AMPEROMETRIC DETERMINATION OF MERCAPTO GROUPS

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Submitted to the faculty of the Graduate School of
the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
DOCTOR OF PHILOSOPHY
May, 1959

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ACKNOWLEDGMENT

The author is extremely indebted to Dr. George Gorin under whose able guidance this work was conducted; to Dr. Otis C. Dermer, and Dr. Paul Arthur, for their critical reading of the manuscript and valuable suggestions; and to his wife sarla, for proof reading and encouragement throughout this study.

The author also wishes to thank the National Institute of Health, Division of Research Grants; The Air Force Office of Scientific Research, Aeromedical Division; and the Research Foundation of the Oklahoma State University for financial support in this research.

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GENERAL INTRODUCTION

Study of the mercapto groups in proteins and other biologically important substances has recently given very interesting and significant results. Not only do these groups show very different degrees of reactivity but their reactivity is further related to the state of denaturation of the protein. Barron and Hellerman (3, 19) have classified the thiol residues of proteins into three types: (I) freely reacting -SH groups which can readily react with nitroprusside, mild oxidizing agents like ferricyanide, or mercaptide-forming agents like iodoacetamide in the native protein, possibly because they are situated at the surface; (II) sluggish -SH groups which react slowly and/or incompletely with the above mentioned reagents, possibly because they are less accessible owing to structural or other reasons; and (III) masked -SH groups which are so well protected in the native protein that mercapto-group reagents do not attack them until the geometric configuration of the molecule has been altered, as in denaturation of the protein.

Many theories regarding the structure of proteins have been formulated in an attempt to explain the varied reactivity of the thiol groups. No definite conclusions can be drawn at present, but it is believed that an important step towards the solution of this problem would involve a thorough understanding of the phenomenon of denaturation. Further, a more detailed study of the reactivity of thiol groups would also aid in the interpretation of the function of such groups in various biochemical

reactions. Barron (3) has discussed in some detail the participation of thiol groups in enzyme reactions and other physiological processes such as cell division, growth, and mutations.

In order to understand these functions more completely and quantitatively, the precise analytical determination of thiol groups is necessary. Quite a few methods for this purpose are known, but their accuracy and applicability to proteins has not been definitely established. There is need to devise new and better methods for the determination of thiol groups at the low concentrations found in proteins and to test the applicability of methods already known.

The purpose of this investigation was twofold: (I) to devise a simple but accurate method for the determination of cysteine and cystine at low concentrations; and (II) to develop suitable methods for the estimation of mercapto groups in proteins. These objectives have been achieved by an adaptation of the "dead-stop" technique, a method of amperometric titration that utilizes polarized electrodes.

The investigation is divided into:

(I) a general study of the response of polarized electrodes;

(II) development of a "dead-stop" end point method for the determination of cysteine and cystine based on the results of (I);

(III) development of a "dead-stop" end point method for the determination of mercapto groups in ovalbumin, a protein which has been widely studied by other methods with respect to mercapto-group content.

PART I

Response of Polarized Metal Electrodes

CHAPTER I

REVIEW OF THE LITERATURE

The use of two polarized electrodes for detecting the end point in electrometric titrations was initiated by Foulk and Bawden (16) in 1926. Two platinum electrodes were dipped in the solution to be titrated, which contained iodine and iodide ion, and a small potential (15 mv) was applied to the electrodes. A current flowed, which was detected by a sensitive galvanometer connected in series with the electrodes. As thiosulfate was added and the iodine consumed, there was noted in the vicinity of the end point a very rapid decrease in the current; at the end point the current had decreased nearly to zero, at which point it remained as excess of thiosulfate was added. Because the end point was indicated by the stopping of the pointer of the galvanometer the method was called "dead stop".

The method can also be applied in the reverse way, that is, by adding standard iodine solution to thiosulfate. In this case, no current flows up to the end point and then the current increases as excess of iodine is added. Kolthoff (25) has called this a "kick off" end point, but it is still often, though erroneously, called "dead stop". The end point in such cases can be obtained by extrapolating the plot of current versus volume of reagent to zero current, and the accuracy of the method depends on how steep and how linear the plot is.

Foulk and Bawden considered that the fundamental condition for the production of the "dead stop" end point is the use of so low a potential

(10-15 mv) between the electrodes that the back electromotive force of polarization balances it and consequently no current flows. The "dead stop" end point occurs when there is a sharp transition from polarization of at least one electrode to complete depolarization of both of them (or vice versa) at the end of the reaction. They assumed that the polarization is due to oxygen adsorbed on the anode and hydrogen adsorbed on the cathode and that the anode can, therefore, be depolarized by a suitable reducing agent and the cathode by a suitable oxidizing agent.

Böttger and Forche (6) contradicted the explanation of Foulk and Bawden. They suggested that the 15 mv. potential applied by Foulk and Bawden was not sufficient for hydrogen formation on the cathode and also showed that when the iodine-thiosulfate reaction is carried out in divided cells, the electrode potential varies with the concentration.

A plausible explanation of the phenomenon was presented by Delahay, (14) who suggested that the "dead stop" end point depends on the substitution of a reversible redox couple for an irreversible redox couple, or vice versa, at the equivalence point. He explained the phenomenon occurring during a "dead stop" titration with the help of polarization curves. According to him, the titration of iodine by thiosulfate by the "dead stop" end point method is feasible because before the end point the solution contains iodine and iodide ion; since the system iodine-iodide ion is reversible a large current is observed, but, when iodine is removed from the system by the addition of thiosulfate, the current decreases rapidly. At the end point the iodine concentration is extremely low and the current is practically zero; after the end point, the current remains very small since the thiosulfate-tetrathionate couple is irreversible.

After the results of Böttger and Delahay, Stone and Scholten (48) presented additional evidence for the view that the "dead stop" phenomenon is not a polarization effect due to adsorption of gas on the electrode surface but is, at least in part, an electrochemical phenomenon based on oxidation at the anode and reduction at the cathode. During their experimental investigations of various systems they found that by applying a suitable potential and using appropriate electrodes the "dead stop" end point method could be used not only in systems containing only one reversible redox couple but in all cases in which the above mentioned electrolytic process (reduction at the cathode and oxidation at the anode) could be made possible.

Quantitative treatments of the phenomenon have been developed by various authors (8, 9, 22, 23); the most important of these papers describe a general theory of "dead stop" titrations, by Bradbury (8, 9), and the calculation of the intensity of current as a function of the concentration of reagent during a "dead stop" titration by Kies (22). These authors have verified their theoretical deductions experimentally. It has been ascertained that the equivalence point in these titrations is more accurately determined at low rather than at very high values of the potential difference between the electrodes, and that accurate titrations with 10^{-4} N reagents should be possible provided they form reversible redox systems in which the standard electrode potentials differ by more than 0.20 volts.

Waddill and Gorin (49) have shown that polarized platinum electrodes can be used to detect the ferro-ferricyanide couple, and utilized this finding in the titration of cysteine with the latter reagent. The possibility of adapting this method of analysis to proteins appeared to be

rather straight-forward. In order to ascertain what physical conditions would give the best results, a brief study was made of platinum electrodes in conjunction with a system that was reversible to them, i.e., iodine-iodide-ion.

It was also desirable to utilize as reagents for the determination of mercapto-groups metal ions such as silver, mercury (II), and copper (11), for the use of which in connection with the "dead-stop" technique there was little precedent. A brief review of this topic is presented on page 23 of this thesis.

