DETERMINATION OF SULFUR AND MERCAPTO GROUPS

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CHAPTER I

INTRODUCTION

Many organic sulfur compounds of biological origin are of great importance in chemistry. While a variety of procedures and techniques are available for their detection and analysis, no method is equally well suited to all problems, and there is therefore a continuing need for novel analytical methods. The present thesis deals with this general problem.

The first part of this thesis deals with the determination of organically bound sulfur. The method of analysis employs a reduction technique, followed by absorption of the hydrogen sulfide and determination of sulfide spectrophotometrically. The amino acid methionine was chosen as a test compound.

The second part of the thesis is concerned with the mercapto group and its determination. One section describes efforts to determine cysteine spectrophotometrically. This was effected by reacting the cysteine with a silver complex of dithizone; cysteine forms the corresponding silver mercaptide and the freed dithizone can be determined spectrophotometrically.

The last section deals with sulfhydryl-containing proteins and efforts to determine their -SH content by the use of ferricyanide. Katyal (35) has shown that ferricyanide is a specific reagent in the case of ovalbumin, but no work with bovine serum albumin or beta-lactoglobulin has been reported using this

oxidizing agent. Before the corresponding disulfide bond can be formed, these proteins must be denatured in order for the sulfhydryl groups to be freely reactive. Efforts to determine the specificity of ferricyanide for the -SH groups concluded experimentation.

CHAPTER II

ANALYSIS OF SULFUR IN BIOLOGICAL MATERIALS (METHIONINE)

The sulfur content of organic compounds is most commonly determined by converting the sulfur to sulfate, which is analyzed gravimetrically as barium, lead or silver sulfate (11). Despite its widespread use, this method is rather time-consuming, and the precipitation of sulfate is one of the most troublesome gravimetric procedures. For these reasons, it seemed of interest to investigate the alternative possibility of converting sulfur to sulfide and determining it in that form. The attempts made to develop such a method are described below. Methionine was chosen as a test compound. Since the results obtained were not sufficiently favorable, the method was not extended to other compounds.

Review of the Literature

The problem involves two parts: (a) conversion of sulfur to the sulfide form, and (b) determination of the sulfide. In the first part of this review will be discussed known methods of effecting both (a) and (b), and in the second part will be described the methylene-blue method for determining sulfide.

Conversion to Sulfide and its Determination

A number of authors have used polarographic techniques in analyzing for sulfides. Trifonov, et al. (64) determined the sulfur content in organic compounds by reducing with Raney nickel,

absorbing the hydrogen sulfide produced in sodium hydroxide, and determining it polarographically. In another direct polarographic method, Andrew and Gentry (2) absorbed the hydrogen sulfide in an ammoniacal solution of cadmium chloride. Using the same absorbent, Roubal, et al. (56) did an indirect polarographic determination by measuring the decrease in the cadmium ion concentration caused by formation of insoluble cadmium sulfide.

By far the most popular method for analyzing the hydrogen sulfide evolved after reduction has been the iodometric method, where the gas is first absorbed in an aqueous solution of a suitable cation that forms an insoluble sulfide. Iodine is added to the sulfide, which is liberated from the precipitate by the addition of strong acid, and the iodine in excess is titrated with standard thiosulfate. The amount of iodine used to oxidize the sulfide is calculated by difference. Heinemann and Rahn (30) used a two-stage reduction to convert sulfur to sulfide. The first was an alkaline reduction with stannous chloride and the second reduction with aluminum metal in hydrochloric acid solution; the hydrogen sulfide was absorbed in ammoniacal cadmium chloride. Shaw (61) applied this method to determine hydrogen sulfide, methyl, ethyl and n-propyl mercaptans in gases. Solid potassium metal in ligroine was used by Zimmermann (72, 73) to achieve quantitative reduction before analyzing the hydrogen sulfide iodometrically. Kurchatov (40) effected reduction with zinc vapors, dissolved the fusion mixture in hydrochloric acid, and collected the hydrogen sulfide in cadmium acetate. Hydrogenation in the presence of a platinum spiral at 850° C. in an electric furnace was the method employed by Yudasina and Vysochina (71); a buffered solution of zinc sulfate was used as absorbent.

All of the iodometric methods described above, although easier and faster to perform than the methylene-blue method next described, afforded limited sensitivity and accuracy; ±3% was the best accuracy attained. The methylene-blue method has a reported accuracy of ±1.0% for hydrogen sulfide samples not exceeding 10 mg. In recent years this method has been perfected for sample sizes in the range 5 - 500 μg., with a total analysis time not exceeding one hour.

Determination of Sulfide as Methylene-Blue

Fischer (21) first called attention to the possibility of using p-aminodimethylaniline as a reagent for the detection of hydrogen sulfide in small quantities. The reaction between this compound and hydrogen sulfide in the presence of an oxidizing agent and hydrochloric acid results in the formation of methylene-blue.

Some years later, Mecklenburg and Rosenkranzer (44) worked out a colorimetric determination for as little as 1.0 - 3.0 μg. of hydrogen sulfide per ml., using ferric chloride as the oxidizing agent. These authors clearly defined the conditions necessary for the best results. Eleven years later Almy (1) adapted the methylene-blue method to the estimation of hydrogen sulfide in proteinaceous food products. A known quantity of protein was subjected to acid hydrolysis, the hydrogen sulfide was swept from the reaction chamber into a zinc acetate solution with carbon dioxide, the p-aminodimethylaniline solution was added, and the intensity of blue color determined two hours after the addition of oxidant. Sheppard and Hudson (62) also employed this method for the

determination of sulfur in gelatin and proteins. These authors found Almy's simple hydrolysis technique too crude for quantitative results. They treated the protein with a solution prepared by dissolving silver chloride in strong ammonium hydroxide, which caused the protein solution to swell; after the protein solution had swelled for one hour, the sample was put in a water bath and heated until the tube was well blackened owing to the formation of silver sulfide, silver oxide and metallic silver, which were then decomposed with concentrated hydrochloric acid. The hydrogen sulfide was removed from the reaction flask by nitrogen and absorbed in a zinc acetate solution.

In trying to find a method for the determination of low concentrations of hydrogen sulfide in natural gas, Sands, et al. (59) successfully adapted the methylene-blue procedure to their problem, and in the process of experimentation did extensive research on all the variables that affect the accuracy of this method, i.e., which salt of the reagent is best, the proper hydrogen sulfide absorbent, aging and temperature effects, etc. Once the proper conditions for a rapid and accurate analysis for hydrogen sulfide were established, this method enjoyed great popularity. It is fast, accurate and easily adapted to micro-determinations. One outstanding virtue is that it is quite specific for hydrogen sulfide. Methyl and ethyl sulfides and mercaptans do not interfere, although they produce a pink color, which may later change to a yellowish or slightly green shade, when added directly to the hydrogen sulfide reagents. Their lack of interference in the test is probably due to the fact that they are not absorbed by zinc acetate solution.

Determination of Methionine

Baernstein (5) outlined the first useful method for determining the methionine content in proteins. It is based on demethylation with hydroiodic acid, oxidation of the resulting homocysteine with sodium tetrathionate, and determination of the thiosulfate formed with iodate after acidification. Some years later, Schormuller and Ballschmieter (60) improved the method and adapted it to microchemical determinations with as little as 50 mg. of protein. Bakay and Toennies (6) were able to formulate a highly specific method of analysis based on the reversible reaction of methionine with iodine. By first blocking the amino groups in the protein hydrolysate and then adding an excess of iodine to this solution the following reaction takes place:

$$CH_3SCH_2CH_2CH(COO^-)\overset{+}{N}H_3 + I_2 \underset{pH\ I\ (B)}{\overset{(A)\quad pH7}{\rightleftarrows}} CH_3\overset{+}{S}CH_2CH_2CH(COO^-)NH + 2HI$$

After the reaction has gone to completion the iodine in excess is removed with a 9:1 mixture of isoamyl alcohol and carbon tetrachloride. Then the pH is lowered and the iodine liberated by reaction (B) is determined spectrophotometrically with the aid of a standard curve. Saito (57) employed a sodium fusion to decompose methionine, reacted the sodium sulfide with sodium nitroprusside, and determined the sulfur content spectrophotometrically.

Introduction to Present Work; General Experimental Detail

From the review of the literature, it appeared that the methylene-blue method might be used to advantage in the determination of sulfide sulfur. Thus the method was tried and adapted to use. First a calibration curve was constructed from sulfide solution of

known strength. Secondly, the removal of hydrogen sulfide from a solution like the reaction mixtures was tested. Finally, samples of methionine were treated with stannous chloride and Raney nickel, and the sulfur removed was determined by this means.

Apparatus

The reaction and absorption assembly was identical to that of Budd and Bewick (15).

Beckman Model DU and DK-1 spectrophotometers were used with 10-mm. Corex absorption cells.

Reagents

Double-Distilled Water. Double-distilled water from an all-glass still was boiled for one-half hour, cooled, stored under nitrogen and used on the day it was prepared.

Stannous Chloride. To 10 g. of C.P. stannous chloride dihydrate was added 10 ml. of hydrochloric acid and the solution was diluted to 100 ml. with double-distilled water. A few pieces of mossy tin were added. The reagent was stable for at least three days at room temperature.

Raney Nickel Catalyst. The catalyst was prepared exactly as prescribed by Pavlic and Adkins (50) and stored under absolute ethanol. It was found to be stable for three weeks.

Aluminum Strips. Sheet aluminum, one sixty-fourth of an inch thick, was cut into strips 0.25 x 1.25 inch. Just prior to use, they were treated with boiling 1:5 hydrochloric acid for 30 seconds, then rinsed with water.

Zinc Acetate. The stock solution was prepared by dissolving 50 g. of C.P. zinc acetate dihydrate in 200 ml. of double-distilled

water. Enough glacial acetic acid was added to effect solution (4-5 ml.) and the whole was made up to 250 ml. The 2% zinc acetate solution was made by diluting the stock solution 10 times with double-distilled water.

Amine Solution. The stock solution was prepared by dissolving 12.7 g. of N,N-dimethyl-p-phenylenediamine monohydrochloride in 35 ml. of 1:1 sulfuric acid and diluting to 50 ml. with 1:1 sulfuric acid. The reagent solution was made by diluting 12.5 ml. of the stock solution to 500 ml. with cold 1:1 sulfuric acid. This solution kept for about a month at 20° C.

Ferric Chloride. This solution was prepared by dissolving 50 g. of C.P. ferric chloride hexahydrate in enough double-distilled water to make 50 ml. of solution.

Iodine. A 0.1 N solution was prepared from "Acculute" concentrate and standardized against standard thiosulfate. One milliliter of this solution was equivalent to 1.6 mg. of sulfur.

Sodium Thiosulfate. Exactly 6.205 g. of C.P. sodium thiosulfate pentahydrate was dissolved in sufficient quantity of double-distilled water to make 250 ml. of 0.1 N solution. This solution was standardized against a standard 0.1 N solution of sodium arsenite.

Starch. One gram of water-soluble starch was made into a paste with cool distilled water and diluted to 100 ml. with hot 50% glycerol solution.

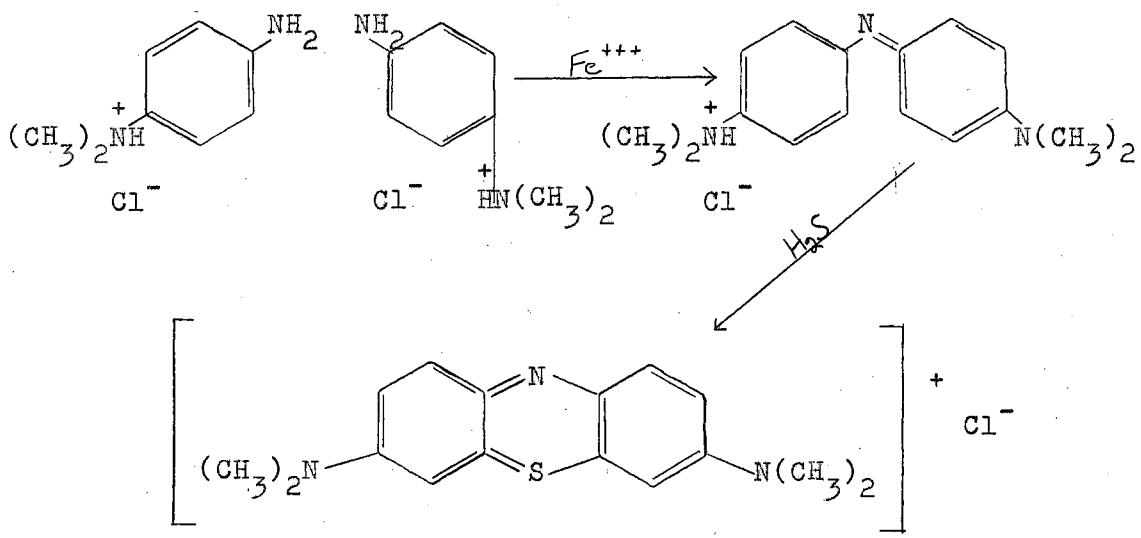
Methionine. Commercial samples of dl-methionine, CfP, were obtained from the California Foundation for Biochemical Research, Los Angeles, California.

