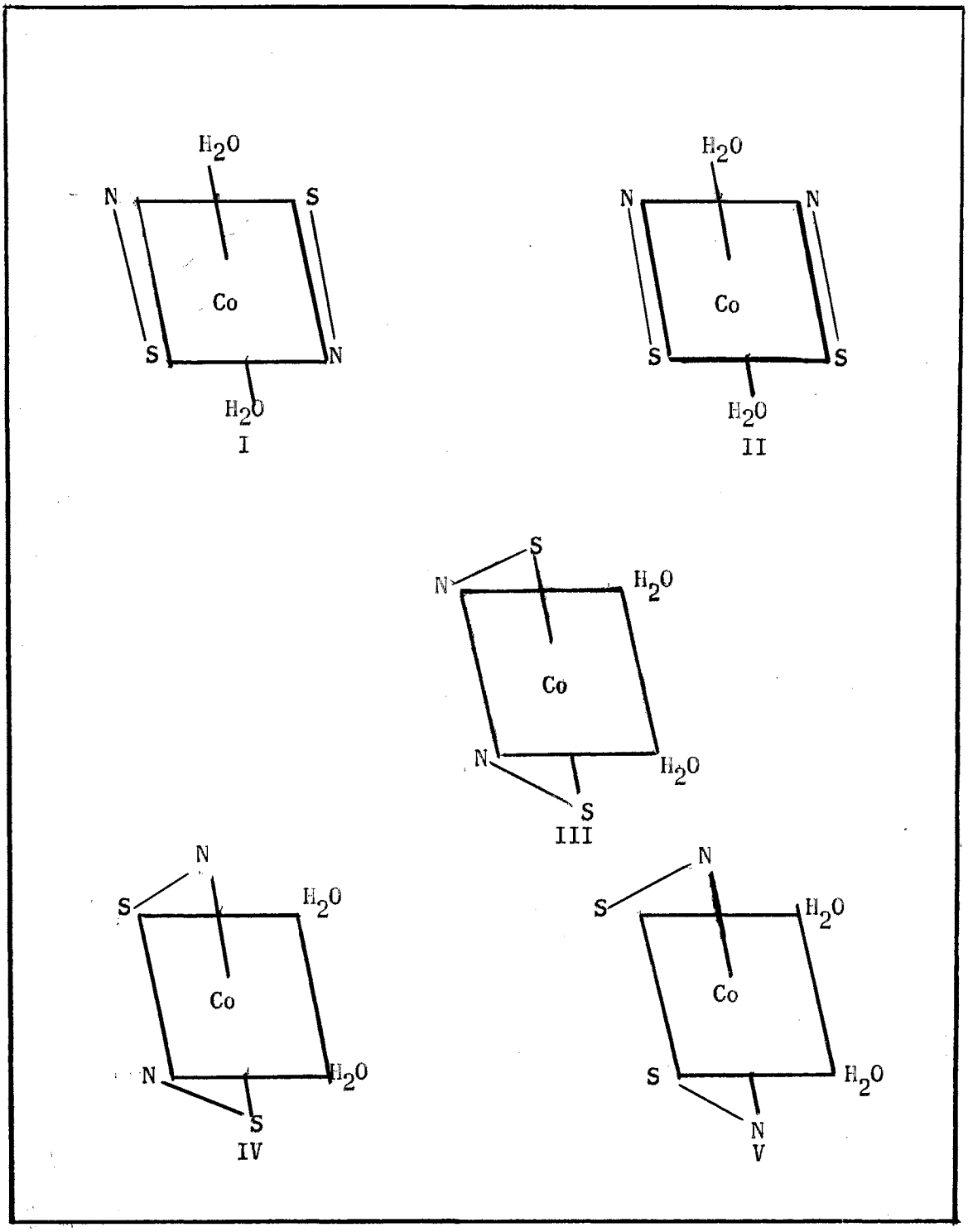


Figure 10. Possible Diaquo Cobalt (III) Biscysteinate Isomers

Also in support of this is that all five materials react with cysteine and base at room temperature to give TNS.