CHAPTER II

EXPERIMENTAL STUDY OF ION ELECTRODE SYSTEMS

Chemicals

All the chemicals were of analytical reagent grade. The concentrations of the solutions used in this study were as follows:

Copper (II) sulfate	1.5 g per liter
Silver nitrate	3.3 g per liter
Sodium sulfite	6.0 g per liter
Sodium nitrite	1.88 g per liter
Tris(hydroxymethyl)aminomethane	0.1 M
Iodine	1.10 g per liter

Apparatus

The apparatus used is shown diagrammatically in Figure 1. It consisted of a pair of dry cells, a 100,000-ohm resistance, a 100,000-ohm decade resistance box, and a pointer galvanometer (sensitivity 0.10 μa per scale division), connected as shown.

The electrodes were pieces of platinum or silver wire, fused into the end of soft glass tubes inside which some mercury was placed to make connection with the rest of the circuit. The potential applied to the electrodes could be adjusted by varying the resistance in the decade box. A Sargent synchronous motor (Type KYC-22) was used to insure constant and uniform stirring of the solution.

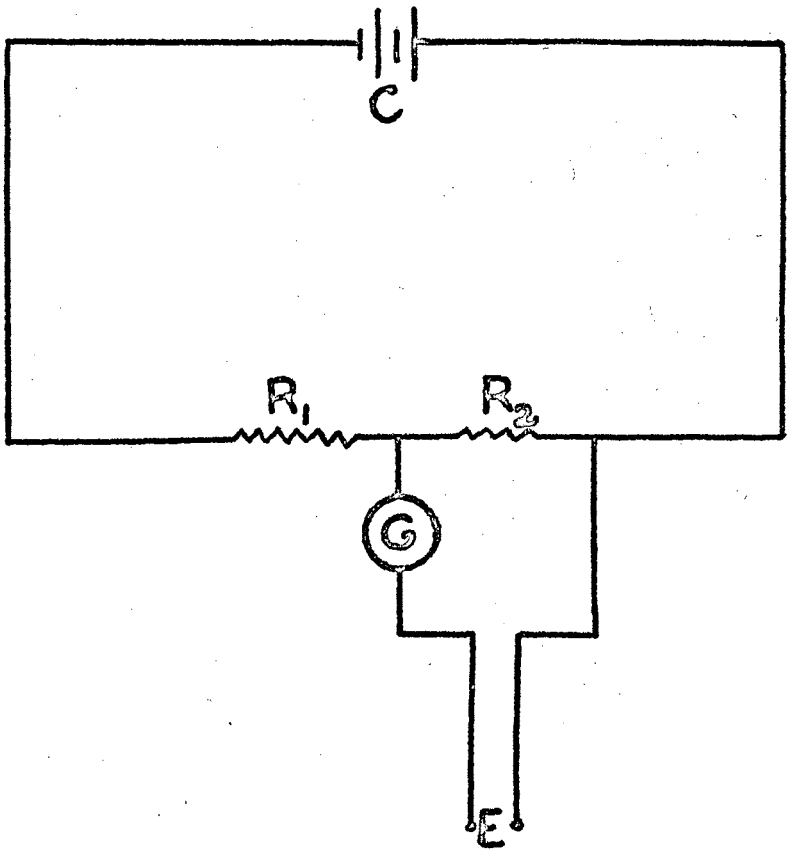

Figure 1

Dead-Stop End-Point Circuit

C = Two 1.5 Volt Dry Cells

R_1 = 100,000-Ohm Resistance

R_2 = 100,000-Ohm Decade Box

G = Galvanometer

E = Metal Electrodes

Method and Results

Effect of Size of the Electrodes and of Stirring Rate. In this study the response of platinum electrodes in a potassium iodide-iodine system was measured. Nitrogen was bubbled through a uniformly stirred potassium iodide solution for about ten minutes and then iodine solution was added gradually from a semimicro buret. The galvanometer readings when plotted against the volume of iodine solution added gave a linear graph up to a sufficient excess of iodine.

The results of varying the size of the electrodes, the distance between the electrodes, and the speed of stirring revealed that:

(I) thick wire electrodes were more sensitive than thin wire electrodes

(II) the longer the wire protruding from the glass, the more sensitive the electrodes; this effect was quite pronounced.

(III) more vigorous stirring and greater distance between the electrodes did not have any pronounced effect, though the former raised the slope of the plots slightly but made them less linear (probably owing to mechanical disturbances), while the latter lowered the slope slightly and made the graphs more linear. However, if the stirring was stopped altogether before taking each reading, the response of the electrodes was very low, showing that the current is diffusion-controlled.

The effect of increasing the potential difference between the electrodes was to increase the slope of the plots, as is predicted by theoretical considerations.

Platinum Electrodes and Silver Ions. In these experiments a potential of about 500 mv. was applied to the electrodes by appropriate adjustment of the decade resistance. The electrodes were dipped in distilled water, and approximately 0.01 M silver nitrate solution was added drop by drop. It was found that the platinum electrodes did not respond to the addition of silver nitrate. This result was in accordance with expectation, because, in the system under consideration, silver ions might be reduced at the cathode, but there is no reaction that can occur at the anode, and consequently no current flows through the solution and no response is obtained.

However, addition to this system of a substance capable of being oxidized at the anode at this potential should produce a response; such a substance might be called an anode depolarizer. Two anode depolarizers were tested in this study, sodium nitrite and sodium sulfite, and both gave fair response. In presence of sodium nitrite, the current increased almost linearly up to a considerable excess of the added silver nitrate. In presence of sodium sulfite the increase was not linear. These results are shown in Figure 2.

In order to study the possibility of carrying out titrations involving silver ions in presence of chloride ion, a coordinating agent was added to the solution which would prevent the precipitation of silver chloride. Two coordinating agents were tried in this study, tris-(hydroxymethyl)aminomethane (hereafter called tris), and ammonia-ammonium nitrate buffer; these reagents were used in presence of sodium sulfite as anode depolarizer. It was found that the addition of the former lowered the response by the pair of platinum electrodes and at the same time made the graphs less linear. On the other hand the addition of ammonia-ammonium