Preparation of Sulfide Solution

Into a 600 ml. beaker was placed 500 ml. of double-distilled water, and into this were passed 80-100 bubbles of hydrogen sulfide gas from a tank cylinder. After vigorous stirring, a 10 ml. aliquot of the hydrogen sulfide solution was pipetted into a 250 ml. volumetric flask which contained 25 ml. of 20% zinc acetate solution, 200 ml. of double-distilled water and 2 drops of glacial acetic acid. The flask was then diluted to volume with double-distilled water. Immediately following withdrawal of the 10 ml. aliquot, 40 ml. of 0.1 N iodine was added to the remaining 490 ml. of hydrogen sulfide solution; the iodine in excess was back-titrated with 0.1 N sodium thiosulfate to a starch end point.

Investigation of Color Development

The formation of methylene-blue may be represented by the following equations:



Methylene blue chloride
(methylthionine chloride)

Methylene-blue crystals have a dark green color with a bronze luster. Their solubility is 1 g. in 25 ml. of water or 65 ml.

of alcohol. The aqueous solution has absorption maxima at 609 and 688 mμ.

Formation of methylene blue is markedly affected by the nature of the anion present. With p-aminodimethylaniline sulfate and chloride of the same concentrations, identical optical densities were obtained. The color development with the chloride salt was more rapid, but the color developed with low sulfide concentrations faded very rapidly. With the sulfide concentrations used in this work there was no fading of the blue color, and the chloride salt was used.

Figure 1 illustrates the optical density vs. wavelength curve from 630 to 770 mμ of a solution of methylene blue. The solution was prepared by pipetting 15 ml. of zinc sulfide solution (3.428 x 10^{-3} mg. of S per ml.) into a 50 ml. volumetric flask, diluting to 35 ml. with 2% zinc acetate solution, adding 1.5 ml. of the amine reagent and shaking the flask gently. After the addition of 5 drops of ferric chloride solution the flask was swirled to mix, the contents were diluted to volume and placed in an ice bath at 10° C. for 15-20 minutes to develop maximum color intensity. Budd and Bewick (15) used the primary absorption maximum at 670 mμ in their experimentation, while Sands, et al. (59) used the secondary absorption maximum at 745 mμ to take their readings. In this work the primary absorption maximum was found at 660 mμ.

Preparation of Standard Curve

Figure 2 represents a composite of at least six separate determinations of hydrogen sulfide. It was constructed by using nine 50 ml. volumetric flasks, that contained 0, 2, 4, 6, 8, 10, 12, 14, and 16 ml. of the zinc sulfide solution (3.428×10^{-3} mg.

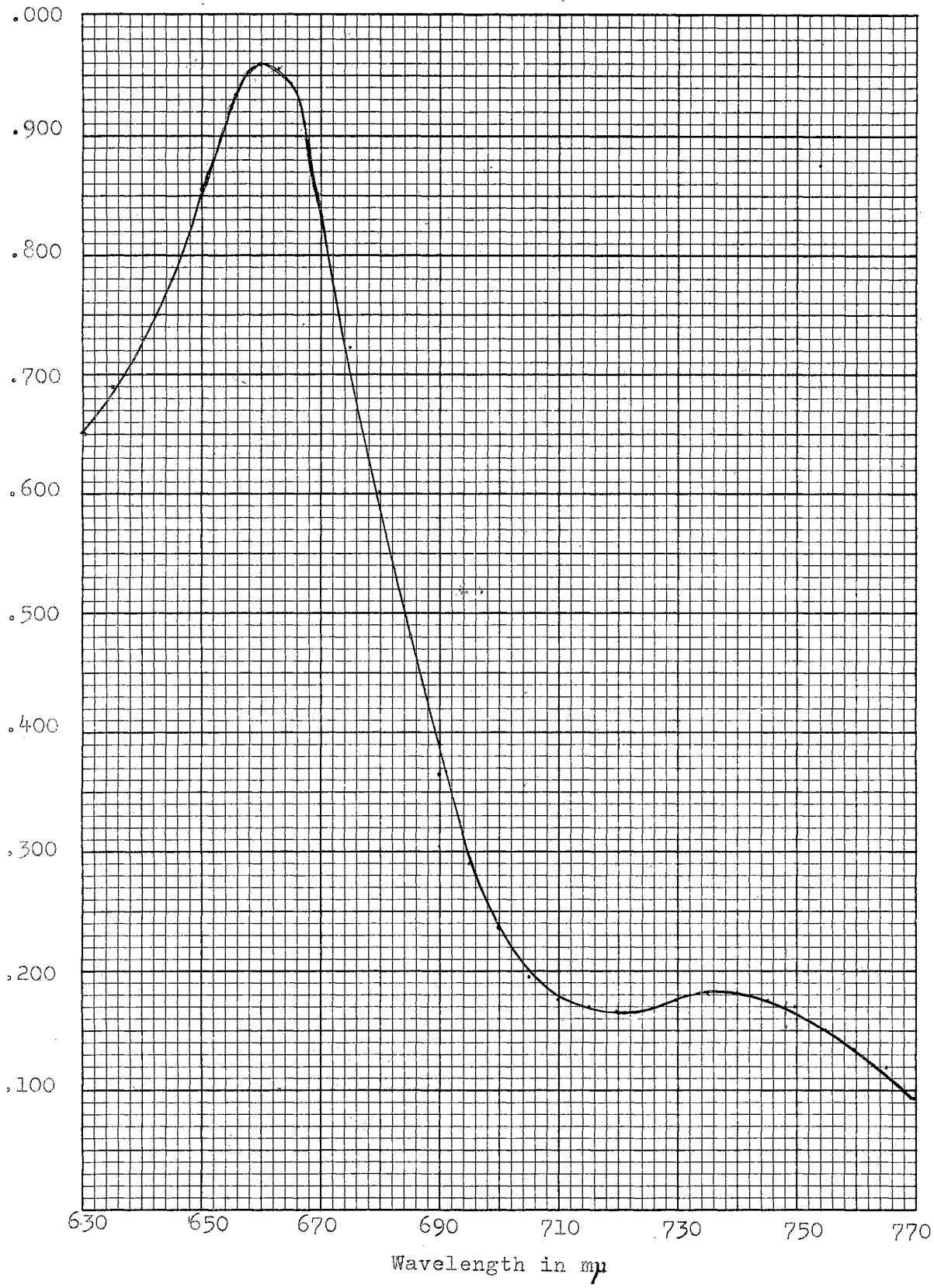


Figure 1. Spectrum of Methylene Blue; Solution Contained 0.05 mg. of S per ml.

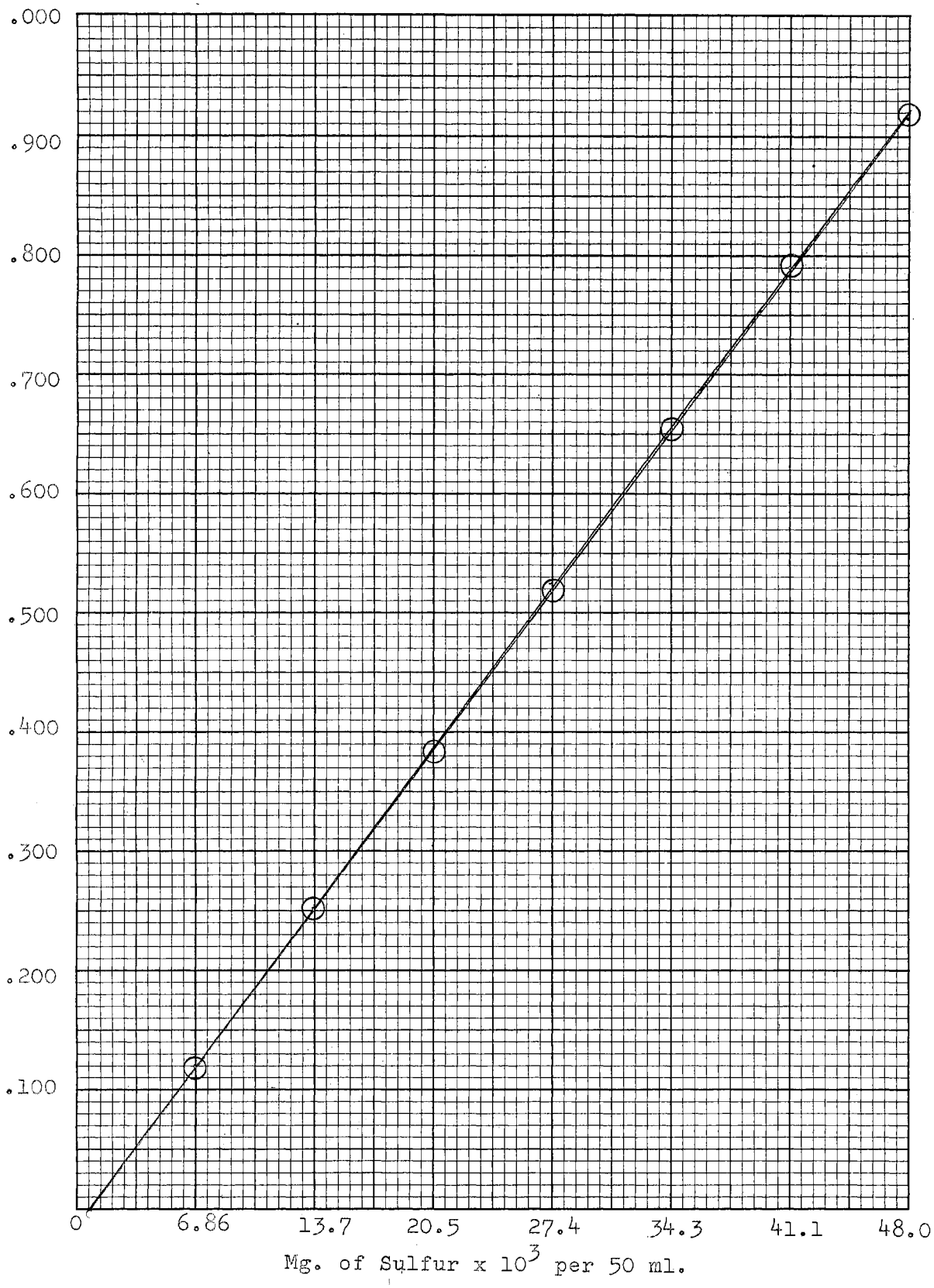


Figure 2. Standard Curve for the Determination of Sulfur as Methylene Blue

of S per ml.), respectively. Each flask was diluted to 35 ml. with 2% zinc acetate solution and then 1.5 ml. of amine reagent and 5 drops of ferric chloride solution were added before the mixtures were brought up to volume with double-distilled water. The standard sulfide curve was determined by allowing the flasks to stand at 10° C. for 20 minutes and then determining optical densities at 660 mμ.