Because product 1 was so easily eluted, it was thought possible that it might not be an anion but a neutral molecule or a cation. The possibility of its being a cation was eliminated when a solution of product 1 was sent through an Amberlite 1R120 Cation Exchange (ammonium form) Resin. The material was not absorbed. Next, the 0.100 M ammonium chloride eluent on the Dowex 1 Anion Exchange Resin was replaced with water and a solution of product 1 was poured on the resin. This time, the resin absorbed product 1. To remove the material from the resin, 0.500 M ammonium chloride eluent was required. The solution was then treated with calcium chloride solution. A brown precipitate resulted. It was also attempted to extract product 1 from the solution with ether, which might be possible if it was neutral. The pH 8 solution, 0.1 M acid, and 0.1 M base were used. None of product 1 was extracted. From this it was concluded that product 1 was an anion but the writer is not sufficiently experienced with displacement chromotography to tell whether the fact that 0.500 M eluent solution was required to displace the material was significant. Only 0.100 M eluent solution was required to elute it.

As the spectrum of product 1 is identical with that of BNS, it seems likely that two molecules of cysteine are coordinated to one cobalt. This results in a charge of minus one. The proton is removed from the carboxyl group of cysteine at a pH of about 3. BNS accepts a proton, becomes neutral and precipitates from solution at a pH of 2. The other proposed cis diaquo isomers do likewise at a pH of about 3. So it would seem unlikely that a biscysteinate complex could become neutral at pH 8

by accepting a proton.

A possible explanation of this can be offered. The affinity of an ion for a resin is greatest whenever the hydrated ion is small and/or highly charged.[22] Product 1 has a low affinity for the resin. As all bis diaquo isomers would have a charge of minus one, the ionic charge is not a factor in the relative ease of elution. So ionic size would be expected to be a determining factor. For product 1 to be more easily eluted than the other cis diaquo isomers, it must be more extensively hydrated. Consulting Figure 10, it is found that the cis isomer III has the negative sulfur atoms trans to each other and thus at opposite ends of the molecule. In IV and V, the sulfur atoms are cis to each other. So III would be the most hydrated species and the one most easily eluted from the resin. For the cis isomers the negative carboxyl group would be about in the same position and so would not be a great factor in the relative ionic size of the hydrated cis isomers. Since in both IV and V the sulfurs are cis to each other, no sharp separation by elution would be expected. This is consistent with the experimental results.

For the trans isomers, I would be expected to be the first eluted as the sulfurs are trans to each other. I also would be expected to be the more stable. This is in keeping with experimental results as the first major portion to be eluted from BNS is the more prevalent by a factor of about 2-1.

For the cis isomers, the two cysteine groups are not in the same plane. So for III, the sulfurs would be trans and the carboxyl groups also more remote from each other than for I. So III would be the more extensively hydrated and thus III would precede I in coming off the resin.

Experimental

1. Preparation of BNS by Schubert's Method:[7] Five grams of cysteine hydrochloride were dissolved in 20 ml. of water. About 1.67 equivalents of base per mole of cysteine hydrochloride or 7.5 ml. of 7.5 M potassium hydroxide were added. Into this solution was poured 16 ml. of 1 M cobaltous chloride solution that contained 4.5 ml. of 7.5 M potassium hydroxide. This was two equivalents of base per mole of cobalt. The mixture was stirred, filtered, and then aerated for an hour.

Next concentrated hydrochloric acid was added dropwise to the solution until a precipitate had formed and had redissolved. Then 100 ml. of alcohol were added and the solution was cooled in an ice bath. The solution was then filtered. The precipitate was dissolved in water and a few drops of concentrated hydrochloric acid. The product was reprecipitated with alcohol and air dried. The product was black and with a formula $H(Co(SCH_2CHCOONH_2)_2\ (H_2O)_2) \cdot 3H_2O$. If precipitation was by acid alone and no alcohol was added, the product was brown and with the formula $H(Co(SCH_2CHCOONH_2)_2\ (H_2O)_2)$ with zero or one molecules of water. In either case the yield was about 65% of theoretical.

2. Preparation of TNS by Schubert's Method:[8] Twenty-five grams of cysteine hydrochloride were dissolved in 52 milliliters of 1 M cobaltous chloride. The solution was cooled by an ice bath to 5^0 C and then was added to 100 ml. of cold 6.6 M potassium hydroxide. The cold mixture was aerated for five hours. Then 200 ml. of denatured alcohol were slowly added and the mixture was filtered through a Büchner funnel and the precipitate was sucked as dry as possible. The precipitate was then dissolved in 150 ml. of water and the solution was filtered. To the filtrate was added 150 ml. of denatured alcohol. The mixture was filtered

and the precipitate was washed with alcohol and ether and then air dried. The yield based on cobalt was about 85%.