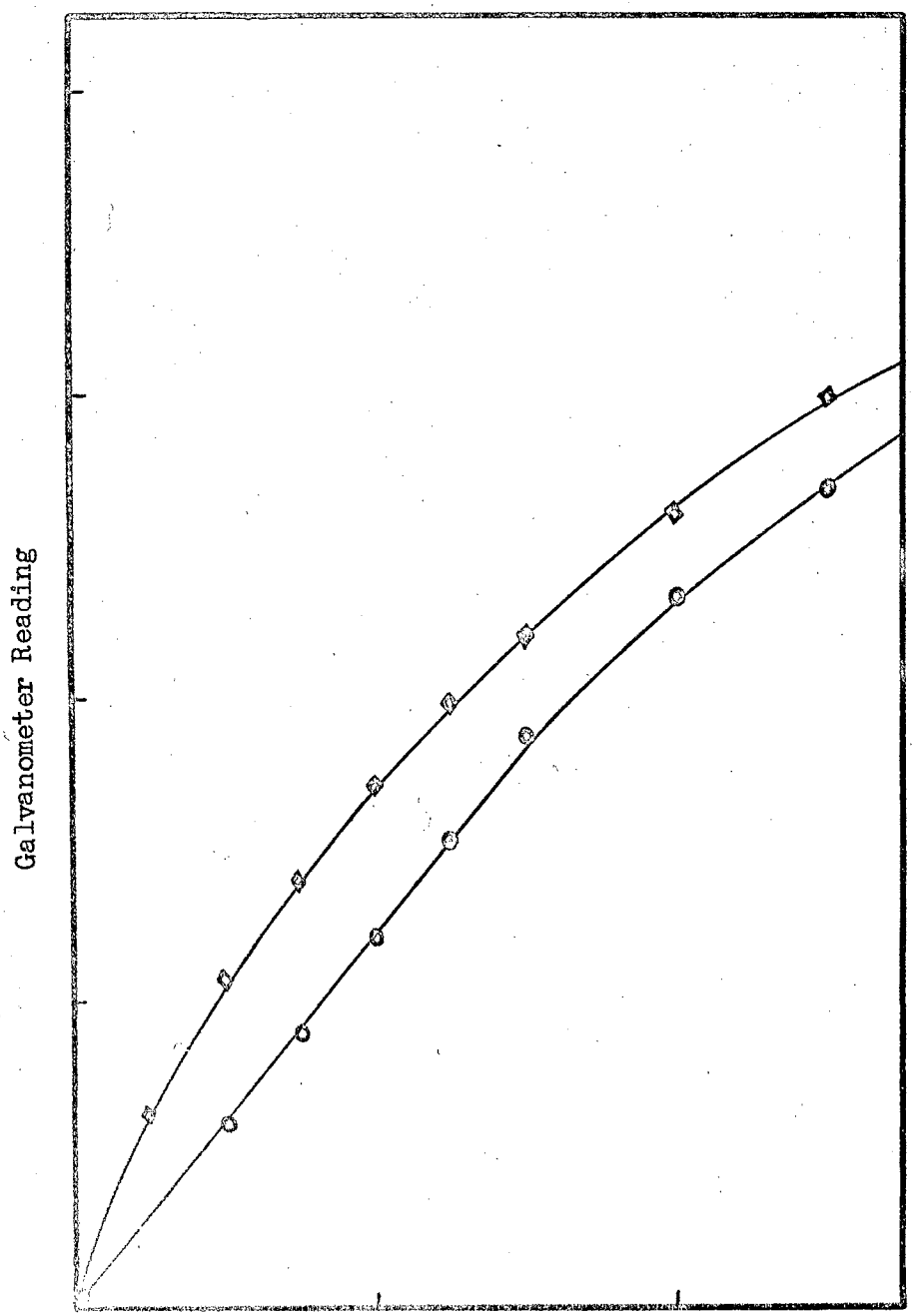

Figure 2

Response of Polarized Platinum Electrodes to Silver Ions

Lower Curve: Sodium Nitrite as anodic depolarizer

Upper Curve: Sodium Sulfite as anode depolarizer

nitrate buffer increased the response by the electrodes tremendously (this response was even more than in case of sulfite alone). The response was still non-linear, however. These observations are difficult to understand, since silver ions are strongly complexed by ammonia and the ammonia-ammonium nitrate buffer should, therefore, decrease the response of the electrodes rather than increase it. Whatever may be the cause, the results are very useful from the practical point of view as they suggest how the end point might be found in titrations involving silver ions in presence of chloride ions.

Silver Electrodes and Silver Ions. When a pair of silver electrodes was used in place of platinum electrodes, the addition of silver ions to the solution produced a large response even at a low potential of 100 mv. Moreover the graphs were linear up to a considerable excess of added silver ions.

Silver-Platinum Electrodes and Silver Ions. When a platinum anode was replaced by a silver wire anode, linear graphs could be obtained even by using sodium sulfite as an anode depolarizer as seen in Figure 3. The reverse combination, silver cathode and platinum anode, on the other hand, did not change the non-linear nature of the graphs. Apparently the electrochemical action at the anode is responsible for the non-linear increase in current observed with two platinum electrodes. It may be that the oxidation of sulfite is irreversible at a platinum anode but more nearly reversible at a silver anode, or it may be that the oxidation of silver to silver ions takes place instead of the oxidation of sulfite when a silver anode is used.

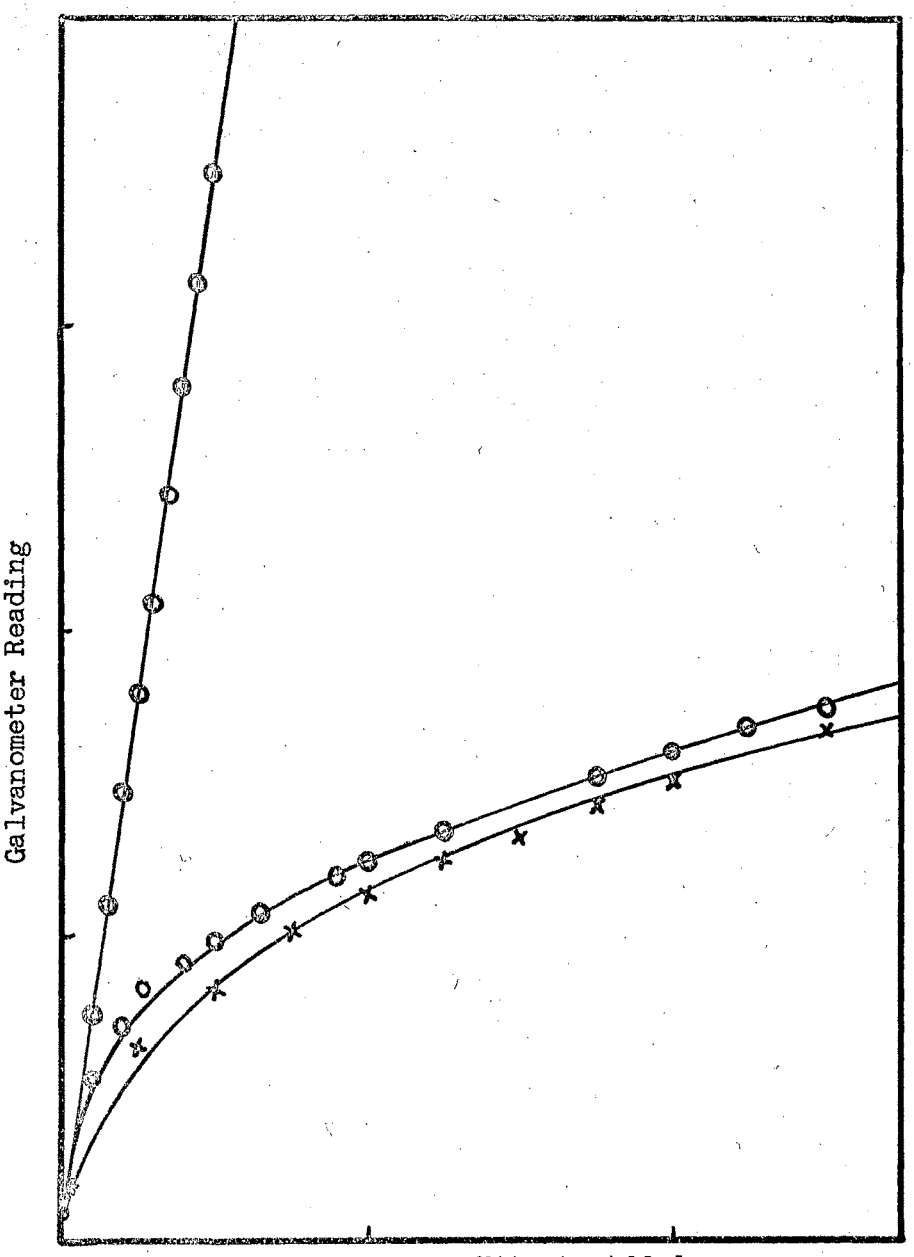

Figure 3

Response of Dissimilar Polarized Electrodes to Silver Ions in Presence of Tris and Sulfite

Lowest Curve: Two Platinum Electrodes

Middle Curve: Silver Cathode, Platinum Anode

Highest Curve: Silver Anode, Platinum Cathode

Platinum Electrodes and Copper (II) Ions. Copper (II) sulfate was used as a source of copper (II) ions. The response in presence of tris as coordinating agent and sodium sulfite as a depolarizer was very poor even at potentials of 250-500 mv. Also, the graphs were non-linear and did not permit easy and exact extrapolation to locate the end point. Some improvement was achieved by employing ammonia-ammonium nitrate buffer instead of tris.