Assay of Hydrogen Sulfide From Reaction Mixture

In a typical determination, 35 ml. of 2% zinc acetate solution was added to the absorption flask, the apparatus was assembled, and the system was purged with purified tank hydrogen, which had previously been deoxygenated with a "Deoxo" purifier. The sulfide solution was added through the side arm of the reaction flask; the hydrogen passing through the flask prevented atmospheric oxygen from entering. Hydrogen was bubbled through the solution for another 15-20 minutes to assure the absence of dissolved oxygen. At the end of this time, the flow of hydrogen was reduced to a rate of 3-5 bubbles per second in the absorption flask. While the solution was being degassed, the funnel was filled with 15 ml. of concentrated hydrochloric acid, which had been treated immediately before by placing in it one or two strips of aluminum for a few seconds and then decanting the acid off. The side arm was opened, two aluminum strips were introduced, the side arm was again securely tightened, and 10 ml. of hydrochloric acid was added dropwise. The hydrogen sulfide formed was swept into the zinc acetate solution. Ten minutes after the addition of the last drop of acid the hydrogen gas was turned off and the absorption flask disconnected. One and one-half milliliters of amine reagent was quickly added to the mixture, swirled, and 5 drops of ferric chloride solution were

added with mixing. The absorption flask was tightly stoppered and placed in an ice bath for 20 minutes. Then the blue solution was transferred quantitatively to a 50 ml. volumetric flask and diluted to volume with double-distilled water. The optical density was measured with the Beckman Model DU spectrophotometer at 660 mμ using the reagents as blank. The sulfur content was determined from the standard curve.

In order to determine the accuracy of the above method, freshly prepared sodium sulfide solutions were used. One aliquot was taken and the sulfide content analyzed by the iodometric method; another aliquot of the same solution was placed in the reaction flask and the evolved hydrogen sulfide determined by the methylene-blue method. In the most favorable cases, the sulfur concentration as determined by the methylene-blue procedure was 96% of the iodometric results.

It was thought that the loss might be due to incomplete absorption by the zinc acetate solution. However the recovery of sulfide was not improved by using stronger concentrations of zinc acetate or by putting another absorption flask in series with the first; no appreciable zinc sulfide formed in the second absorption flask. The shape of the reaction flask was also modified on three occasions with no effect on the recovery.

Reduction of Methionine

Methionine (4-methylmercapto-2-aminobutanoic acid) has a molecular weight of 149.15. The concentration used in these reductions was 1×10^{-3}M, which was equivalent to 3.2×10^{-2} mg. of sulfur per ml.

Stannous Chloride Reduction

Concentrated aqueous solutions of stannous salts become turbid owing to oxidation and hydrolysis. The formation of the basic salt can be prevented by adding hydrochloric acid,

$$6SnCl_2 + O_2 + 2H_2O \rightleftarrows 4Sn(OH)Cl + 2SnCl_4,$$

and oxidation can be prevented by keeping the acidic solution in contact with a few pieces of tin. Thus the stannous chloride solution used was 10% with respect to concentrated hydrochloric acid and contained a few pieces of mossy tin.

In a series of experiments 100 ml. of $1x10^{-3}$M methionine solution was refluxed under purified nitrogen with quantities of stannous chloride solution ranging from 10 to 100 ml. for times ranging from 1 to 8 hours. After all these reductions a certain amount of yellow insoluble precipitate had formed, which, upon analysis for sulfide content by the methylene-blue method, always gave negative results. The possibility of the product being stannous sulfide was ruled out. It has been reported by Oka and Matsuo (47) that stannic sulfide decomposes in the presence of excess stannous chloride, producing free sulfur. It was ascertained that the precipitate was in fact elemental sulfur, that may have been formed in this way. In any case, the formation of sulfur precluded the quantitative determination of the sulfur in methionine by this method.

Raney Nickel Reduction

In a series of experiments 100 ml. of $1x10^{-3}$M methionine were reduced with a pinch of the catalyst at a slow reflux rate.

The time necessary for reduction was found to be at least 1 hour. After reduction, the reaction mixture contained unreacted Raney nickel as well as nickel sulfide. The initial attempts to analyze for sulfide involved transferring an aliquot portion of the reaction mixture and adding it to the gas-evolution apparatus. Due to the presence of precipitated material it was impossible to obtain a truly representative aliquot. An alternative attempt to analyze for sulfide involved adding an excess of iodine to the entire reaction mixture and acidifying. It was found that the results were consistently high, owing to reduction of the iodine by the unreacted Raney nickel.

A third alternative would be to carry out the reduction of methionine in the gas-evolution apparatus. This could have been done by increasing the size of the reaction vessel and decreasing the amount of methionine used. However, the procedure would be more cumbersome. This and the fact that the recovery of sulfide samples had not been complete convinced us that the method would not be practical, and further investigation of it was not undertaken.

SUMMARY AND CONCLUSIONS

The attempted reduction of the sulfur in methionine to sulfide ion with stannous chloride was an absolute failure because sulfur was formed as well as tin sulfide.

With Raney nickel catalyst, the production of a nickel sulfide from methionine solutions was easily accomplished. But the poor reproducibility and low accuracy attained over-all indicated that the method would not be a good one for estimating the sulfur content of methionine and related compounds.

As concerns the determination of sulfide by the methylene-blue method, it is absolutely necessary to remove all oxygen from the apparatus and metallic sulfide solutions before releasing the hydrogen sulfide. The methylene-blue method is fast, reasonably accurate for semimicro quantities of reducible sulfides and requires no special techniques in the preparation of reagents.

Using the volumes and concentrations of reagents reported herein, the maximum amount of sulfur that can be determined at 660 mμ is 60 μg., but by taking the optical density readings at 745 mμ the range can be extended to nearly 320 μg.

CHAPTER III

COLORIMETRIC DETERMINATION OF CYSTEINE WITH SILVER DITHIZONATE

Review of the Literature

The analysis of cysteine (2-amino-3-mercaptopropanoic acid) has occupied the time of many investigators and much information is available in the literature. Malisoff, et al. (43) published an early review of mercaptan chemistry, which included methods of analysis for the thiol group. The biological importance of thiol groups is the subject of a review by Barron (7), who also discussed methods of analysis. Chinard and Hellerman (17), in their review, classified the various reagents used in thiol determinations into three main divisions, namely, oxidizing, alkylating and mercaptide forming agents. Nogare (46) recently published a review of methods for the analysis of sulfur-containing organic compounds; one section is devoted to mercaptans and to detailed methods for their determination. A later review by Patterson and Lazarow (49) deals with glutathione, but the authors explicitly make clear that some of the methods are applicable to the determination of cysteine. The last two reviews are complementary, as different methods are chosen for detailed discussion. An account of more recent methods is given by Waddill (66), who himself devised an analytical method of the "dead stop" amperometric type.

A quick survey of any one of the above reviews might indicate that another method for the determination of cysteine is superfluous;

yet, the number of methods suitable for analyzing microgram quantities of sulfhydryl-containing compounds is limited, and a method of high precision and accuracy could be quite beneficial. Some experience with the silver salt of dithizone indicated that it might be used for the spectrophotometric determination of microgram quantities of cysteine, and this matter was therefore looked into.

Diphenylthiocarbazone (I), or dithizone, was first synthesized by Emil Fischer (20) during an investigation of the structure and reactions of phenylhydrazine. The preparation is comparatively simple, but the product is seldom obtained in a pure state. The commercial product usually contains as impurities sulfur, the carbazide, and yellow to brown oxidation products, mainly diphenylthiocarbadiazone(II). This is insoluble in acid and basic aqueous

$C_6H_5NHNHCSN{=}NC_6H_5$ (I)

$C_6H_5{-}N{=}NCS{-}N{=}N{-}C_6H_5$ (II)

solutions, but readily soluble in chloroform and carbon tetrachloride; the other impurities are also insoluble in dilute ammonium hydroxide, while dithizone dissolves readily. Dithizone can therefore be purified by dissolving it in chloroform, shaking the solution with dilute ammonium hydroxide, decanting the aqueous layer, and acidifying it to precipitate the pure reagent. The dithizone is then taken up in carbon tetrachloride, to which it imparts a green color. Cooper and Sullivan (19) used the ratio of the optical density at 620 mμ to that at 450 mμ as a criterion of purity of the sample; the purest dithizone they obtained had a ratio of 1.68. Impurities increase the absorption at 450 mμ.

Dithizone can exist in a thioketo and an enethiol form. It is believed that the primary absorption maximum at 620 mμ is due

$$C_6H_5NHNHCSN{=}NC_6H_5 \rightleftarrows C_6H_5NH{-}N{=}C(SH){-}N{=}N{-}C_6H_5$$

keto form enol form

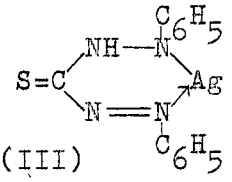
to the former and the secondary absorption maximum at 450 mμ to the latter, in carbon tetrachloride.

Dithizone forms strongly colored complexes with many metal ions, but no attempt to use it as an analytical reagent was made until Hellmut Fischer took up the study of the metallic dithizonates and their possible applications in analytical chemistry in 1925. To him goes most of the credit for the present knowledge and usefulness of this organic compound (22). Another more critical review of Hellmut Fischer's work was written by White (69).

When a purified solution of dithizone in carbon tetrachloride is shaken with an aqueous solution of a metallic cation, a complex salt is formed which is usually soluble in the organic liquid and insoluble in neutral and acidic aqueous solutions. The quantitative formation of dithizonates is markedly affected by the hydrogen-ion concentration of the aqueous phase, and the pH governs the order in which metal ions are extracted. Generally the more "noble" a metal is, the lower the pH for optimal extraction (70). The ions of gold, platinum, palladium, silver, mercury, tin(II), copper, bismuth, zinc, cobalt, nickel, lead, thallium and cadmium react with dithizone in immiscible solvents more or less consecutively as the aqueous phase is progressively changed from strongly acid through weakly acid, neutral, ammoniacal and alkaline, up to a concentration of 5% sodium hydroxide. Dithizone is therefore not a specific reagent, but under proper conditions it can be used to

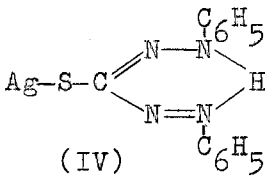
extract a whole group, or to separate subgroups and determine the individual members thereof.

According to Hellmut Fischer, et al. (23), when a heavy metal cation such as silver reacts with dithizone, the hydrogen atom of the imido group is replaced by the metal forming a "keto" dithizonate.



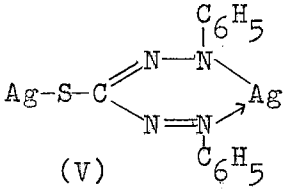
(III) "keto" dithizonate

However, in recent years, the structure of this compound has been the subject of much debate. Buch and Koroleff (14), who studied the spectrum of silver dithizonate spectrophotometrically, and Chemistskaya (16), who used photographic techniques, agreed that structure IV represented the "keto" dithizonate. Irving and Bell (34) added that since it is the hydrogen from the thiol group that imparts acidic character to dithizone, it should be replaced first in the formation of primary silver dithizonate.



(IV) "keto" dithizonate

Some metals form another dithizone complex which is commonly called the "enol" dithizonate. It is now realized that the "enol" complexes are formed by replacing the other hydrogen atom with another cation(V).



(V) "enol" dithizonate

Generally, the keto forms of all metal dithizonates exist, but many of the enol forms are not known; the keto form is preferentially formed in an acid or neutral solution, while the enol modification is formed either in an alkaline medium or with a large deficiency of dithizone. The enol form is transformed into the keto form by treating with an acid.

Dithizone reacts quantitatively with aqueous solutions of silver ion up to 0.5 N in mineral acid to form the keto dithizonate, which is soluble in carbon tetrachloride and chloroform, to which it imparts a yellow color. In neutral or alkaline solutions, the enol modification is formed. This complex imparts a red-violet color to chloroform solutions in which it is sparingly soluble; it separates as a fine violet precipitate from carbon tetrachloride in which it is insoluble (68).

It is interesting to note that silver keto dithizonate is stable in the presence of alkalies, and, once formed in an acid solution, is not appreciably converted to the enol form even on shaking the carbon tetrachloride solution with 5% sodium hydroxide. Therefore, after reaction of the silver ions with dithizone, excess of the latter can be removed from the carbon tetrachloride solution by shaking with dilute ammonia.

The idea of using silver dithizonate for the analysis of cysteine was supported by the work of Babko and Philipenko (4), who established that reaction (1) would go to completion, if the instability constant of the metallic dithizonate were at least 400 times larger than the solubility product constant of the metallic mercaptide formed. Since the instability constant of silver

(1) $MDz + RSH \rightarrow RSM + HDz$ (M = any metal; Dz = dithizone)

(in CCl_4) (in H_2O) (ppt.) (in CCl_4)

dithizonate is far more than 400 times greater than the solubility product of the silver mercaptide, this reaction should proceed quantitatively for thiol groups. Mercury(II) also gives an easily prepared, stable dithizonate, which does not have an absorption maximum around 620 mμ, but the silver keto dithizonate was preferred to the mercuric dithizonate for these reasons: silver keto dithizonate can easily be obtained uncontaminated by enol form, and is more soluble in carbon tetrachloride; divalent mercury ion might form both simple and double mercaptides.