3. Preparation of TNS from Hexamminecobalt (III) chloride: Sodium hydroxide solution, 0.100 M, was boiled and deaerated. To this was added enough dry hexamminecobalt (III) chloride and cysteine hydrochloride to give solutions, 10^{-3} M in cobalt and 3 x 10^{-3}M, 4 x 10^{-3} M, and 5 x 10^{-3} M in cysteine. The solutions were stoppered and allowed to stand two hours. They were then examined spectrophotometrically. To better protect the solutions from air, it was found expedient to calibrate the flask so it could be completely filled with liquid. By comparing the absorbancies of these solutions with one prepared by weighing out TNS directly, the yield based on cobalt was found to be 100%.

4. Preparation of TNS from BNS: To deaerated 0.100 M sodium hydroxide solution was added enough BNS to give a 10^{-3} M solution and 1.1 moles of cysteine per mole of BNS. The solution was heated at 70^{0} for twenty minutes, stoppered, and let cool. It was then examined spectrophotometrically.

5. Lability of TNS Solutions: Three series of TNS solution were prepared with concentrations 10^{-2} M, 10^{-3} M, and 10^{-4} M. One series was in water, the second was in 1 M base and the third was in 1 M base which had also been made 0.11 M in cysteine. From 250 ml. Erlenmeyer flasks containing water, 10^{-4} M solution, 10^{-3} M solution and 10^{-2} M solution were arranged in that order. The purpose of the flask filled with water was to minimize evaporation of the complex solution by humidifying the air passed through the flasks. The last flask was directly connected to an aspirator and air was sucked through the solutions at a rate of about 12 ml. per second.

The reaction was followed by measuring the change in absorbancy of the solutions at 442 mμ. The pure complex had a molar absorbancy of 570 while the decomposition product has an absorbancy of about double that value; thus the increase in absorbancy of the solution gave an indication of the extent of decomposition of the solution.

TABLE XI

ABSORBANCIES OF AERATED INS SOLUTIONS IN WATER

Time of Aeration Hours	Absorbancy at 442 Mμ 10^{-4} M	10^{-3} M	10-2 M
0	.086	.620	.523 x 11
0.25	.108	.661	.520 x 11
0.50	.121	.702	.522 x 11
0.75	.118	.753	.555 x 11
1.25	.127	.838	.592 x 11
1.75	.120	.910	.645 x 11
2.25	.130	.984	.720 x 11
2.75	.132	1.050	.780 x 11
3.25	.153	1.128	.860 x 11
3.75	.147	1.177	.908 x 11
5.75	.138	1.375	1.055 x 11
21.00	.176	1.636	1.340 x 11

The 10^{-2} M solutions were diluted to one-eleventh strength to obtain more convenient absorbancy values. "Time zero" was when the aspirator was turned on. In the course of the setting up the experiment, the 10^{-4} M and 10^{-3} M solutions had already undergone some decomposition.

The 10^{-2} M solution was not affected for about 30 minutes but once the reaction started, it proceeded readily. The pH of the solutions was below 10 and this factor alone would cause decomposition. The initial pH's of the solutions were 6.0, 8.0, and 9.5 for the 10^{-4} M, 10^{-3} M, and 10^{-2} M solutions respectively.

In another set of experiments, three more solutions were prepared with 1 M sodium hydroxide as the solvent instead of water. The results of these runs are shown in Table XII.

TABLE XII

ABSORBANCIES OF AERATED TNS SOLUTIONS IN 1M SODIUM HYDROXIDE

Time of Aeration Hours	Absorbancy at 442 mμ 10^{-4}M	1.33×10^{-3} M	1.32×10^{-2}M
0.00	.073	.790	.693 x 11
0.33	.081	.830	.712 x 11
0.67	.089	.848	.729 x 11
1.00	.094	.855	.740 x 11
1.50	.101	.860	.761 x 11
2.00	.106	.872	.810 x 11
2.50	.108	.880	.876 x 11
3.00	.117	.924	.970 x 11
3.50	.123	.990	1.032 x 11
4.00	.133	1.025	1.133 x 11
4.50	.138	1.097	1.255 x 11
5.00	.140	1.113	1.273 x 11

Next investigated was the effect of the presence of an excess of cysteine. To each sample was added sodium hydroxide and cysteine to give concentrations of 1 M and 0.11 M respectively. The results of this run are shown in Table XIII.