However, it was found that the combination of silver anode and platinum cathode gave good results, especially when a small quantity of copper (I) ion was added to the solution to form a cupric-cuprous ion couple. The response was further improved in the presence of ammonia-ammonium nitrate buffer and the plots were quite linear.

Platinum Electrodes and Mercury (II) Ions. Mercury (II) chloride was used as a source of these ions, with sodium sulfite as an anode depolarizer. No response could however be obtained in this case up to a potential of 500 mv. On the other hand, current flowed easily when a small mercury pool was employed as a cathode. It has been reported that mercury (II) ions form a strong complex with sulfite and it appears that the mercury-sulfite complex is not easily reduced at the platinum cathode.

Conclusions

The observations reported above indicate that by employing a suitable pair of metal electrodes and an anode depolarizer, one can detect the presence of silver, copper (II), and mercury (II) ions, (presumably, other ions as well) by the flow of a current which increases linearly with increasing concentration of those ions up to a considerable excess.

Further, the versatility of the method is enhanced by the fact that its sensitivity can be adjusted by varying such factors as the potential applied to the electrodes, the size of the electrodes, and the sensitivity of the galvanometer.

PART II

DETERMINATION OF CYSTEINE AND CYSTINE

CHAPTER III

INTRODUCTION

Several methods for the determination of cysteine and thiol groups are known. All but the most recent ones are discussed quite completely by Block and Bolling (5) and in a more critical manner by Chinard and Hellerman (10). These methods can be classified according to the type of reaction undergone by the thiol group, namely oxidation, thioether formation, and mercaptide formation.

Oxidation methods have been widely used for the determination of cysteine in the past. The general reaction in this case can be represented by the following equation:

$$2\ R\text{-}SH + \text{Oxidizing reagent} \longrightarrow R\text{-}SS\text{-}R + \text{reduced reagent}$$

Many oxidizing agents have been suggested for this purpose, but excepting cystine, which possesses other disadvantages, their specificity for the thiol groups is open to question; most of them are found to react with tyrosine or tryptophan if the conditions are not carefully controlled. Iodine has been commonly used in the past, but it is capable of oxidizing cysteine beyond the disulfide stage, and gives high results unless special precautions are taken. Lavine (32) and Pilmer (43) have suggested procedures which would avoid this overoxidation, but the accuracy of these methods has not been satisfactorily established.

It has been claimed that porphyrindin is specific under specified

conditions (17), but this has been questioned (2, 30). At any rate, control of conditions is essential, and, furthermore the reagent is expensive and difficult to standardize because of its instability.

The use of ferricyanide for the assay of cysteine samples was first suggested by Mason (37). Anson (1) carried out a quantitative study of the reaction between cysteine and ferricyanide and found it to be specific and stoichiometrically exact at pH 6.8. Both he and Mirsky (1,40) employed an excess of ferricyanide and determined the amount of ferrocyanide produced colorimetrically by adding ferric sulfate to form Prussian blue. Waddill and Gorin (49) utilized the reaction between cysteine and ferricyanide in a sensitive and precise method that employs two polarized platinum electrodes.

Hellerman et al (19) have employed o-iodosobenzoate ion as a sulfhydryl reagent and claim that its action is specific at pH 7. According to their method an excess of standard reagent added to the cysteine sample is subsequently reduced by iodide, and the iodine liberated is titrated with standard thiosulfate.

Thioether formation is exemplified by the use of iodoacetate and its amide. The reaction can be represented as follows:

$$R\text{-}SH + ICH_2COOH \longrightarrow R\text{-}S\text{-}CH_2COOH + HI$$

The reaction, however, is not specific.

Of the mercaptide-forming reagents, p-chloromercuribenzoate ion (1) has been found to be most specific. In some cases it has been shown to react with mercapto groups not attacked by mild oxidizing agents. Its reaction with mercapto groups is reversible and this permits its use in the study of mercapto-enzymes as well as of denatured proteins. The

course of reaction may be represented by the equation:

$$R\text{-}SH + ClHg\text{-}R\text{-}COOH \longrightarrow R\text{-}S\text{-}Hg\text{-}R\text{-}COOH + HCl$$

Metal ions that form stable mercaptides have recently gained importance as methods have been developed for determining the end point amperometrically. The determination of cysteine and thiol groups with silver, copper (II), and mercury (II) ions has been described by Kolthoff and coworkers (27,28,29). These methods are quite sensitive but employ a rotating platinum electrode, a suitable reference electrode, and a salt bridge, an assembly that is more complicated and cumbersome than would be needed with polarized electrodes.

In this investigation, a method that utilizes polarized electrodes has been developed; this makes it possible to dispense with the reference electrode and the salt bridge.

CHAPTER IV

A METHOD FOR THE DETERMINATION OF CYSTEINE AND CYSTINE

A method for the determination of cysteine and cystine by titration with polarized electrodes has been developed, and has been described in a paper intended for publication. This paper is transcribed here as a part of this thesis, with the exception that the authors' names and summary have been omitted and that the references have been included with the rest at the end of the thesis, and numbered appropriately. Since the style required for publication is more concise than is desirable in a thesis, a more detailed description of some experimental details, as well as of related experiments, will be given in the subsequent chapter.

Determination of Cysteine and Cystine by Amperometric Titration with Copper (II) Ions Using a Pair of Dissimilar Polarized Electrodes

Cysteine and cystine are substances of great importance in biochemistry; accordingly, there is continuing interest in new and better methods for their determinations. Methods that involve titration with certain metal ions to an amperometric end point have been introduced in the past few years and have met with rather wide acceptance, which attests to substantial advantages of accuracy and convenience at the levels of concentration commonly encountered. These titrations have been carried out with a non-polarizable anode and a rotating platinum or dropping mercury cathode. Silver ions have been used most extensively (27), and

copper (II) (28) or mercury (II) (29) ions to some extent.

This paper demonstrates that in the titration with copper (II) ions the end point can be detected with a pair of dissimilar metal electrodes, a silver anode and a platinum cathode, polarized with a potential of about 50 mv. From the practical point of view, use of the polarized metal electrodes simplifies the apparatus required in many respects and renders the procedure more convenient. Furthermore, the use of such an electrode system to detect metal ions is of potential utility in many other connections; although similar applications have occasionally been made, it does not seem that the potentialities of the technique have been adequately exploited up to now.

Some of the principles and experimental results which led to the choice of electrodes employed in this method will be discussed first; then application to the determination of cysteine and cystine will be described.

Principle of the Method and Choice of the Electrode System

Most applications of polarized electrodes made to date have involved a pair of similar inert electrodes (platinum) and a redox couple that is reversible to them. A small potential is applied to the electrodes and when the solution contains both members of the redox couple, oxidation of the reduced form takes place at the anode and reduction of the oxidized form at the cathode with a consequent flow of current that is indicated by a rather sensitive galvanometer. In an analytical procedure, one of the members of the redox couple does not accumulate to an appreciable extent either before or after the end point, which is consequently marked by the cessation or onset of current flow. For a more detailed and quantitative discussion of the general principles of the method, reference might be made to a paper by Kolthoff (25) or a book by Delahay (15).

On the basis of these considerations it would seem possible to detect metal ions with a pair of polarized electrodes made of the reduced metallic form of that ion. At the cathode the metal ion would be reduced to metal and deposited; at the anode the metal would ionize and go into solution, and a current would flow as a result. Some use of metallic electrodes of this kind has been made, notably by Masten and Stone (38) in the argentimetric determination of halide mixtures, and by Kies (24) in the titration of various anions with mercury (I). In both of these cases, however, the reaction occurring at the anode does not appear to be a simple oxidation of the metallic electrode, and current is observed to flow both before and after the end point; however, there was a more or less sharp minimum at the end point, which can thus be located.