Recently, Fridovich and Handler (26) have described the use of dithizone in determining the sulfhydryl content of proteins. In their method, the protein was treated with an excess of p-chloromercuribenzoate and the excess mercurial was reacted with a dithizone solution. Formation of mercuric dithizonate decreases the absorbancy of dithizone at 625 mμ, and this decrease was found to be proportional to the excess mercurial. In this way they calculated the amount of mercurial bound to the sulfhydryl groups of the protein.

Introduction to Present Work, General Experimental Detail

The method to be described represents a more direct approach than that of Fridovich and Handler. In this method an aliquot of aqueous cysteine solution is added to a solution of silver dithizonate and the concentration of freed dithizone is determined at its primary absorption maximum. It may be expected that the concentration of dithizone formed be directly proportional to the

concentration of cysteine added, and this would then serve for the microanalysis of sulfhydryl groups.

Reagents

Carbon tetrachloride. Reagent-grade carbon tetrachloride was distilled over calcium oxide and the middle fraction retained. Used carbon tetrachloride could be recovered by the method of Biddle (10). One batch of carbon tetrachloride was purified according to Geiger and Sandell (27), but did not behave differently from that distilled from calcium oxide so their tedious method of purification was not used.

All other organic solvents were of reagent grade, and were not further purified.

Mineral Acids. Reagent-grade concentrated acids were used. The desired concentrations were obtained by dilution with double-distilled water.

Ammonium Hydroxide. Concentrated C.P. ammonium hydroxide was diluted 1:100 with double-distilled water.

Silver Nitrate. Reagent-grade silver nitrate was used to make 1×10^{-2} M solutions by accurately weighing 169.9 mg. and dissolving with enough double-distilled water to make 100 ml. of solution.

Cysteine. Commercial samples of cysteine hydrochloride monohydrate were obtained from the California Foundation for Biochemical Research, Los Angeles, California and stored in a dessiccator near 0° C. One-hundredth molar solutions were prepared by dissolving a weighed amount of cysteine hydrochloride monohydrate in deoxygenated double-distilled water.

Dithizone. The "White-Label" product of the Eastman Kodak Company, Rochester, New York, was used. It was purified according

to Sandell (58) with slight modifications. One-half gram of dithizone was dissolved in 50 ml. of chloroform and the solution filtered through a coarse fritted-glass funnel to remove any insoluble material. The filtrate was shaken in a separatory funnel with four successive portions of 1:100 ammonia, each 50 to 75 ml. in volume. The aqueous extracts were separated from the chloroform and filtered through a small plug of cotton to remove droplets of chloroform. Carbadiazone present as impurity was discarded with the chloroform solution. The ammoniacal solution was then made slightly acidic with a dilute solution of sulfur dioxide gas to precipitate dithizone. Any semicarbazide present would remain in the acidic aqueous solution. Sulfur dioxide also serves to reduce any residual carbadiazone. The precipitated dithizone was then extracted with 2-3 portions of carbon tetrachloride, each having a volume of 100-150 ml., and the combined carbon tetrachloride extracts were shaken twice with an equal volume of double-distilled water. This wash was used to remove traces of the inorganic salt formed by acidifying the alkaline solution. The solution was stabilized by covering with a 0.1 M solution of sulfur dioxide, whose volume was one-tenth the volume of the dithizone solution. The dithizone solution prepared in this way and stored in a brown glass-stoppered bottle at 5° C. in the dark was kept for at least 6 months with no apparent change in strength.

Silver Dithizonate. Fifty milliliters of the dithizone solution were poured into a separatory funnel and washed with double-distilled water until the washings were no longer acid to litmus. A small amount of this solution was then drawn off and 3 ml. diluted with carbon tetrachloride until the optical density could be determined. The absorbance read at 620 mμ divided by the molecular

absorptivity (34.6×10^3) gave the molar concentration. From this value the amount of silver nitrate needed to give a slight excess of silver ion could be calculated and the corresponding volume of 1×10^{-2}M silver nitrate in 0.1 N sulfuric acid solution was added to the separatory funnel. The funnel was shaken vigorously for 3 minutes, and the carbon tetrachloride layer was allowed to separate. The silver dithizonate solution was then ready for dilution and addition to volumetric flasks. If the spectrum of the silver dithizonate solution showed any absorbance at 620 mμ due to excess dithizone, this could be removed by washing the carbon tetrachloride solution a few times with an equal volume of 1:100 ammonia.

Determination of Cysteine in Mixed Carbon Tetrachloride-*tert*-Butyl Alcohol

Before aqueous cysteine solutions could be added to carbon tetrachloride solutions of silver dithizonate a third component had to be found that would allow the solutions to mix. This component when added to a carbon tetrachloride solution of silver dithizonate should not cause decomposition or precipitation of the complex, and should allow addition of as much as 1 ml. of aqueous cysteine solution to 10 ml. of the reagent. After many solvents had been tried, the solvent mixture that behaved most favorably was one composed of 50% carbon tetrachloride and 50% *tert*-butyl alcohol.

Analytical Method

To a given volume of silver dithizonate in carbon tetrachloride was added an equal volume of butanol and the whole diluted to an optical density of 2.0 at 460 mμ with 50% carbon tetrachloride-50% butanol. Into a series of 10-ml. volumetric (or 25-ml. volumetric)

flasks were pipetted 5 ml. of the diluted silver dithizonate solution, and varying volumes of $1x10^{-2}$M cysteine solution were added to each flask by means of micro-pipettes. The maximum amount of cysteine solution of this concentration that could be used was approximately 0.5 ml. The flasks were shaken and the amount of free dithizone formed was measured with the Beckman Model DU spectrophotometer at the primary absorption maximum for dithizone in this ternary system, namely 600 mμ. By plotting concentration of cysteine used vs. optical density a straight line results which shows that silver dithizonate and the sulfhydryl group react in a ratio of 1:1.

Experimental Results

Figure 3 illustrates the spectra of dithizone and silver dithizonate in a 50% carbon tetrachloride-50% butanol mixture. The concentration of the solutes are not accurately known, and exact molecular extinction coefficients cannot be given. The primary absorption peak for dithizone occurs at 600 mμ while the secondary absorption maximum is at 440 mμ. The absorption maximum for silver dithizonate occurs at 460 mμ. Pel'kis (51) gives the spectra of dithizone in ten other common organic solvents.

Figure 4 illustrates typical results obtained in the initial experiments with cysteine. In these experiments various volumes of $1x10^{-2}$M cysteine solution were added to 25 ml. of silver dithizonate reagent whose absorption was 1.885 at 460 mu and 0.077 at 600 mμ. It can be seen that the plot is linear above 0.075 ml. of cysteine solution, but becomes curved below this value and does not pass through the origin. The steep slope of the curve is indicative of the high sensitivity of this method. The readings were taken 5

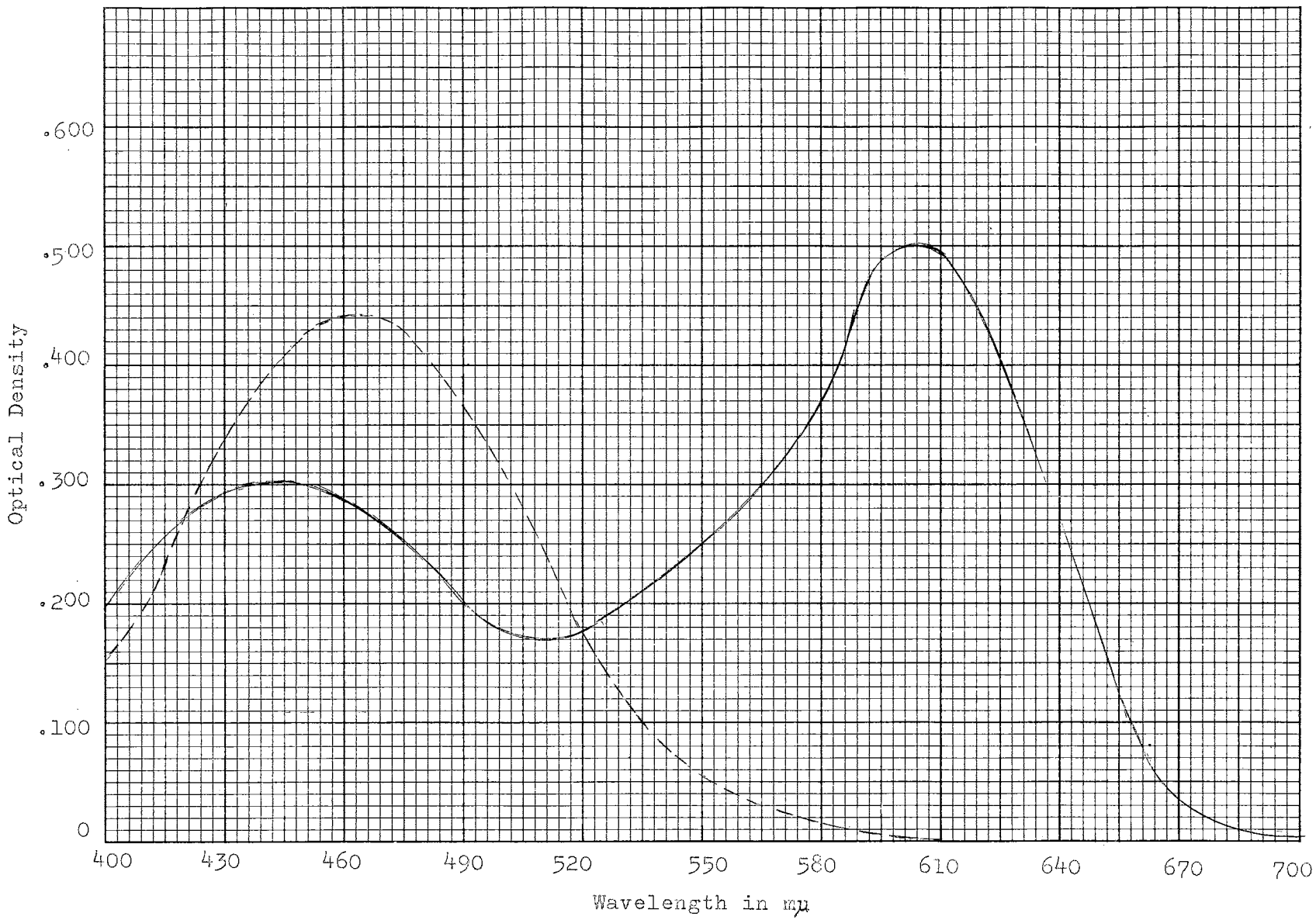


Figure 3. Spectra of Dithizone and Silver Dithizonate in a 50:50 Mixture of CCl_4 and t-Butanol