TABLE XIII

ABSORBANCIES OF AERATED TNS SOLUTIONS IN 1 M SODIUM HYDROXIDE AND 0.11 M CYSTEINE

Time of Aeration Hours	Absorbancy 1.11×10^{-4} M	1.68×10^{-3} M	1.19×10^{-2} M
0.00	.033	.960	.625 x 11
0.33	.045	.960	.615 x 11
0.67	.053	.975	.640 x 11
1.00	.067	.996	.67- x 11
1.50	.064	1.008	.702 x 11
2.00	.068	1.019	.742 x 11
2.50	.072	1.042	.767 x 11
3.00	.085	1.060	.791 x 11
3.50	.091	1.078	.814 x 11
4.00	0.93	1.073	.845 x 11
4.50	.091	1.081	.884 x 11
5.00	.097	1.102	.908 x 11
15.00	.198	1.187	1.235 x 11

The absorbancy of the 1.11×10^{-4} M solution was abnormally low, but when it reached a normal value, the solution was fairly stable for 1.5 hours. Cysteine stabilized all solutions; however it had the

least effect on the 1.19×10^{-2} M solutions.

6. Qualitative Test for Cobalt: The solution was made alkaline with ammonia and then sodium sulfide solution was added. A black precipitate indicated the presence of cobalt.

7. Spectral Work: All spectrophotometric work was done using a Beckman Model DU Spectrophotometer. The solutions were usually diluted quantitatively to give absorbancies below 1.1. Samples sensitive to air were measured in cells with polyethylene stoppers and the cells were filled completely to avoid air spaces.

8. Decomposition of TNS by Acid: TNS was dissolved in water and concentrated hydrochloric acid was added dropwise with stirring until the solution was distinctly acid. The solution was then allowed to stand about five minutes. Then an equal volume of alcohol was added to the solution and 20% sodium hydroxide solution was added dropwise until a precipitate formed. This was around pH 3. The solution was filtered and the precipitate was washed with water and alcohol and dried in air. Then the precipitate was redissolved in water and a few drops of concentrated hydrochloric acid. An equal volume of alcohol was added and the product was reprecipitated with a few drops of 20% sodium hydroxide solution. The olive-brown precipitate was filtered and washed with water, alcohol, and ether. It was then dried in air.

By dissolving a weighed amount of this material in base, an absorption spectrum could be obtained and by assuming the material to be an isomer of BNS, molar absorbancies could be calculated. So by acidifying a TNS solution and measuring the absorbancy of the solution, the maximum possible yield was about 65%. Actual yields were 50-55%, for if one attempted to completely precipitate the product from an acidified TNS

solution, the product was contaminated with a white material - probably cystine.

9. Experiments with Ethylenediamine: BNS and air-decomposed TNS were dissolved in 0.01 M alkali. One milliliter of ethylenediamine (Eastman Practical Grade) was added to each solution. The BNS solution was 10^{-4} M and the decomposed TNS solution was 10^{-3} M. The solutions were examined spectrophotometrically at 350, 442, and 580 mμ over a period of six hours. At the conclusion of the experiment, both solutions were examined over the range 320-760 mμ. No evidence of reaction was noted.

10. Ion Exchange: The ion-exchange column was made from a three-foot length of 25 mm. diameter glass tubing. At the bottom of the column was a 2 cm. glass-wool plug and a stopcock. The resin bed was about 53 cm. long. On top of the resin was another 2 cm. glass-wool plug, to prevent the resin bed being agitated by liquids poured onto it.

The resin bed was prepared by taking Dowex-1 anion exchange resin, 200-400 mesh, in the chloride form and pouring the dry resin into a beaker full of distilled water. The suspension was stirred and then allowed to settle for fifteen minutes. Any suspended material at that time was decanted and discarded. The remaining resin was washed into the column with water. Once the resin was in the column, it had to be kept wet.

Dr. William T. Reiman III had recommended that the maximum sample size be 6×10^{-3} of the capacity of the resin and that the maximum flow rate be 0.7 centimeters of column length per minute. The stated minimum capacity of the wet resin was 1.3 milliquivalents per ml. and the volume of the column was about 200 ml. Hence the maximum sample size would be 1.56 milliequivalents and the maximum flow rate 3 ml. per minute. As

the flow rate through the column by gravity alone was 2.8 ml. per minute, no suction was necessary.

Before use the column was washed with 125 ml. of the eluent to be used, which initially was a solution 0.100 M in ammonium chloride and 0.015 M in ammonia. Other eluent solutions used contained 0.200 M, 0.300 M, 0.500 M, and 1.000 M ammonium chloride and a proportionate amount of ammonia. The pH of these solutions was about 8. The pH of the eluent solution was convenient for keeping samples in solution but yet keeping the hydroxide-chloride equilibrium on the resin at a negligible level. If 500 ml. of a particular eluent solution had not separated the desired materials, then a more concentrated eluent solution was used.