Another type of application of polarized electrodes to titrations with metal ions was made by Foulk and Clippinger (12) in the determination of halides with silver ions; in this case a reducing agent, nitrite ion, is added to the solution as an "anode depolarizer," and a current begins to flow as excess silver ions are added after the end point. The electrode reactions presumably are oxidation of nitrite at the anode and deposition of silver at the cathode.

The titration of cystine or cysteine with copper (II) ions by the method of Kolthoff and Stricks (28) is carried out in the presence of excess sulfite ion; it seemed likely that this ion also could act as an anode depolarizer, making it possible for a current to flow when an excess of copper (II) ions was added after the end point. This is found to be the case, but with a pair of platinum electrodes the current does not increase quite linearly with the amount of excess reagent added; a plot typical of those obtained is shown in Figure 4. Because of the slight

curvature in the vicinity of the end point, this cannot be located accurately by extrapolation. Varying the potential applied to the electrodes changes the magnitude of the current but not the shape of the plots.

The results of the exploratory investigation upon the response of polarized metal electrodes to copper (II) ions, reported on page 15 of this thesis, suggested that a combination of silver anode and platinum cathode should give a better and more linear response in this titration than a pair of platinum electrodes; this expectation was realized. A typical plot is shown in Figure 4. It can be seen that extrapolation can be carried out with accuracy as the plot is quite linear up to a sufficient excess of copper (II) ions.

It is especially noteworthy that with the electrode system just described a current flows in the presence of excess copper (II) ions even if no outside potential is applied. The current which flows is, however, small and a correspondingly greater uncertainty would attach to the extrapolation by which the end point is determined. While a more sensitive galvanometer might be used, it has seemed preferable to retain a more rugged and somewhat cheaper instrument described below, and to apply a potential of 50 mv. to the electrodes. This value should be generally useful. It should be remembered, however, that this potential might be varied by a considerable amount as may be required to give convenient galvanometer readings in systems of different characteristics.

Apparatus

The apparatus has been described previously (49). The silver electrode is about 1.0 cm. long, made by sealing 22-gauge silver wire in a soft glass tube, and melting some De Khotinsky cement on the inside of the joint to

make it water-tight. A 5-ml. semimicro buret with 0.01-ml. divisions is used for determining quantities of cysteine down to 1.5 mg., and a 0.10-ml. Kirk ultramicroburet for determining smaller amounts.

Reagents

All reagents were of A. C. S. Reagent grade unless otherwise specified.

Buffer-Electrolyte Solution was prepared from 8.0 g. ammonium nitrate, 7.0 ml. concentrated ammonium hydroxide, and enough water to make 1.00 l.

Copper (II) Sulfate Solution was prepared from accurately weighed amounts of cupric sulfate pentahydrate. The titer of the solution was checked electrogravimetrically.

L-Cysteine Hydrochloride Hydrate (purified grade) was obtained from the California Foundation for Biochemical Research, Los Angeles 63. Stock solutions for analysis were prepared by dissolving accurately weighed quantities in 0.1 M hydrochloric acid made with deionized, deaerated water, and were kept in stoppered containers under nitrogen for no longer than six hours. It was found convenient to distribute the solution immediately after mixing into a number of small vials, which, after being opened to the air for withdrawal of a sample, could be discarded.

L-Cystine was obtained from Schwarz Laboratories, Mount Vernon, New York (C. P. grade, $(\alpha)^{D}_{25} = 208^{\circ}$ (c = 1 in 1 M HCl). Some cystine was purified by recrystallization. Standard solutions were prepared by dissolving accurately weighed quantities in 5 ml. of 1 M hydrochloric acid and diluting to the required volume. The solutions were kept in the dark and the titer was stable for several days.

Water used in preparing all solutions was deionized and deaerated as described previously (49).

Nitrogen was of commercial grade, water- or oil-pumped. In most experiments it was used without further purification, it having been established that no significant difference was made by passing the gas through a solution of vanadous ion to remove oxygen; however, this purification was applied when the amount of cysteine or cystine present in solution was less than 2 mg.

Procedure

Between ten and fifty milliliters of buffer-electrolyte solution and an equal volume of 0.25 to 0.3 M sodium sulfite are placed in the titration beaker. Nitrogen is passed through a wash bottle containing some of the titration medium and then is bubbled through the sample until the galvanometer reading attains a constant value. The nitrogen bubbling tube is raised above the surface of the solution, and a stream of gas is played over it throughout the titration. Through a hole in the stopper are added 2.0 ml. of 0.001 M cadmium sulfate or patassium iodide solution and sufficient sample to give preferably a concentration between 5×10^{-4} and 5×10^{-5} M in cysteine or cystine. The buret tip is then placed below the surface of the solution, and the copper sulfate solution is added with stirring. The addition must be rather slow, (at a rate that does not cause too much fluctuation of the galvanometer). After the galvanometer readings have begun to rise, three or four very small portions of the reagent are added in excess, and the galvanometer reading is recorded after each addition. Because of the time required for adding the reagent, it is convenient to use relatively concentrated solutions

(0.02 to 0.05 M) and correspondingly small volumes of reagent.

Determination of Cysteine

The reaction of cupric ion with cysteine varies depending on the conditions. In the conditions employed by Harris (18), copper (II) oxidizes one mole of cysteine to cystine and the copper (I) combines with another mole of cysteine to give a precipitate of copper (I) cysteinate:

$$2\ Cu^{++} + 4\ RS^{-} \longrightarrow RSSR + 2\ RSCu \qquad (i)$$

The cysteine is represented as an anion, RS^{-}, in which the thiol group has been ionized; this may not represent the actual state of ionization of the group at the beginning of the reaction, but this is of no importance in the presence of excess buffer mixture, which is more than enough to neutralize the hydrogen ion in question. RSSR represents cystine.

As Clark (11) first showed, sulfite ion reacts with cystine according to equation (ii):

$$RSSR + SO_3^{=} \longrightarrow RS^{-} + RSSO_3^{-} \qquad (ii)$$

Accordingly, the reaction of copper (II) ion with cysteine in the presence of sulfite should be expected to consume more metal ion; combination of equations (i) and (ii) gives:

$$2Cu^{++} + 3RS^{-} + SO_3^{=} \longrightarrow 2RSCu + RSSO_3^{-} \qquad (iii)$$

i.e. a stoichiometry of two-thirds of a gram-ion of copper (II) per one mole of cysteine. Results approximating this stoichiometry were observed when the reaction was carried out in high ammonia (0.5 M) and low sulfite (0.05 M) concentrations.

In 0.05 M ammonia and 0.1 to 0.15 M sulfite, however, the stoichiometry is two gram-ions of copper (II) per mole of cysteine. Kolthoff and

Stricks (28) have suggested that under these conditions there is further reaction between the copper (I) cysteinate and copper (II) ion; the overall equation is given as (iv):

$$2\ Cu^{++} + RS^{-} + SO_3^{=} \longrightarrow 2\ Cu^{+} + RSSO_3^{-} \qquad \text{(iv)}$$

While the stoichiometry does not vary within the range of sulfite concentrations indicated, higher concentrations of sulfite give high results if the concentration of ammonia is not increased correspondingly. This may be due to reduction of copper (II) ion by sulfite, and/or to formation of complex ions.

Attempts to determine an amount of cysteine smaller than 1 mg. gave consistently low results; use of the method is limited to amounts greater than this. Kolthoff and Stricks (28) were able to determine by their amperometric method amounts so small as 0.1 mg. of cysteine, as long as they took adequate precautions against oxidation by oxygen dissolved in the reagent solution. Although oxidation is no doubt a contributing factor to the low results obtained in this work, no systematic improvement was obtained by using an ultramicroburet and much smaller volumes of reagent solution; it is therefore believed that amounts smaller than the limit specified cannot be determined satisfactorily by the method described here.