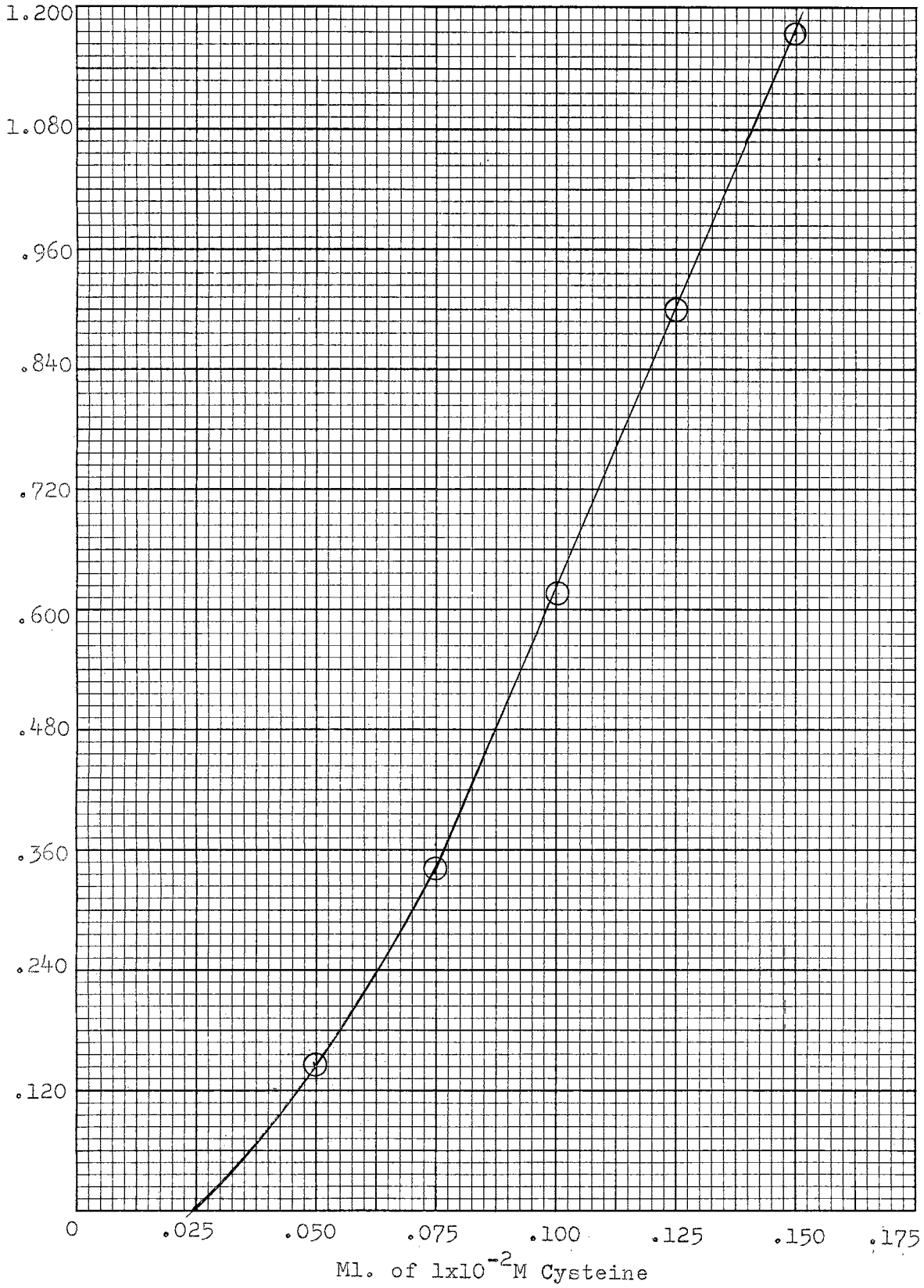


Figure 4. Optical Density of Free Dithizone at 600 mμ.

minutes after the cysteine solution was added to the silver dithizonate solution.

One reason why the curve did not go through the origin was likely that the cysteine or the free dithizone formed were partially oxidized. After many trials, it was thought that decreasing the volume of silver dithizonate solution would reduce such oxidation, as the solvent would contain less dissolved oxygen. The next series of experiments involved the addition of various volumes of 1×10^{-2}M cysteine solution to 5 ml. of silver dithizonate in a 25 ml. volumetric flask. The flasks were swirled and after 5 minutes additional 50% carbon tetrachloride-50% butanol solution was added to the mark. Figure 5 represents typical results. It can be seen that these trials did not substantially improve the method. Another series of experiments was performed identically to that described above, except the mixtures were diluted to the 25 ml. mark after addition of cysteine solution with silver dithizonate solution. This also did not improve the results.

Attempted Use of Other Solvent Systems

Carbon Tetrachloride-Ethanol System

To a cherry-red solution of silver dithizonate in carbon tetrachloride (silver dithizonate appears this color in concentrated solutions) was added 95% ethanol until the solution was homogeneous. Carbon tetrachloride and ethanol are miscible in all proportions but silver dithizonate is not very soluble in the latter; accordingly only a dilute solution could be obtained containing 1 part of a carbon tetrachloride solution of silver dithizonate to 99 parts of ethanol. The resulting reagent did

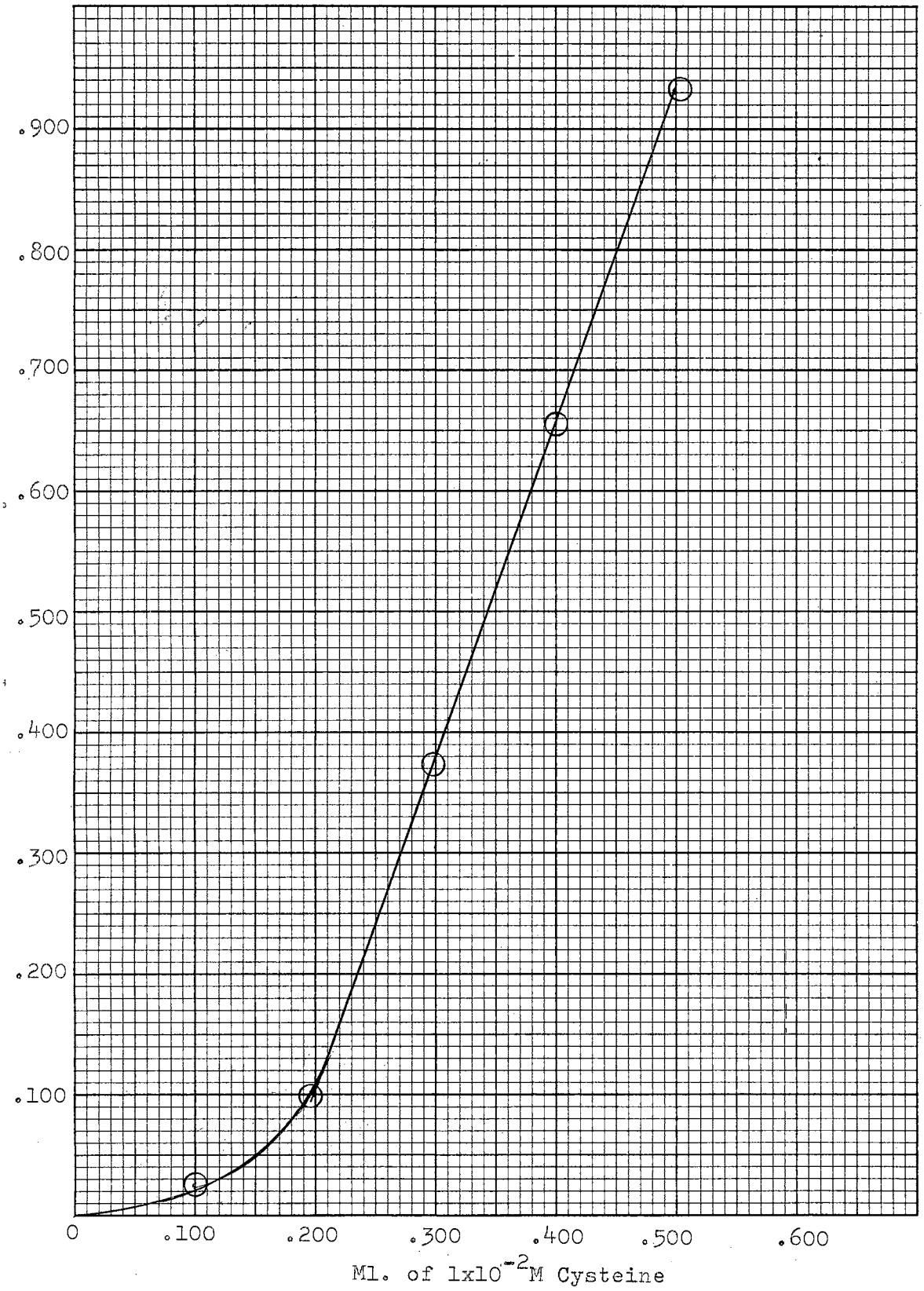


Figure 5. Optical Density Developed at 600 mμ.

not contain a sufficient amount of silver dithizonate. Also, the reaction in this mixed solvent was very slow. A better solvent system was therefore sought.

Carbon Tetrachloride-2-Propanol System

Since silver dithizonate was not sufficiently soluble in ethanol, it was thought that 2-propanol solvent might serve better. The best reagent, found after many experiments, contained equal volumes of a carbon tetrachloride solution of silver dithizonate and 2-propanol. This system was completely miscible when up to 1 ml. of aqueous cysteine solution was added to 10 ml. of the reagent. The concentration of free dithizone obtained upon reaction with $1x10^{-2}$M aqueous cysteine was not directly proportional to the amount of the latter; the plot of absorptions vs. amount of cysteine was curved upwards and the curve itself did not pass through the origin. Therefore this system was abandoned, after the failure of many attempts to improve the reagent.

Carbon Tetrachloride-Acetic Acid-Water System

Since cysteine is readily soluble in acetic acid it was thought a reagent could be prepared with this organic acid. The first attempt to make a reagent involved the ternary: 81.6% carbon tetrachloride solution of silver dithizonate, 17.7% glacial acetic acid and 0.70% water, all percentages by weight. The resulting reagent was clear and could easily be diluted to proper absorbancy. But 10 ml. of the reagent was only miscible with up to 0.05 ml. of $1x10^{-2}$M aqueous cysteine solution so it was discarded.

The second attempt involved a ternary with a much lower proportion of carbon tetrachloride. It was composed of the following

percentages by weight: 16.7% carbon tetrachloride solution of silver dithizonate, 68% glacial acetic acid and 15.4% water. This reagent was initially clear but after standing for 30 minutes the silver dithizonate precipitated out.

The third attempt was a compromise between the two extremes described above and involved a ternary composed of the following percentages by weight: 30.6% carbon tetrachloride solution of silver dithizonate, 67.8% glacial acetic acid and 1.56% water. This ternary was also initially clear, but faded quite rapidly.

Carbon Tetrachloride-Acetic Acid-2-Propanol System

In the investigation of a substitute for the water in the last system 2-propanol was tried and after many attempts the following ternary system proved most useful; 77% isopropanol, 18% glacial acetic acid and 5% carbon tetrachloride solution of silver dithizonate, all percentages by volume. The primary absorption maximum for free dithizone in this system was shifted from 620 to 590 mμ. By placing 3 ml. of this reagent in 10-ml. volumetric flasks, adding 0.01-0.06 ml. of 1×10^{-2}M aqueous cysteine solution and mixing, a straight line passing through the origin was obtained by plotting the absorbancies vs. the amount of cysteine. However the silver dithizonate concentration was rather low, so that large cysteine samples could not be analyzed. The carbon tetrachloride content and silver dithizonate concentration could only be increased by increasing the acetic acid content proportionally. This resulted in rapid fading of the color, so this system was not of much value.

Carbon Tetrachloride-Ethanol-Ethyl Acetate System

Kirsten (36) used silver dithizonate to determine chloride, bromide, iodide and sulfide in aqueous solutions. His reagent

consisted of 2 ml. of 85% phosphoric acid dissolved in 150 ml. of ethyl acetate. To 5 ml. of the reagent he added 2 ml. of silver dithizonate in chloroform and various volumes of the aqueous anion solution. He measured the liberated dithizone at 598 mμ. The analysis of chloride, bromide and iodide was reported to be quantitative but the sulfide analysis was only qualitative.

A reagent similar to Kirsten's was achieved by adding 5 ml. of a carbon tetrachloride solution of silver dithizonate to 100 ml. of a solution composed of 50% ethyl acetate and 50% ethanol, which had been made 1% with respect to concentrated phosphoric acid. This reagent was miscible with the desired volumes of aqueous cysteine, and the reaction was essentially complete after 5 minutes. However the reagent did not contain sufficient silver dithizonate to permit analysis of samples as large as desired. Even when the proportion of silver dithizonate solution was increased to 10% of the reagent only 0.2 ml. of 1×10^{-2}M aqueous cysteine solution freed all the available dithizone in 5 ml. of the reagent. The proportion of silver dithizonate solution could not be increased over 10% because the reagent precipitated. Use of larger volumes of the 10% reagent did not improve the method. Neither the substitution of 2-propanol for ethanol nor varying the percentage of ethyl acetate had beneficial effects, and this reagent was therefore abandoned.

SUMMARY AND CONCLUSIONS

Silver dithizonate in carbon tetrachloride has been used as a reagent in the analysis of cysteine; this binds the silver and the liberated dithizone is determined spectrophotometrically.