The sample was dissolved in the minimum amount of solvent and poured on the resin. The solution coming from the bottom of the column was collected and its absorbancy at 350 or 442 mμ was measured. The material eluted with 0.100 ammonium chloride eluent passed through the column rapidly and it was usually completely collected in a 25 ml. volume. As this material often composed 25-30% of the total sample and the total volume of all eluents was about 2000 ml., this solution, relatively, was very concentrated.

The absorbancy was plotted against the milliliters of eluent. Volume zero was taken as the point at which the sample solution was added. If the concentration of the solution were changing rapidly, smaller volumes of solution were collected. Thus for the material eluted with 0.100 M ammonium chloride eluent, 2-3 ml. volumes were collected. At other inflection points 10 ml. samples were collected. Where the concentration was not appreciably changing, volumes as large as 50 ml. were collected. The absorbancy of each solution was measured. If the volume

collected, for example, was at 1000-1050 ml., its absorbancy was plotted on the graph at the midpoint or 1025 ml.

Each component of the sample had a characteristoc peak on the absorbancy vs. ml. plot. Thus by choosing an appropriate volume, a sample largely composed of one component of the sample could be obtained and examined. The solutions were too dilute to make precipitating solids feasible. So only solutions of the various components were used.

Better results were obtained when the elution could be continuously carried out. If the elution was stopped, the eluent in the column had a longer period of contact with the resin and thus had a higher concentration of material dissolved in it. This would result in spurious peaks. A continuous run required about 15 hours.

11. Reactions of Various Eluted Fractions with Cysteine in Basic Solutions: The solutions of the five components resulting from the elution of decomposed TNS and a solution of BNS were made 0.5 M in sodium hydroxide. About 0.5 of cysteine hydrochloride was added to each solution and the containers were stoppered and let stand. The solutions were cooled with tap water so that they would not be warmed by the dilution of the base or the reaction of base and cysteine hydrochloride. All solutions except that of BNS gave a green solution that spectrophotometrically was shown to be a solution of TNS. Room temperature was about 30^0 C.

12. One liter of a solution of 0.8 moles of sodium hydroxide and 0.10 moles of cysteine hydrochloride hydrate was prepared and allowed to come to room temperature. 6×10^{-4} moles of TNS were dissolved in 5 ml. of water and 3 ml. of concentrated hydrochloric acid were added. This mixture was stirred three minutes and then poured into the alkaline

cysteine solution. A green color almost immediately resulted. The spectrum of the solution was identical with that of a solution of TNS prepared by Schubert's method.

To a similar alkaline cysteine solution was added 6×10^{-4} moles of BNS. No immediate reaction was noted but after standing for 1.75 hours, the solution was green with vestiges of a brown color remaining. The spectrum of the solution had peaks at 350 and 580 mu and the spectrum seemed intermediate between that of BNS and TNS. The spectrum was redetermined after 9 and 15 hours standing but little further change was noted. The change that did occur appeared to be in the direction of the formation of TNS. Vestiges of brown color still remained.

If the solution after standing 15 hours, were heated to 70° C for ten minutes, the spectrums of the resulting solution was identical with that of a solution of TNS.

CHAPTER IV

THE REACTION OF CYSTEINE AND COBALTOUS ION WITH OXYGEN

Results and Discussion

A brief investigation was made of the kinetics of the reaction of cysteine and cobaltous ion with atmospheric oxygen.

In these experiments cysteine and cobalt were mixed in buffers of various pH values and shaken with oxygen. The reaction was stopped by adding acid, and the amount of cysteine remaining was then determined by Lavine's method.[23]

The first series of experiments was conducted with cobalt in excess to determine the order of reaction with respect to cysteine. Samples were run at pH's of 5.30, 6.20, 7.97, and 9.30. In all cases, a plot of the log cysteine concentration vs. time gave a straight line, which meant that the reaction appeared to be first order with respect to cysteine. From the slope of the line, the first-order reaction rate constant and half-life were calculated. The data were plotted in Figures 11 and 12. The rate constant and half-lives are listed in Table XIV.