Commercial samples of cysteine are not analytically pure; indeed, the preparation of pure samples is a very difficult task because of the properties of the substance, particularly its susceptibility to oxidation. In this work a commercial sample of cysteine was used without further purification. The precision of the method is demonstrated by the consistency of the results, calculated as percentage of the theoretical amount of cysteine for aliquots of different size taken from the same sample.

Table I reports results obtained by taking known volumes of standard stock solutions, 0.01-0.04 M; one set of determinations is given in detail and others are summarized. The precision is seen to be of the order of 1%.

The value found for the percentage purity of the sample is a reasonable one, but the accuracy of the method can only be ascertained by comparison with the results of other methods. Table II reports the results obtained by the present method, by the ferricyanide titration method of Waddill and Gorin (49), and by the method of Kolthoff and Stricks (28). It is seen that they agree within experimental error. The results obtained by copper (II) titration are consistently lower than those obtained with ferricyanide. It may be that the end point in the copper method comes about 1% too soon. However, the discrepancy is no larger than the precision to be expected of the method, and therefore hardly significant.

Other amino acids, with the exception of cystine which will be discussed below, do not interfere; aspartic acid, tyrosine, leucine, tryptophan, serine, lysine, and methionine were tested. In the presence of certain amino acids, however, the slope of the current-versus-volume of reagent line is substantially lowered, and the accuracy of locating the end-point by extrapolation is adversely affected; this might be counteracted by increasing the potential applied to the electrodes.

Determination of Cystine

According to stoichiometric relationships already discussed, cystine should react with copper (II) ion in the presence of sulfite according to equation (v):

$$RSSR + 2SO_3^{=} + 2Cu^{++} \longrightarrow 2Cu^{+} + 2RSSO_3^{-} \qquad (v)$$

TABLE I

DETERMINATION OF CYSTEINE IN A COMMERCIAL SAMPLE

Stock Solution	Approximate Amounts of Cysteine taken mg.	% Cysteine Found	Deviation From Mean %
I	8.0	96.4	0.2
		96.2	0.4
	3.7	96.8	0.2
		96.9	0.3
	2.0	97.0	0.4
		95.8	0.8
	0.8	97.0	0.4
		97.0	0.4
	Mean	96.6	Average Deviation 0.4
			Standard Deviation 0.48

	Number of Determinations			Average Deviations
II	5	3.5-0.9	96.6	0.3
III	7	8.0-2.0	96.5	0.4
IV	7	5.0-0.9	96.7	0.5
V	3	7.5	96.4	0.3
VI	6	4.9-1.2	95.7	0.6
VII	7	6.0-2.0	96.1	0.3

TABLE II

DETERMINATION OF CYSTEINE IN A COMMERCIAL SAMPLE
COMPARISON OF METHODS

	Number of Determinations	% Cysteine
Present Method	43	96.4
Waddill and Gorin (49)	10	97.5
Kolthoff and Stricks (28)	6	96.6

TABLE III

DETERMINATION OF CYSTINE IN A COMMERCIAL SAMPLE
PRESENT METHOD

Stock Solution		Amount of Cystine mg	% Cystine Found mg	Deviation From Mean %
I		7.5	96.2	0.7
		4.9	96.9	0.4
		2.5	97.2	0.7
		1.2	95.5	1.0
			Mean 96.5	Average Deviation 0.70
			Standard Deviation = 0.75	
	Number of Determinations			Average Deviations
II	3	3.4-1.1	96.5	0.6
III	13	5.4-1.3	95.5	0.9
		Kolthoff and Stricks Method		
III	13	5.4-1.3	96.5	0.6

Kolthoff and Stricks (28) analyzed a sample of cystine in this way by their amperometric method with good results.

Table III reports results obtained with a commercial sample of cystine by the present method and by the method of Kolthoff and Stricks (28); the results are seen to be in agreement with each other within the limit of experimental error, and the precision is within 1%. However, the absolute percentage of cystine found appeared to be rather low, since cystine is a stable substance and high purity is not too difficult to obtain.

In order to check the absolute accuracy of the method, a sample of cystine was purified by crystallization to constant titer. The percentage

cystine found increased to 98.5% after three crystallizations, but did not increase further after two more crystallizations. The discrepancy is only a little greater than experimental error, but is considered to be significant; in other words, the method, as applied to cystine, gives results 1 to 2% low. A small empirical correction might be applied to the results but the method, without correction, should be sufficiently accurate for most applications of practical importance. Where greater accuracy is desired, this method is not recommended.

When both cysteine and cystine occur in a sample, the method will measure the total molar concentrations of both, but not the relative concentration of either. This can be obtained by determining cysteine separately by the ferricyanide titration method (49), or by reducing the cystine to cysteine with sodium amalgam, as described by Kolthoff and Stricks (27).