Mixed solvent systems must be used to mix with aqueous solutions. A reagent composed of silver dithizonate in 50% carbon tetrachloride-50% tert-butanol was found to have the most desirable characteristics. Even in this case, results were not favorable since the standard curve was not linear and would not pass through the origin. The method of analysis is limited to 0.30 ml. of 1×10^{-2}M cysteine solutions. The sensitivity is very high, but the useful range is so narrow that the method would not be generally useful.

Other reagents were tried with varying degrees of success, but in all cases the free dithizone or cysteine was readily oxidized by dissolved oxygen in the reagent.

CHAPTER IV

MERCAPTO-GROUP CONTENT OF SOME PROTEINS

Of the chemically active groups of proteins and biological substances the mercapto (sulfhydryl) groups possess the broadest reactivity. Their determination is, accordingly, of special interest. This section of the thesis deals with the mercapto-group content of two proteins, bovine serum albumin and beta-lactoglobulin. A peculiar problem attaches to the determination of mercapto-groups, namely, that their reactivity and consequently the number found in some conditions varies with the state of denaturation of the protein. Constant reference will therefore be made to the process of denaturation in what follows.

Review of the Literature

Denaturation

While in the older literature it is not uncommon to find reference to "denaturation" as if it were a well-defined process, it is now realized that denaturation is a complicated phenomenon and may involve a number of separate processes. For this reason, it cannot be discussed in detail here. However, it may be said in general that denaturation results in the protein assuming a looser and more random arrangement; there is an unfolding of the molecule. With respect to the oxidation of mercapto groups this process is of importance because it may permit distant groups to come into close contact, thus

making possible their oxidation with the formation of a disulfide bond.

Neurath, et al. (45) have written an excellent review on the nature of the denaturing reaction, the kinetics and thermodynamics of protein denaturation, and the properties of denatured proteins. Other sources containing valuable material on protein denaturation include Anson's (3) review on the reversibility of denaturation, the work of Putnam (52, 53) on the role of synthetic detergents as denaturants, and Chinard and Hellerman's (17) review of the action of various sulfhydryl reagents on native and denatured proteins.

Bovine Serum Albumin

The first analysis for the sulfhydryl content of this protein was performed by Greenstein (28). He used porphyrindin, an oxidizing agent, and his method consisted of adding larger and larger amounts of porphyrindin until sodium nitroprusside no longer gave a sulfhydryl test. In the protein denatured with 8 or 16 M guanidine hydrochloride (GHCl) he found 1.94 -SH groups per mole of albumin (M.W. 69,000). All subsequent data will be reported using this molecular weight. Later it was shown that porphyrindin is not specific for thiol groups, as it also oxidizes the hydroxyphenyl groups of tyrosine under the same conditions.

A number of authors have determined the sulfhydryl content of serum albumin with mercaptide-forming agents. Boyer (12) measured spectrophotometrically the increase in absorbancy accompanying mercaptide formation with p-chloromercuribenzoate, and found 0.89 -SH groups per mole. Fridovich and Handler (26) treated the protein with p-chloromercuribenzoate and employed dithizone to determine the excess of mercurial; the decrease in dithizone concentration

was measured at 625 mμ by means of a spectrophotometer. They found 1.05 -SH groups per mole. Another spectrophotometric method was devised by Horowitz and Klotz (31), who used a colored azomercurial to effect mercaptide formation. By calculating the amount of dye bound by the protein they reported the thiol content of serum albumin to be 0.67 -SH groups per mole. Simpson and Saroff (63) employed methylmercuric iodide in excess, and after mercaptide formation titrated the excess mercury compound with dithizone until the first excess of dithizone imparted a green color to the solution. At a pH of 3.0 they found 0.50 -SH groups per mole of albumin.

The most popular method used in determining the sulfhydryl content of bovine serum albumin has been amperometric titration with aqueous solutions of silver nitrate. The method was first used by Benesch and Benesch (8) in 1948, who found 0.80 -SH groups per mole in aqueous solutions and 0.71 -SH groups in aqueous solutions after denaturation with guanidine hydrochloride. Using the same titrant, Rosenberg, et al. (55) found no essential difference in the thiol content of serum albumin denatured with alcohol, urea or guanidine hydrochloride. In all cases they reported an average of 1.77 -SH groups. Benesch, et al. (9) improved their amperometric method by conducting the titrations in a neutral "TRIS" buffer where formerly an ammonium hydroxide-ammonium nitrate buffer of high pH had been used. These authors found 0.67 -SH groups per mole of native albumin and 1.04 -SH groups after the albumin had stood for 30 minutes in 8 M urea.

Kolthoff, et al. (39) used mercuric chloride or acetate and reported this titrant gave clearer end points than silver ion. They reported 0.65 -SH groups per mole of serum albumin. With 4 M guanidine

hydrochloride as the denaturing medium, and mercuric chloride as titrant, Kolthoff, et al. (38) found 0.68 -SH groups per mole of albumin, the same value they reported earlier with native albumin. These authors stated that the denatured protein was easily oxidized whereas in the native state it was perfectly stable toward oxygen. They showed that at pH 7.0 all the sulfhydryl could be determined, but that at pH 9.0 the results were low even when great care was taken to exclude oxygen completely.

The latest attempt to determine the sulfhydryl content of bovine serum albumin is that reported by Roberts and Rouser (54), who employed N-ethylmaleimide in dilute aqueous solutions at pH 6.0 and measured the decrease in absorbancy of this reagent at 300 mμ; this corresponded to 0.60 -SH groups per mole.

While the results of several investigators are not in good agreement, it is seen that most of them are close to 0.66 -SH groups per mole. The fact that a non-integral value is found has been justified by reference to the results of Hughes (32) with human serum albumin. This investigator demonstrated that this protein was not homogeneous, and he isolated and crystallized a fraction, called mercaptalbumin, which contains 1.0 -SH group per mole of protein. Hughes found that human serum albumin contains 66% mercaptalbumin or 0.66 -SH groups per mole of human serum albumin. While many investigators apparently assume that bovine serum albumin is exactly similar to human albumin with respect to mercaptalbumin content, there is little published evidence to support this. deVrekar and Lontie (65) determined the mercaptalbumin content of bovine serum albumin by light-scattering measurements and found it to be 77%.

It is interesting to note that out of the dozen or more determinations of the sulfhydryl content in bovine serum albumin only the initial attempt by Greenstein utilized an oxidizing agent. This perhaps can be attributed to the fact that porphyrindin was found to be unspecific for sulfhydryl groups, and this may have led many researchers to believe that all oxidizing agents were unspecific.

Beta-Lactoglobulin

It has been twenty-five years since beta-lactoglobulin was first crystallized by Palmer (48). During this time there have been many articles devoted to the chemistry of this protein, but only a superficial inspection of the literature is needed to show that there is much discrepancy between sets of experimental data. As an example, the values reported for the molecular weight of this protein have varied from as low as 35,000 to as high as 42,000, and there is no agreement at present on a correct value. In this work, the value of 40,000 will be adopted, and data on the sulfhydryl content of beta-lactoglobulin will refer to this value.

The initial evidence on the sulfhydryl content of beta-lactoglobulin was reported by Brand, et al. (13); they used a photometric method employing phosphotungstic acid on the protein hydrolysate and found 3.67 mole -SH per mole of protein. Five years later, Larson and Jenness (41) adapted the "dead stop" amperometric titration of Foulk and Bawden (24) to this analysis. They titrated with o-iodosobenzoate and found 4.3 mole -SH in the native protein; when the protein was first denatured in 8 M GHCl the value decreased to 4.23 mole -SH per mole of protein (42). Groves, et al. (29) also employed an oxidizing agent, porphyrindin. They used solutions buffered at pH 7.2 and 5 M with respect to GHCl and found, first of all, that

denaturation was practically instantaneous by measuring the increase in optical rotation. The sulfhydryl content in the same medium was found to be 1.82 mole. The only other oxidizing agent used for this analysis was ferricyanide, which was employed by Christensen (18). The resulting ferrocyanide concentration was measured colorimetrically as Prussian Blue. In a medium composed of 1% protein and 38% urea, he found 1.13 mole -SH after denaturing at 30° C. and 4.4 mole -SH after denaturing at 0° C. Since the latter value was more in accordance with Brand's data, he reported this as the sulfhydryl content of beta-lactoglobulin. His data were not extensive and he did not offer any realistic hypothesis for the large difference in sulfhydryl content found at the two temperatures.

By the use of mercaptide-forming agents a number of authors have found a substantially lower -SH titer. Fraenkel-Conrat, et al. (25) used p-chloromercuribenzoate in GHCl-denatured solutions and found 2.0 mole -SH per mole of protein. They also reported 2.8 mole -SH with the alkylating agent, iodoacetamide, but considered the former method more specific. Boyer (12) and Horowitz and Klotz (31) reported 2.48 and 1.83 mole -SH respectively. The former used p-chloromercuribenzoate and the latter used a colored azomercurial to effect mercaptide formation as in the experiments with bovine serum albumin.

The only reported use of an amperometric method is the work of Hutton and Patton (33), who used silver nitrate as titrant and found 1.75 mole -SH.

The foregoing review of the literature is complete to the writer's knowledge and illustrates the complete lack of agreement among the results of various workers. The purpose of this study

is not merely to add another value to this list, but to evaluate the utility of ferricyanide as a specific reagent for the mercapto groups of proteins.

Introduction to Present Work; General Experimental Detail

Katyal (35) has shown that ferricyanide is a specific reagent in the case of ovalbumin. He employed a spectrophotometric method and the "dead stop" amperometric titration in his successful determination of sulfhydryl content in this protein. The same two analytical procedures will be used in this work to determine the thiol content of bovine serum albumin and beta-lactoglobulin.

Reagents

Cupric Sulfate. Exactly 61.6 mg. of reagent-grade cupric sulfate pentahydrate were dissolved in enough deionized water to make 100 ml. of solution. One milliliter of this solution is equivalent to 24.64×10^{-4} mmole of sulfhydryl or 250 mg. of bovine serum albumin containing 0.68 -SH groups per mole of protein.

Potassium Iodide. A 1×10^{-3}M solution was prepared by dissolving 16.6 mg. of reagent-grade potassium iodide in sufficient deionized water to make 100 ml. of solution.

Potassium Ferricyanide. A 4×10^{-3}M solution was prepared by dissolving 131.7 mg. of reagent-grade potassium ferricyanide in enough deionized water to make 100 ml. of solution. This solution was made fresh daily.

Potassium Ferrocyanide. A 4×10^{-3}M solution was prepared by dissolving 169 mg. of reagent-grade potassium ferrocyanide trihydrate in sufficient deionized water to make 100 ml. of solution.

Phosphate Buffer. Reagent-grade monopotassium and dipotassium phosphate, and sodium hydroxide were used in preparing 3.5×10^{-2}M buffers ranging in pH from 6.8 to 9.0.

Deionized Water. Distilled water was passed through a column of analytical-grade Amberlite MB-1, a sulfonic acid-quaternary amine resin mixture; the deionized water was used in all experiments.

Guanidine Hydrochloride (GHCl). C.P. guanidine hydrochloride was recrystallized from methanol according to Kolthoff, et al. (38) and dried at 50° C. in vacuo for about 5 hours.

Urea. A concentrated aqueous solution of reagent-grade urea was purified according to Benesch, et al. (9) and dried in vacuo at 50° C. for about 5 hours.

Sodium Lauryl Sulfate (SDS). This anionic detergent, U.S.P., obtained from Fisher Scientific Company, Fairlawn, New Jersey, was not subjected to further purifications.

Trimethyldodecylammonium Chloride (DAC). This cationic detergent was a product of Armour and Company, Chicago 9, Illinois. It was an especially purified product and was not subjected to further purification.

p-Chloromercuribenzoate. A 5×10^{-3}M solution was prepared by dissolving an accurately weighed sample of sodium p-chloromercuribenzoate in the minimum volume of 0.5 M sodium hydroxide and diluting to the desired volume with phosphate buffer of pH 9.0.

Crystallized Bovine Plasma Albumin. This product was obtained from the Armour Laboratories, Chicago 11, Illinois. It was found to contain 6.3% moisture by heating in vacuo at 110° C. to constant weight.

Crystallized Bovine Beta-Lactoglobulin. Two sources of this protein were used. Sample I was the product of Nutritional Biochemicals Corp., Cleveland, Ohio and was found to contain 11% moisture; Sample II was obtained from Pentex, Inc., Kankakee, Illinois and had a moisture content of 5.3%. All experiments were performed using accurately weighed fresh samples.