The second set of samples were prepared with cysteine in excess, to determine the order of the reaction with respect to cobalt. Runs were made at pH's of 4.68, 6.96, 7.93, 8.80, 9.74, and 0.5 M sodium hydroxide. In all of these runs the reaction was very fast and essentially complete within five minutes or less. No detailed measurements of the rate could therefore be carried out. At different pH's, varying

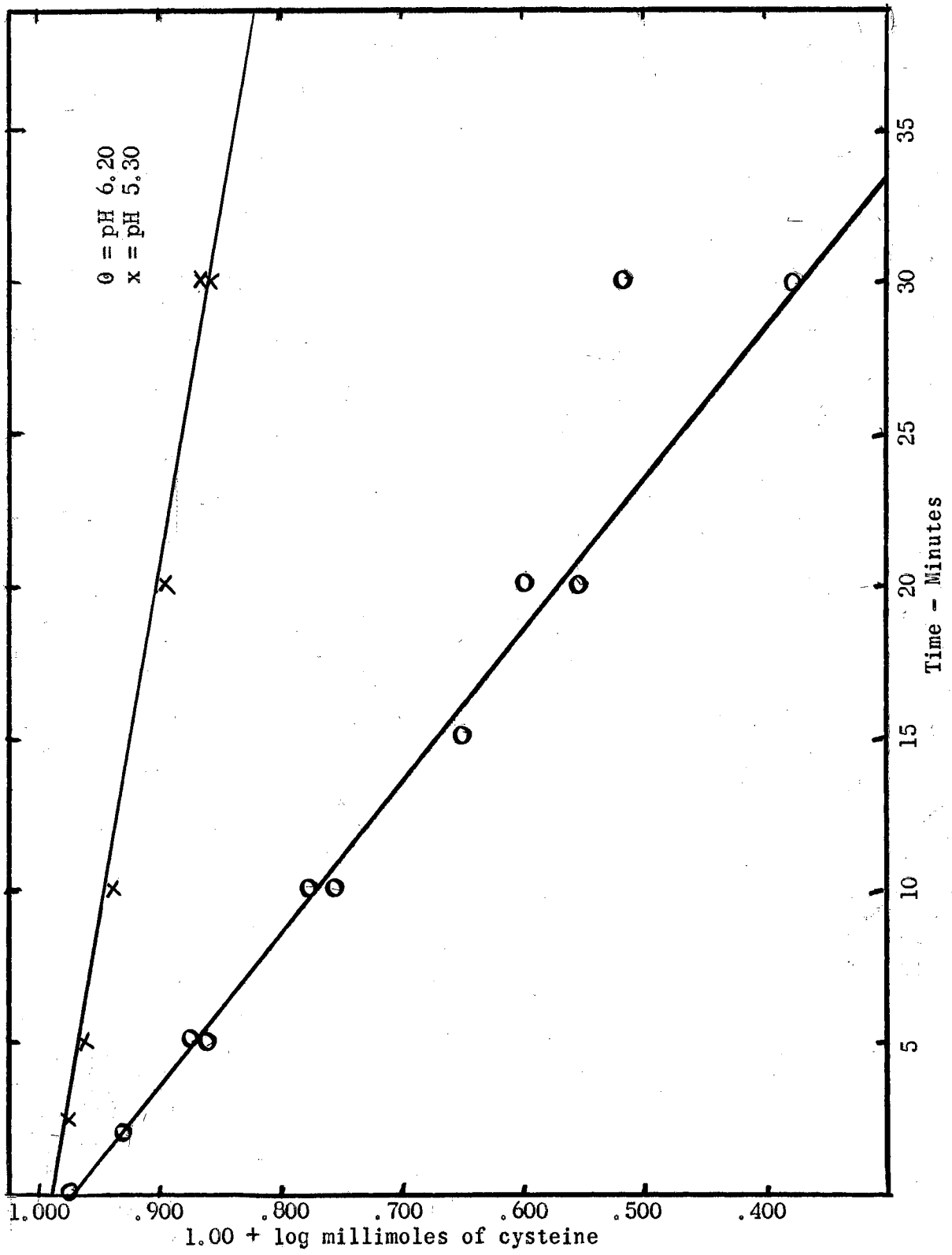


Figure 11. The Reaction of Cysteine and Cobalt with Oxygen I

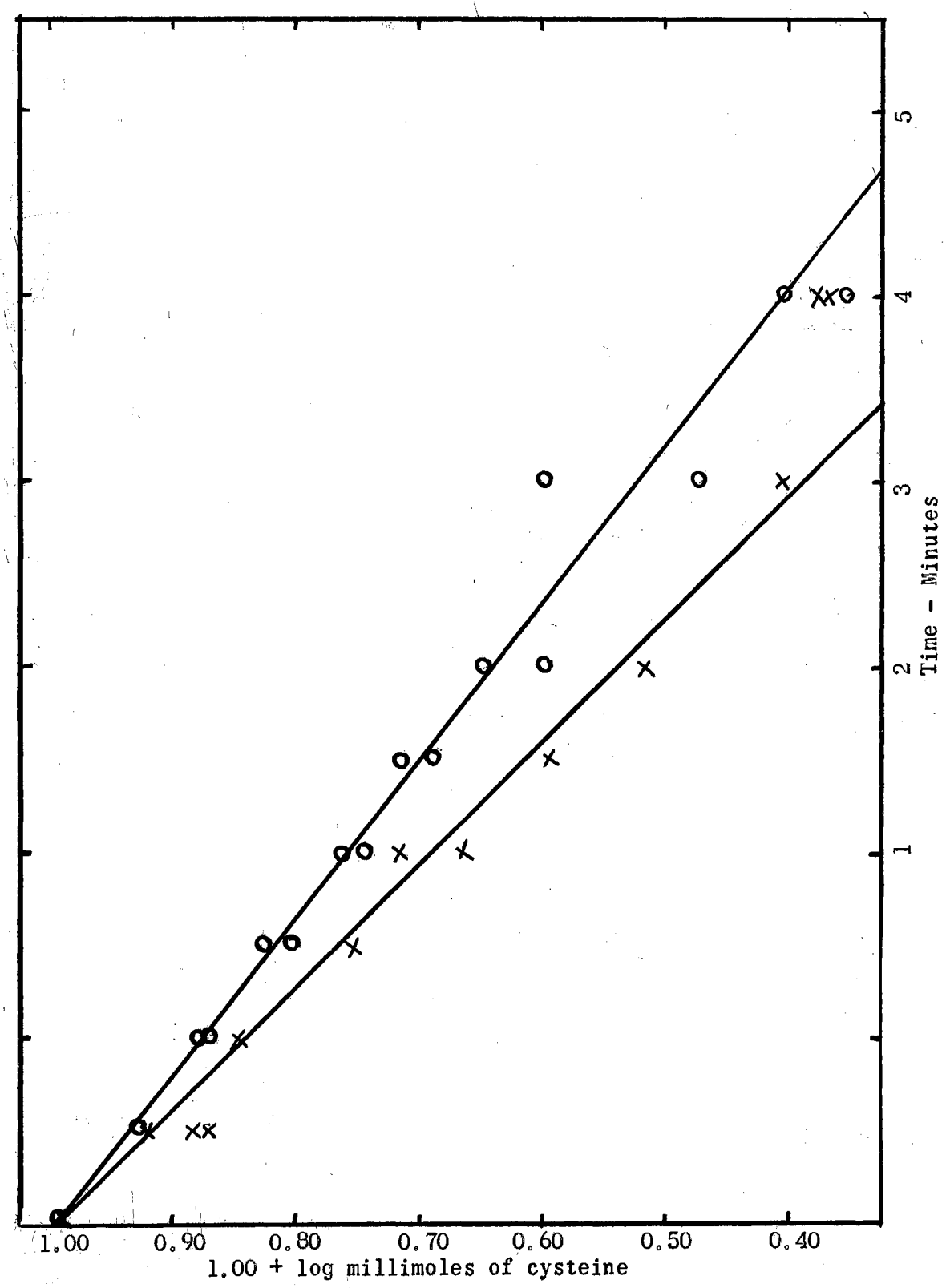


Figure 12. The Reaction of Cysteine and Cobalt with Oxygen II
(O = pH 7.97, x = pH 9.30)

quantities of cysteine reacted per mole of cobalt. These data are listed in Table XV.

TABLE XIV

EXPERIMENTAL HALF-LIVES AND RATE CONSTANTS IN THE PRESENCE OF EXCESS COBALT

pH	Rate Constant, Minutes^{-1}	Half-life, Minutes
5.30	.0102	68
6.20	.0506	13.7
7.97	.275	2.52
9.30	.311	2.33

TABLE XV

MOLES OF CYSTEINE CONSUMED PER MOLE OF COBALT PRESENT

pH	Cysteine Consumed per Cobalt
6.96	2.46
7.93	2.57
8.80	2.66
9.74	2.86
0.5 M NaOH	2.53

The product formed in this range is BNS, and this requires only two moles of cysteine per mole of cobalt. Therefore it is clear that side reactions also take place and that their extent is pH dependent. From

Schubert's work,[7] the excess cysteine is probably converted to cystine.