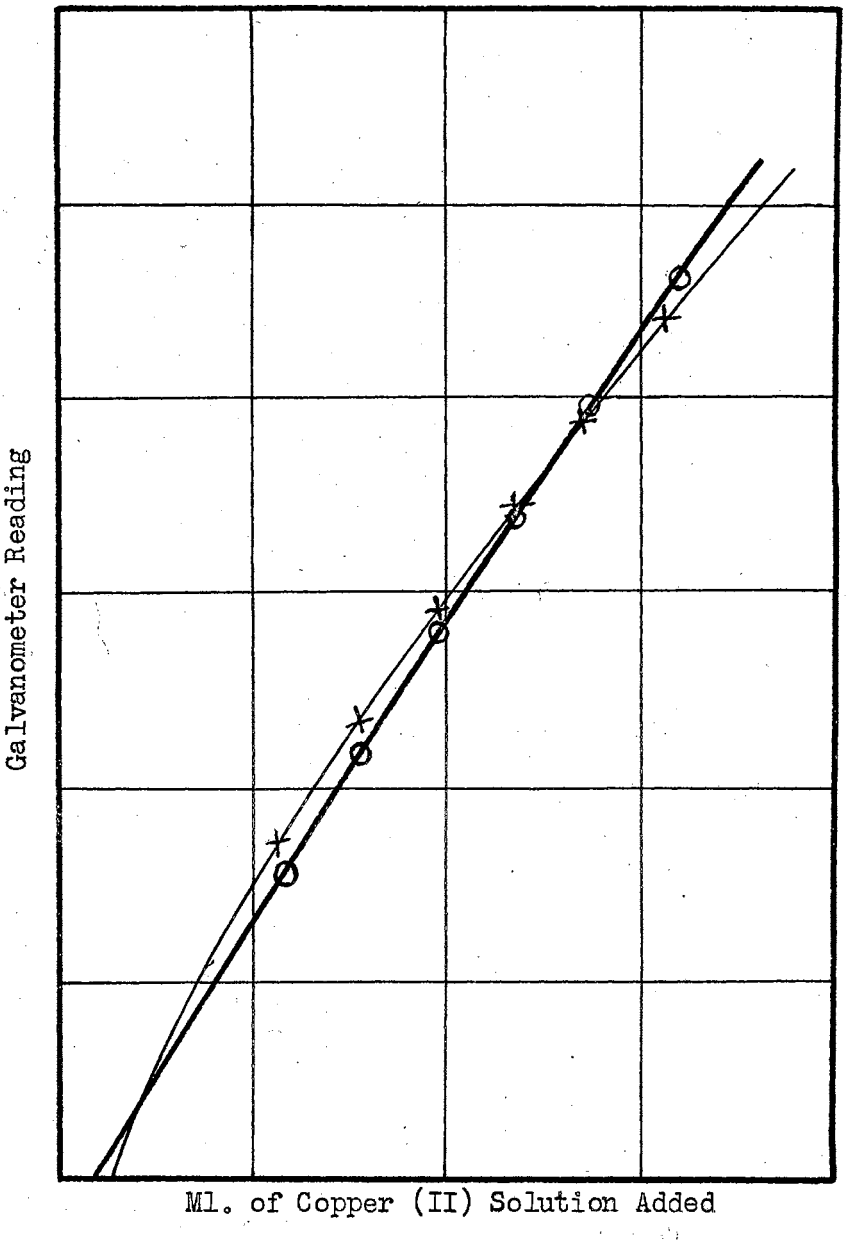

Figure 4

Titration of Cysteine With Copper (II) Ions Using

X Two Platinum Electrodes

O Silver Anode and Platinum Cathode

CHAPTER V

EXPANDED EXPERIMENTAL SECTION AND RELATED EXPERIMENTS

Detailed Analysis of Cysteine Samples

Preparation and Handling of the Samples: Dilute solutions of cysteine are unstable and suffer a rapid loss in titer on standing at room temperature. Therefore, special precautions had to be taken in the preparation and storage of stock solutions of this material. The concentration of the solutions used in this study varied from 0.01 M to 0.04 M and these were prepared by dissolving an accurately weighed quantity of cysteine hydrochloride hydrate in 0.05 to 0.1 M hydrochloric acid made with deionized, air-free distilled water. The addition of hydrochloric acid is recommended as it was found that the cysteine solutions prepared in hydrochloric acid were much more stable than those prepared in water alone. After preparation the stock solutions were immediately transferred into a number of air-free 5-ml. vials, which were stored in a cool, dark place. Each vial was discarded after being opened to the air for withdrawal of a sample. Stock solutions prepared and stored in this manner gave a practically constant titer when used within six hours of their preparation.

Detailed Results: Sample I and sample II were commercial cysteine hydrochloride hydrate (purified grade, green label) obtained from the

California Foundation for Biochemical Research. Detailed analytical results for them are shown in Tables IV and V; each set of determinations was done on a different solution.

TABLE IV

CYSTEINE DETERMINATION AT VARIABLE CONCENTRATIONS OF SAMPLE I BY TITRATION WITH 0.02 TO 0.1 M COPPER SULFATE IN PRESENCE OF 0.15 M SULFITE AND 0.05 M AMMONIA-AMMONIUM NITRATE BUFFER USING SILVER ANODE AND PLATINUM CATHODE

Amount of Cysteine Taken (mg)	Amount of Cysteine Found (mg)		% Cysteine Found		Deviation from Mean
3.592	3.465		96.5		0.1
3.592	3.462		96.4		0.0
1.796	1.750		97.4		1.0
1.697	1.640		96.6		0.2
0.898	0.863		96.1		0.3
6.015	5.788		96.2		0.2
6.015	5.806		96.5		0.1
4.010	3.865		96.4		0.0
4.010	3.857		96.2		0.2
2.005	1.923		95.9		0.5
2.005	1.927		96.1		0.3
1.907	1.823		95.6		0.8
12.94	12.450		96.2		0.2
8.63	8.396		97.3		0.9
4.32	4.195		97.2		0.8
2.16	2.080		96.4		0.0
		Mean	96.4	Mean Deviation	0.4

TABLE V

CYSTEINE DETERMINATION AT VARIABLE CONCENTRATIONS OF SAMPLE II BY TITRATION WITH 0.02 TO 0.1 M COPPER SULFATE IN PRESENCE OF 0.15 M SULFITE AND 0.05 M AMMONIA-AMMONIUM NITRATE BUFFER USING SILVER ANODE AND PLATINUM CATHODE

Amount of Cysteine Taken (mg)	Amount of Cysteine Found (mg)	% Cysteine Found	Deviation from Mean
8.04	7.82	97.2	0.9
6.04	5.84	96.7	0.4
6.04	5.81	96.2	0.1
4.02	3.89	96.8	0.5
4.02	3.88	96.5	0.2
2.01	1.92	95.5	0.8
2.01	1.94	96.4	0.1
5.04	4.91	97.4	1.1
4.03	3.91	96.8	0.5
4.03	3.89	96.6	0.3
3.02	2.92	96.7	0.4
2.02	1.96	97.1	0.8
2.02	1.94	96.4	0.1
0.96	0.92	95.6	0.7
4.90	4.72	96.4	0.1
3.68	3.53	96.1	0.2
2.45	2.34	95.5	0.8
2.45	2.34	95.5	0.8
1.23	1.17	95.4	0.9
1.23	1.17	95.4	0.9
	Mean	96.3	Mean Deviation 0.6

Analysis of Small Amounts of Cysteine: For analysis of solutions containing less than 1.5 mg. of cysteine a 0.10 ml. Kirk ultramicroburet and a concentrated, approximately 0.1 M copper (II) sulfate solution were used. The titration mixture was prepared by mixing together 10 to 15 ml. of the phosphate buffer, an equal volume of 0.25 to 0.30 M sodium sulfite, and 1 to 2 ml. of 0.001 M cadmium ion. The mixture was de-aerated with nitrogen which had been passed through a series of wash bottles containing vanadous ion, distilled water, and the titration medium respectively. The titration was then completed as already described in Chapter IV.

It was found that the results obtained were low in samples containing less than 1 mg. of cysteine; this is exemplified in Table VI.

TABLE VI

TITRATION OF SMALL AMOUNTS OF CYSTEINE
WITH 0.1 M COPPER (II) SULFATE
USING MICROBURET

Amount of Cysteine Taken (mg)	Amount of Cysteine Found (mg)	% Cysteine Found
0.554	0.527	95.1
0.660	0.626	94.9
0.330	0.307	93.0
0.250	0.236	94.5
0.359	0.334	93.0
	Average	94.1 %

Detailed Analysis of Cystine Samples

Purification of Cystine: A 5-g. sample of cystine obtained from Schwarz Laboratories was dissolved in 90 ml. of approximately 1.5 M hydrochloric acid. The solution was filtered through a Whatman filter paper No. 1 and titrated with approximately 0.6 M ammonia to a pH of 5. The separated cystine was filtered off and thoroughly washed with hot distilled water till the washings were free of chloride and ammonium ions. The material was dried overnight in an oven at 45°C and then allowed to stand in contact with air for a few hours to bring it in equilibrium with atmospheric moisture. The same procedure was employed for recrystallization. Table VII shows the results of analysis of the commercial sample, while Table VIII summarizes the analysis of the purified samples obtained after two, three, and five crystallizations respectively.

TABLE VII

DETERMINATION OF CYSTINE IN COMMERCIAL SAMPLE 5501
BY 0.02 TO 0.1 M COPPER (II) SULFATE

Stock Soln.	Amount of Cystine Taken (mg)	Amount of Cystine Found (mg)	% Cystine Found	Mean Deviation
II	1.13	1.08	95.7	0.7
	3.40	3.29	96.8	0.4
	3.40	3.30	97.1	0.7
III	5.39	5.25	97.5	1.1
	5.39	5.26	97.8	1.4
	2.69	2.60	96.5	0.1
	2.69	2.57	95.6	0.8

TABLE VII (Con't)

Stock Soln.	Amount of Cystine Taken (mg)	Amount of Cystine Found (mg)	% Cystine Found	Mean Deviation
	3.77	3.60	95.4	1.0
	3.77	3.59	95.3	1.1
	3.23	3.15	97.5	1.1
	3.23	3.16	97.8	1.4
III Con't	1.35	1.29	95.6	0.8
	4.31	4.14	96.0	0.4
	4.31	4.15	96.2	0.2
	2.16	2.06	95.5	0.9
	2.16	2.06	95.5	0.9
	7.45	7.24	97.1	0.7
IV	7.45	7.17	96.2	0.8
		Mean	96.4	Mean Deviation 0.8

TABLE VIII

TITRATION OF PURIFIED CYSTINE SAMPLES WITH 0.02 TO 0.1 M COPPER (II) SULFATE IN PRESENCE OF 0.15 M SULFITE

No. of Crystallizations	Amount of Cystine Taken (mg)	Amount of Cystine Found (mg)	% Cystine Found
	7.00	6.81	97.2
2	3.50	3.40	97.1
	1.75	1.69	96.8
		Mean	97.0
	5.00	4.93	98.6
3	2.50	2.46	98.5
	1.25	1.23	98.2
		Mean	98.4

TABLE VIII (Con't)

No. of Crystallizations	Amount of Cystine Taken (mg)	Amount of Cystine Found (mg)	% Cystine Found
5	4.20	4.15	98.7
	2.10	2.07	98.4
		Mean	98.5

Effect of Increasing Sulfite Concentration

As already pointed out, the stoichiometry of the reaction between copper (II) ion and cysteine is two ions of copper (II) per mole of cysteine in 0.05 M ammonia and 0.1 to 0.15 M sulfite solution. The effect of increasing the sulfite concentration with and without increasing the corresponding ammonia concentration was determined and the results are shown in Table IX.