BOVINE SERUM ALBUMIN

Spectrophotometric Determination

Analytical Method

Ferricyanide has a broad absorption peak about 410 mμ, and its reduction product, ferrocyanide, does not absorb in this region. Hence the amount of ferricyanide consumed is measured by the decrease in absorbancy compared to a "reagent" blank, which contains no protein. Such a blank is necessary because a small amount of ferricyanide may be consumed even when protein is absent. The optical density of the sample and "reagent" blank are compared to the "spectral" blank, consisting of deionized water.

A recently weighed sample of bovine serum albumin, 250 mg., was transferred to a 25 ml. volumetric flask and dissolved in 5 ml. of 3.5×10^{-2}M phosphate buffer of pH 6.9. To this clear solution was added 2.0 ml. of 4×10^{-3}M potassium ferricyanide. The denaturant was added and whole diluted to the mark with deionized water. The mixture was allowed to stand in a 37° C. water bath. At various intervals an aliquot portion was transferred to a spectrophotometer cell, and the absorption of the "reagent" blank and sample were measured at 410 mμ on a Beckman Model DU spectrophotometer. The amount of

ferricyanide consumed by the albumin could be calculated from the decrease in absorption.

Sample Calculation

After standing at 37° C. for 780 minutes a particular sample and reagent blank had the following optical density at 410 mμ.

	O.D.
Sample	0.209
Reagent Blank	0.319

Since the reagent blank contains 2 ml. of 4×10^{-3}M ferricyanide, the amount of unreacted ferricyanide is;

$$8 \times 10^{-3} \times \frac{.209}{.319} = 5.24 \times 10^{-3} \text{ mmoles}$$

The amount of ferricyanide consumed by the denatured albumin is:

$$8 \times 10^{-3} - 5.24 \times 10^{-3} = 2.76 \times 10^{-3} \text{ mmoles}$$

The bovine albumin sample contains 6.3% moisture.

$$(250 \text{ mg.}) \times (100-6.3) = 234 \text{ mg. dry wgt.}$$

$$\frac{234.25}{69,000} = 3.395 \times 10^{-3} \text{ mmole of BSA}$$

$$\frac{2.76 \times 10^{-3}}{3.395 \times 10^{-3}} = 0.81 \text{ mole -SH per mole BSA}$$

Experimental Results

No oxidation of sulfhydryl was found in native bovine serum albumin. This is in accord with the findings of Kolthoff and Anastasi (37). These authors also reported that an equivalent amount of copper(II) (1.5 ml. of copper(II) of the concentration

used is equivalent to 250 mg. of BSA on the mole basis) catalyzes the oxidation of the sulfhydryl group in BSA by ferricyanide.

Since the copper(II) catalyst and ferrocyanide might be expected to react and form copper ferrocyanide, some preliminary experiments were done with ferricyanide, ferrocyanide, copper(II) and BSA, with the results shown in Table I. Each 25 ml. volumetric flask contained 5 ml. of 3.5×10^{-2}M phosphate buffer of pH 6.9, various volumes of copper(II) solution (0.62 mg./ml.) and 1 ml. of 4×10^{-3}M ferrocyanide or ferricyanide solution. The flasks were diluted

TABLE I

OPTICAL DENSITY OF FERRO- AND FERRICYANIDE IN PRESENCE OF COPPER(II)

Sample	1	2	3		4		5	6
Wave Length, mμ →	410	410	470	410	470	410	410	410
Time in Minutes ↓ 30	0.175	0.180	.090	.070	.229	.185	.042	.082
240	0.174	0.178	.080	.050	.297	.274	.061	.089

to the mark with deionized water and aliquot portions taken for spectrophotometric analysis. Sample 1 contained 1.5 ml. of the copper(II) solution and 1 ml. of the ferricyanide while sample 2 contained 2.5 ml. of copper(II) solution. It can be seen that the optical density of ferricyanide at 410 mμ is independent of copper(II) concentration. Sample 3 contained 1.5 ml. of copper(II) solution and 1 ml. of ferrocyanide, while sample 4 contained 3.5 ml. of copper(II) solution; neither of the original reactants absorb at 410 mμ, and the absorption noted is due to copper ferrocyanide, which is readily formed at

this pH, and shows a broad absorption peak about 470 mμ. Sample 4 contained an excess of copper(II) and it should be noted that the absorption at 410 mμ is nearly identical to the absorption of sample 2 after 30 minutes. Sample 5 was identical to sample 3 but in addition contained 250 mg. of BSA. Sample 6 was identical to sample 4 but in addition it also had 250 mg. of BSA present. In the presence of BSA copper(II) is bound by the protein in such a fashion that it is not available for the formation of copper ferrocyanide. The small absorbance of samples 5 and 6 at 410 mμ is due to the protein present.

Table II is comprised of data illustrating the oxidation of denatured BSA as a function of time. The BSA was denatured by adding 12 gm. of GHCl to the 25-ml. volumetric flasks; this is equivalent to 5 M GHCl. Samples 1 and 2 were mixtures of phosphate buffer, pH 6.9, BSA and ferricyanide in the concentrations specified under "analytical method". Although the reaction was carried out mainly at 37° C., the volumetric flasks were taken out of the water bath when aliquots were taken for analysis; hence the measurements of reaction rates are only roughly quantitative. The pH of the reagent blank in sample 1 and 2 was 5.38, while the protein-containing sample had a pH of 5.34 after 24 hours. This decrease in pH can be expected from the following equation:

$$2(Fe(CN)_6)^{+++} + 2RSH \rightarrow 2(Fe(CN)_6)^{++} + R\text{-}S\text{-}S\text{-}R + 2H^+$$

Sample 3 illustrates the effect of 1×10^{-3} M potassium iodide on the ferricyanide oxidation.

In order to determine the specificity of ferricyanide for the sulfhydryl group of BSA, the -SH groups were blocked by adding 1 ml.

TABLE II

MOLE OF -SH FOUND PER MOLE OF BSA IN GHCl DENATURING-MEDIUM

Time of Denaturation at 37° C. in Minutes	Sample 1 pH 5.38-5.34			Sample 2 pH 5.38-5.34			Sample 3 pH 5.38-5.34		
	O.D. of Blank	O.D. of Sample	Mole-SH/ Mole BSA	O.D. of Blank	O.D. of Sample	Mole-SH/ Mole BSA	O.D. of Blank	O.D. of Sample	Mole-SH/ Mole BSA
20				.330	.267	0.45			
55							.321	.227	0.685
120	.320	.231	0.68						
190	.322	.229	0.686						
300	.323	.225	0.715						
320							.319	.197	0.905
490							.318	.190	0.954
600	.319	.209	0.812						
740				.324	.213	0.81			
780	.315	.207	0.81						
935				.323	.206	0.85			
1115				.323	.200	0.90			
1155							.318	.180	1.03
1440				.323	.189	0.98			
2940				.320	.162	1.16			

TABLE II (Continued)

Time of Denaturation at 37° C. in Minutes	Sample 4 pH 5.66-5.40 +1.0 ml. of 0.15% PCMB			Sample 5 pH 6.90-5.85			Sample 6 pH 6.70-5.90 +1.0 ml. of Copper(II)		
	O.D. of Blank	O.D. of Sample	Equiv. of Feic Consumed/ Mole BSA	O.D. of Blank	O.D. of Sample	Mole-SH/ Mole BSA	O.D. of Blank	O.D. of Sample	Mole-SH/ Mole BSA
55	.317	.320	0	.318	.220	0.74	.335	.250	1.00
320	.318	.280	0.28	.310	.165	1.14	.345	.227	1.34
490	.314	.257	0.43	.313	.157	1.20	.354	.225	1.44
1155	.310	.231	0.60	.306	.144	1.30	.356	.220	1.49

of 0.15% p-chloromercuribenzoate (PCMB) to a buffered solution of BSA. This enough PCMB to tie up 1.09 mole of sulfhydryl at the albumin concentration used, namely 250 mg. Sample 4 illustrates the results when PCMB was added to the reagent blank and sample buffered with phosphate buffer of pH 6.9. The pH of blank and sample after 24 hours was 5.60 and 5.40, respectively. After 55 minutes, no ferricyanide had been consumed although after 1155 minutes the decrease in ferricyanide concentration amounted to 0.60 equivalents per mole of BSA. It appears from this that ferricyanide reacts rapidly with the sulfhydryl groups of BSA, but that this is accompanied by reaction with other groups in the molecule as well.

Samples 5 and 6 in Table II illustrate the effect of raising the pH of the albumim samples. In these samples 5 ml. of 3.5×10^{-2}M phosphate buffer of pH 9.0 was substituted for the pH 6.9 buffer. In addition sample 6 contained 1.0 ml. of copper(II). After denaturation for 24 hours at 37° C. the pH of sample 5 was 6.9 and 5.85 for the reagent blank and protein sample respectively, while the corresponding pH for sample 6 was 6.70 and 5.90. It can be concluded from these data that, as the pH is raised, the reaction becomes more unspecific.

Table III is comprised of data illustrating the oxidation of BSA denatured with other agents, as a function of time. Samples 1 and 2 were denatured by adding 12 gm. of urea to the 25 ml. volumetric flask, making the solution 8 M with respect to urea. Sample 3 was denatured by adding 10 ml. of 10% SDS to the 25 ml. volumetric flask, making this solution 0.14 M with respect to SDS concentration, and an equivalent mole quantity of copper(II) was also added. All

TABLE III

MOLE OF -SH FOUND PER MOLE OF BSA IN UREA AND SDS DENATURING-MEDIUM

Time of Denaturation at 37° C. in Minutes	Sample 1 (Urea) pH 7.4-7.18			Sample 2 (Urea) pH 7.47-7.02			Sample 3 (SDS)		
	O.D. of Blank	O.D. of Sample	Mole-SH/ Mole BSA	O.D. of Blank	O.D. of Sample	Mole-SH/ Mole BSA	O.D. of Blank	O.D. of Sample	Mole-SH/ Mole BSA
20				.321	.328	0			
120	.324	.291	0.24				.363	.341	0.14
190	.320	.272	0.35				.367	.331	0.23
300	.316	.249	0.50				.391	.320	0.43
600	.316	.212	0.78				.434	.307	0.69
740				.314	.213	0.76			
780	.313	.202	0.83				.443	.293	0.80
935				.313	.206	0.80			
1115				.310	.194	0.88			
1440				.307	.173	1.03			

values for the sulfhydryl content of BSA in these tables were converted to the moisture free basis. After 24 hours the pH of the reagent blank in sample 2 was 7.47 while the protein sample had a pH of 7.02. Again it can be seen that the action of ferricyanide on BSA is unspecific when either urea or SDS are used as denaturants. The cationic detergent DAC was also used as a denaturant in these studies but was found to be of no use due to the increase in absorbancy of DAC-denatured albumin samples instead of a decrease.

In comparing denaturants, the action of GHCl is fastest, followed by urea, while the slowest reaction takes place with SDS-denatured samples. But it must be concluded that in all the above cases the action of ferricyanide on denatured albumin involves oxidation of other groups besides sulfhydryl.

Electrometric Determination

Analytical Method

The apparatus used has been described (63). In this work a Leeds and Northrup lamp and scale galvanometer was used (Model No. 2430-A) which had a sensitivity of 0.41 uv/mm; the internal resistance was 17 ohm. A 5 ohm resistance was connected in parallel with the internal resistance, giving a final sensitivity of approximately 0.10 ua/mm. The potential impressed upon the electrodes was 100 mv., as determined with the aid of a Beckman Model "G" pH meter.

In a typical electrometric titration procedure, the protein was taken from the refrigerator and allowed to come to room temperature before weighing. The accurately weighed sample, 250 mg., was placed in a 100-ml. tall-form beaker and dissolved in 10 ml. of 3.5×10^{-2}M phosphate buffer of pH 6.9. This was followed by the

addition of 1 ml. of $1x10^{-3}M$ potassium iodide or 1.5 ml. of copper(II) solution. Then 1.0 ml. of $4x10^{-3}M$ potassium ferricyanide was added, followed by the proper amount of denaturant, and the whole diluted to 25 ml. with deionized water. The beaker was then placed in a 37° C. water-bath until oxidation was complete. A reagent blank was prepared containing no protein; this solution was also placed in the water-bath and was used to measure the residual current. The extent to which the reaction had gone to completion was periodically checked by immersing the electrodes in the solutions, stirring with a glass stirrer rotating at 600 r.p.m., and noting the current on the galvonometer. Since the blank contained no ferrocyanide its reading was constant. In certain favorable cases, the current produced in the sample was proportional to the concentration of ferro- and ferricyanide present. Thus, when small portions of $4x10^{-3}M$ ferricyanide solution were added from a 5-ml. semi-micro buret the current increased. The values of the current were plotted as a function of total ferricyanide added and the line connecting these points was extrapolated to the residual current line; this measured the amount of ferricyanide present in the sample before the additional small portions had been added. The difference between the amount present at that time and that initially added gives the amount consumed by the protein.