It is possible in these experiments for cysteine to be in three forms; free cysteine, cobaltous cysteine complexes, and cobaltic cysteine complexes. In the runs with an excess of cysteine, the cobalt was first precipitated as cobaltous hydroxide which then rapidly dissolved. As cobaltous hydroxide is sparingly soluble in water,[24] the cobalt must have gone into solution as a cobaltous cysteine complex. At this point, the cysteine consumption, as determined by titration, was still low. So the cysteine titrated by the Lavine method[23] must include the cysteine present as cobaltous cysteine as well as free cysteine. (It is of course possible that at pH 0-1, the cobaltous cysteine complex decomposes to cobaltous ion and cysteine.) The cysteine consumed in the reaction as determined by titration is in the form of cobaltic cysteine or oxidized to cystine.

It was found that between 2.46 and 2.86 moles of cysteine were consumed per mole of cobalt present. The only stable cobaltic cysteine complex formed under these conditions is BNS. So between 0.46 and 0.86 moles of cysteine per atom of cobalt are consumed by another reaction. From the work of Michaelis and Guzman-Barron[3] and Kendall and Holst,[6] it seems that this cysteine is converted to cysteine.

The oxidation of cysteine under these conditions proceeds at a much faster rate than if the oxidation were by oxygen alone; therefore one must seek a reaction path involving cobaltic ion. Cobaltic ion cannot be formed in solution by the oxidation of cobaltous ion with oxygen unless a complex is formed.

BNS is stable. TNS, under these conditions, is not stable but it cannot be an intermediate because the cobalt complexes resulting from

its decomposition (Chapter 3) are not found when cobalt and cysteine react directly with oxygen. The intermediate complex, like BNS and TNS, should be formed by sulfhydryl-amino chelation. The suggested possibility is a cobaltic monocysteinate.

This complex could undergo a reaction with a second molecule of cysteine to give BNS or it might undergo self-oxidation-reduction to cobaltous ion and cystine. The former reaction would be promoted by a high cysteine concentration while the latter reaction would be favored by a low cysteine concentration. Thus in a high cysteine concentration, the cysteine consumption would approach a lower limit of two moles per atom of cobalt and the oxygen consumption, 0.5 atoms per mole of cobalt. This is consistent with the oxygen consumption data of Neville.[12]

Experimental

1. Preparation of Buffered Cysteine Solutions: A solution of cysteine hydrochloride in a buffered medium was prepared and the pH was measured with a pH meter. The solutions were 1 M with respect to buffer or saturated if a 1 M solution could not be prepared, and 1 M with respect to potassium iodide. This was to make the ionic strength factor reproducible and to provide a large buffer capacity. Buffer systems used were disodium phosphate and monosodium phosphate, sodium carbonate and sodium bicarbonate, and potassium acid phthalate and sodium hydroxide.

2. Kinetic Runs: An aliquot of the cysteine solution was taken and a cobaltous ion solution was quickly added. This was "zero time." The solution was then continuously shaken. The reaction was effectively

stopped by adding concentrated hydrochloric acid in excess of the amount required to react with the buffer completely. At low pH's, any reaction was negligibly slow. The time of reaction was the interval between the time of the addition of cobaltous ion and the addition of hydrochloric acid. The remaining cysteine was then determined by the Lavine method[23] with the modification that an external starch indicator on a spot-test plate was used. The reason for this was that, in the Lavine procedure, the original solution is colorless and starch is not added until the iodine has been almost completely titrated and the solution is a pale yellow. This is done because in strong acid solutions, the starch-iodine equilibrium is sluggish. In this work, a brown cobaltic cysteine was formed so the solutions were colored and this technique was not possible. The solutions were 1 M in potassium iodide as this material was necessary for the Lavine titration.

The initial cysteine concentration was determined by running a blank to which no cobalt had been added. The air oxidation of cysteine did not proceed at a competing rate as blanks run as the first sample of a run were in excellent agreement with blanks run as the last sample of a run.

The first series of experiments was conducted with cobalt in excess. The solutions were 0.090 M in cobalt nitrate and 0.0454 M in cysteine. Each sample was shaken for a given length of time and then 25 ml. of 12 M hydrochloric acid were added. Then 50 ml. of 0.0500 N iodine were added. After shaking the solution, the unreacted iodine was titrated with 0.0500 N sodium thiosulfate. The milliequivalents of iodine less the milliequivalents of thiosulfate gave the milliequivalents or millimoles of cysteine. Duplicate runs were carried out at all pH's and they were in good agreement.

The second set of experiments was carried out with cysteine in excess. The solutions were 0.227 M in cysteine and 9.09×10^{-3} M in cobaltous ion. The same procedure was followed as had been carried out in the previous set of experiments. Duplicate runs were made at all pH's except 7.93 and 8.80 where only one run at each pH value was carried out.

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