Experimental Results

As noted earlier, the denaturants guanidine hydrochloride and urea serve best in denaturing BSA. But it was reported by Katyal (35) that urea could not be used as a denaturant in the electrometric method because it reduces the response of the electrodes to small amounts of ferricyanide. This was found to be true, even

after extensive purification of the urea. He also stated the GHCl denatured solutions have erratic and high galvanometer readings; however GHCl was successfully used as a denaturant in this study after careful recrystallization.

In the first series of experiments with GHCl 12 g. of it was added to the buffer solution of albumin, which also contained 1 mole of copper(II) per mole of albumin. After various periods of standing at 37° C., 0.62 ml. of 4×10^{-3}M ferricyanide was added and, when the galvanometer reading had reached a steady value, small additional amounts of ferricyanide were added. It was thus learned that the amount of ferricyanide consumed decreased with increasing time of standing in the GHCl solution. For example, if the denatured albumin solution was allowed to stand at 37° C. for only 15 minutes before the addition of ferricyanide, 0.47 mole -SH per mole albumin was found; after a 120-minute period of denaturation, 0.36 mole -SH was found. This difference can probably be attributed to air-oxidation of the denatured protein, as no precautions were taken to exclude oxygen.

If the GHCl was added after a small excess of ferricyanide has been mixed with the protein, different results were obtained; in this case, the galvanometer readings reached a maximum after 20 minutes and then began to drift downwards. If more ferricyanide was added to the solution before the readings start to decrease, the galvanometer did not respond linearly to these additions. Therefore, the amount of ferricyanide reduced could not be determined quantitatively. It can only be concluded some reduction of ferricyanide takes place in these conditions.

In the next series of experiments, 10 ml. of 10% SDS was used as denaturant. In the first set of experiments, the reaction mixture consisted of 250 mg. of BSA, 10 ml. of 3.5×10^{-2}M phosphate buffer of pH 6.9, 1 ml. of 10^{-3}M iodide ion, 1.0 ml. of 4×10^{-3} M ferricyanide and 10 ml. of 10% SDS, added in that order. The volume was adjusted to 25 ml. in all cases with deionized water. The purpose of the iodide ion was to stabilize the galvanometer readings. In these experiments it was learned that no ferrocyanide was produced even after denaturation for 24 hours at 37° C.

The next set of experiments was identical to those above, except that they were conducted in the presence of 1 mole of copper(II) per mole of albumin (1.5 ml. of solution), in place of the iodide ion. After denaturation for 260 minutes at 37° C., one sample gave a reading of 27 on the galvanometer, where initially the reading was only 5. Upon addition of small increments of ferricyanide to this sample it was noted that the galvanometer readings increased linearly. Extrapolation to the value of the current found in the reagent blank indicated 0.77 mole -SH per mole of albumin. Upon longer standing it was found that the galvanometer readings slowly drifted downwards. Another sample showed 0.78 mole -SH per mole of albumin after denaturation for 168 minutes, but again the galvanometer readings slowly drifted downward, indicating further consumption of ferricyanide. More experiments were conducted with copper(II) ion and SDS, but in most cases the readings were quite erratic, and the amount of ferricyanide consumed was not calculated.

In order to estimate in another way what concentration of ferrocyanide had been formed in these experiments, a mixture was prepared with 10 ml. of 3.5×10^{-2}M phosphate buffer of pH 6.9, 1.0 ml. of

4×10^{-3}M ferricyanide, 10 ml. of 10% SDS, and 1.5 ml. of copper(II) solution, i.e., the same mixture as used above but without the BSA. This solution was allowed to come to 37° C. and small amounts of 4×10^{-3}M ferrocyanide were then added. As might be expected from the spectrophotometric experiments, the added ferrocyanide precipitated as copper ferrocyanide and there was no appreciable rise in the galvanometer readings when up to 1.0 ml. of the ferrocyanide was added. Thus it was not possible to ascertain ferrocyanide concentration in the presence of copper(II).

By substituting 10 ml. of 10% DAC for the anionic detergent, the electrode response obtained after reaction with denatured BSA samples was too feeble to be measured.

BETA-LACTOGLOBULIN

Spectrophotometric Determination

Experimental Results

The method of analysis for the sulfhydryl content of beta-lactoglobulin (BLG) was identical to that used for BSA. Preliminary experiments with urea and guanidine hydrochloride as denaturants were done to ascertain whether or not this protein would also show a slow but steady decrease in absorbancy as a function of time, as BSA did. It was surprising to find that with 5 M GHCl and 8 M urea as denaturants the reaction was complete in less than 30 minutes, with no further decrease in the optical density of the reagent blank or sample in up to 8 hours. To illustrate, a sample of urea-denatured BLG showed an uptake of ferricyanide equivalent to 2.32 mole -SH per mole of protein after 150-minutes incubation, and 2.45 mole -SH

after 480 minutes. A corresponding sample of GHCl-denatured BLG showed 3.15 mole -SH after 150 minutes and 3.27 mole -SH after 480 minutes, at 37° C.

Six determinations done with both samples of BLG gave, for the urea-denatured samples, 2.44±0.10 mole -SH per mole of BLG, and for the GHCl-denatured samples 3.12±0.10 mole -SH per mole of BLG.

A series of experiments were then designed to show the effect of blocking the -SH groups of BLG in the presence of these two denaturants. The -SH groups were blocked by an appropriate concentration of PCMB prior to the addition of denaturant and ferricyanide. To a series of 25-ml. volumetric flasks were added 50 mg. of BLG and solution was effected by adding 5 ml. of 3.5×10^{-2}M phosphate buffer of pH 6.9. To these solutions was added 1.5 ml. of 0.15% PCMB, which was sufficient to block 4.75 -SH groups in the protein used. After a period of 20 minutes had elapsed, 2.0 ml. of 4×10^{-3}M ferricyanide was added, followed by the appropriate amount of denaturant, and the volume was then brought to 25 ml. The reagent blank for these solutions contained everything but the BLG. The blanks and samples were incubated at 37° C. and at suitable time intervals aliquot portions were analyzed spectrophotometrically. The results of these blocked-sulfhydryl experiments are shown in Table IV. The results indicate that, with BLG, ferricyanide is quite specific for sulfhydryl groups in the presence of urea. However, when GHCl was used as denaturant the optical density decreased from the beginning, showing that ferricyanide was not specific.

These results explain in part the higher results for mercapto-group content found in the presence of GHCl, but the decrease found in the PCMB blocked samples after 30 minutes is not sufficient to

TABLE IV

EFFECT OF FERRICYANIDE ON THE BLOCKED -SH GROUPS OF BLG

Time of Denaturation at 37° C. in Minutes	Sample 1 (Urea) O.D. of Blank	O.D. of Sample	% Decrease in O. D. Blank	Sample	Sample 2 (GHCl) O.D. of Blank	O.D. of Sample	% Decrease in O. D. Blank	Sample
5	.310	.311	0	0	.320	.320	0	0
10	.312	.311	0	0	.320	.309	0	3.4
20	.316	.315	0	0	.320	.307	0	4.0
30	.314	.316	0	0	.320	.303	0	5.3
60	.314	.313	0	0	.320	.301	0	5.9
120	.306	.307	1.6	1.6				
150	.297	.299	4.2	3.9	.320	.288	0	10.0
300					.319	.273	0.3	14.7
345	.275	.273	11.3	12.2	.318	.268	0.6	16.2
540	.268	.265	13.6	14.8	.318	.236	0.6	25.8

explain the difference fully. Attempts to raise the pH of the GHCl samples only resulted in a much higher -SH titer.

Determinations using detergents as denaturants were not successful. A SDS-denatured BLG sample showed only a 20% decrease in absorbancy at 410 mμ after 24 hours incubation at 37° C. The DAC-denatured protein solution showed a slow but steady rise in optical density. Thus further work with the synthetic detergents was abandoned.

Electrometric Determination

Experimental Results

The sulfhydryl groups of BLG, like those of BSA, are not oxidized by ferricyanide in the native state. Thus all attempts to determine the thiol content are dependent on the ability of denaturants to make the -SH groups freely reactive.

Into a 100-ml. tall-form beaker was placed 50 mg. of BLG, 5 ml. of 3.5×10^{-2}M phosphate buffer of pH 6.9, 1 ml. of 4×10^{-3}M ferricyanide, and 12 gm. of GHCl, in that order. The final volume was diluted to 25 ml. with deionized water and placed in a 37° C. water bath for 20 minutes. Then the electrodes were placed in solution which was stirred by a glass stirrer rotating at 600 r.p.m. The buret tip was placed below the surface of the solution in the beaker, small increments of 4×10^{-3}M ferricyanide were added, and the increase in galvanometer deflections was noted. By extrapolation to the residual current line, it was found that the amount of ferricyanide consumed after 20 minutes was equivalent to 3.02 mole -SH per mole of protein. But the longer the solution was

incubated at 37° C., the more ferricyanide was consumed until, after 4 hours, there was no yellow color left. Thus it must be again concluded that ferricyanide is unspecific under these conditions.

It has been reported by several investigators that SDS binds BLG and does not serve to denature it. Groves, et al. (29) found that at a pH of 8.0 and 25° C. BLG was about 40% denatured after 250 hours, while the SDS derivative was denatured to a smaller extent under the same conditions. Nevertheless, a series of experiments were set up to show the effect of SDS and DAC on this protein. Into a 100 ml. tall-form beaker was placed 50 mg. of BLG, 5 ml. of 3.5×10^{-2}M phosphate buffer, 1.5 ml. of 4×10^{-3}M ferricyanide and 10 ml. of a 10% solution of the detergents. The final volume was diluted to 25 ml. In the case of SDS the current rose from its initial value of 22 to a final value of 74 after 7 hours, and continued to rise after that time. This would indicate some reduction of ferricyanide but the process of denaturation was too slow to follow electrometrically. In the case of DAC the results were opposite. After only 100 minutes of incubation at 37° C. the current went off the scale and it was not until 17 hours later that the current came back on the scale, slowly drifting downward. Unfortunately, this interesting phenomenon could not be investigated further, for lack of time.

SUMMARY AND CONCLUSIONS

The reaction of ferricyanide with bovine serum albumin and beta-lactoglobulin has been studied. Ferricyanide has been used successfully for the determination of mercapto groups in denatured ovalbumin and it was wished to see whether this reagent could be used with other proteins. The proteins were tested both in the native state and after denaturation with urea, guanidine hydrochloride, sodium lauryl sulfate and trimethyldodecylammonium chloride.

The reaction of ferricyanide with bovine serum albumin was investigated spectrophotometrically and electrometrically. Spectrophotometric measurements indicated no reaction with the native protein and a slow reaction in 5 M guanidine hydrochloride or 8 M urea. The reaction did not come to a definite end, and it appears that ferricyanide reacts with other groups in this protein besides sulfhydryl. Initial treatment with p-chloromercuribenzoate did not inhibit the reaction. Electrometrically, there was found 0.77 mole of sulfhydryl per mole of protein in some favorable cases. Reproducibility was not easy to obtain, however, and the significance of this result is uncertain.

Spectrophotometrically, urea-denatured beta-lactoglobulin samples were shown to react stoichiometrically with ferricyanide at pH 7.0. The amount of -SH groups found was 2.44 ± 0.10 per 40,000 g. of protein. Samples denatured with guanidine hydrochloride,

on the other hand, gave a -SH titer of 3.12±0.10 groups per mole of protein at pH 5.4. It was found that p-chloromercuribenzoate inhibits the reaction in urea, but only partly the reaction in guanidine hydrochloride. The unspecificity of ferricyanide in the presence of this denaturant may be responsible for the high results.

Synthetic detergents were very poor denaturants for both proteins.

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