TABLE IX

EFFECT OF THE COMPOSITION OF ELECTROLYTE ON THE PERCENTAGE OF CYSTEINE FOUND

No. of Determinations	Composition of Electrolyte		% Cysteine Found
	Sulfite M	Ammonia M	
3	0.10	0.05	96.1
5	0.15	0.05	96.2
3	0.20	0.05	96.7
4	0.25	0.05	99.2
2	0.40	0.05	100.9
3	0.40	0.15	96.8
2	0.50	0.05	103.6
2	0.50	0.20	97.1

The data show that the apparent amount of cysteine found does not vary with the concentration of sulfite in the range 0.1 to 0.2 M but that there is a general increase beyond 0.2 M sulfite concentration when the ammonia concentration is not correspondingly increased. At a concentration of 0.25 M sulfite and 0.05 M ammonia, the average percentage of cysteine found was 99.2, which is much higher than the results obtained by Kolthoff or ferricyanide methods for the same sample. The concentration of sulfite was, therefore, maintained below 0.2 M throughout this investigation.

Desirable Precautions

The following precautions with respect to the electrodes and the reagent solutions should be observed in order to get good results:

1. The electrodes should be stored in distilled water when not in use. Any oxide or sulfide film that may form over the surface of silver wire upon long storage must be scraped off cautiously before use.
2. The first two or three readings with freshly prepared electrodes are erroneous and should be rejected.
3. Frequent checking of electrodes is desirable, as any cracks or leaks in electrodes give unsteady readings of the galvanometer.
4. Because of the instability of dilute cysteine solutions, concentrated stock solutions should be prepared and used within six hours of their preparation.

Determination of Cysteine with Silver Ions Using a Pair of Polarized Electrodes

Experiments were carried out to explore the possibility of using silver ions and polarized platinum electrodes for the determination of cysteine and cystine. In these experiments the same apparatus was employed but the potential applied was 500 mv. Twenty-five milliliters of 0.1 M tris solution and 10 ml. of 0.8 M sodium sulfite were placed in a 100-ml. beaker, and nitrogen was bubbled through the solution until the galvanometer reading attained a constant value. The nitrogen bubbling tube was then raised above the surface of the solution and known volume of cysteine sample added. The titration was carried out with standard approximately 0.05 M silver nitrate solution. In these titrations, a small residual current flowed when the cysteine sample was added, and this current showed a slight decrease as the first part of the titration proceeded; then the current again increased. The galvanometer readings plotted against the volume of the silver nitrate added thus gave two lines, the point of intersection of which was taken as the end point for the titration. It was difficult to locate this exactly, because the current in the vicinity of the minimum was very small; moreover, the response of the galvanometer was poor and the plots were nonlinear. Table X shows the results obtained in this investigation, and it is seen that the precision of the method is poor, as might be expected.

Substitution of the galvanometer by a more sensitive lamp and scale instrument (Leeds and Northrup, sensitivity 0.05 μa/mm) and use of ammonia-ammonium nitrate buffer instead of tris improved the response by the electrodes but gave unsteady readings of the galvanometer.

TABLE X

TITRATION OF CYSTEINE WITH 0.05 M SILVER NITRATE IN PRESENCE OF SODIUM SULFITE AND AMMONIA-AMMONIUM NITRATE BUFFER USING A PAIR OF PLATINUM ELECTRODES

Number	Amount of Cysteine Taken (mg)	Amount of Cysteine Found (mg)	% Cysteine Found	Deviation From Mean %
1	39.67	38.94	98.3	2.0
2	28.29	26.17	92.5	3.8
3	29.75	29.04	97.6	1.3
4	9.92	9.68	97.6	1.3
5	19.27	17.73	92.0	4.3
6	47.15	46.87	99.4	3.1
7	49.59	49.59	100.0	3.7
8	37.72	34.86	92.5	3.8
		Mean	96.3	Mean Deviation 2.9

A set of determination was then carried out with a pair of silver electrodes and tris or ammonia-ammonium nitrate buffer. In this case, the plot of galvanometer readings against the volume of the silver nitrate added showed an even shallower minimum and no reproducible results could be obtained, owing to the difficulty in locating the end point.

PART III

DETERMINATION OF MERCAPTO GROUPS IN OVALBUMIN

CHAPTER VI

INTRODUCTION

Ovalbumin has been the most widely studied protein with respect to its mercapto group content. It has also been employed in studies of many aspects of the denaturation of proteins. The uncovering or increase in availability of sulfhydryl groups which accompanies denaturation of ovalbumin was first detected by Arnold (2) by the use of the nitroprusside reaction. A number of other reagents such as porphyrindin (17), cystine (39), and ferricyanide (1, 40) have subsequently been used on this protein for the same purpose. These reagents do not indicate the presence of any mercapto groups in the native protein. However, the existence of preformed sulfhydryl groups in ovalbumin is indicated by the fact that iodine, acetamide, and p-chloromercuribenzoate (PCMB) do react with the native protein. The availability of these groups thus depends on the type of reagent that is used to demonstrate their presence. For denaturation, urea, guanidine hydrochloride, and the anionic detergent Duponol have been most extensively studied (1, 17, 40). Anson (1) has shown that the maximum amount of -SH liberated in egg albumin is the same for all the three denaturing agents mentioned above, but that when these are compared on an equimolecular basis, Duponol is the most effective denaturant. This observation and the availability of a large number of ionic detergents should encourage exploration of the

action of these compounds on proteins, which may subsequently help to elucidate the structure of the protein molecule. Quantitative data on the denaturing action of cationic detergents are almost entirely lacking at present.

The mercapto-group content of ovalbumin was first measured quantitatively by Mirsky and Anson (39), using cystine as an oxidizing agent. Greenstein (17) and, later, Anson and Mirsky (1, 40) employed the oxidizing agents porphyrindin and ferricyanide, respectively, for the same purpose. The latter workers also introduced the use of p-chloromercuribenzoate to estimate the maximum number of sulfhydryl groups liberated in urea, guanidine hydrochloride, or in Duponol. Many other workers have also used this reagent. In addition o-iodosobenzoate has been used, and, recently amperometric methods (4, 26) which employ a rotating platinum electrode have been employed. Unfortunately the results of these methods, summarized in Table XI, do not agree with each other.

An investigation was, therefore, undertaken to study the release of -SH groups by the action of ionic detergents and also to develop the amperometric ferricyanide method of Waddill and Gorin (49) for the determination of sulfhydryl groups in ovalbumin. Two ionic detergents, were used in this study, sodium dodecyl sulfate, designated as SDS, and dodecyltrimethylammonium chloride, DAC. A modification of the ferricyanide colorimetric method used by Lontie and Beckers (35) was also applied. The results of this study were presented in a paper read at the 133rd National Meeting of the American Chemical Society at Chicago on September 8, 1958; this paper is transcribed in the next chapter, except that the authors' names and summary have been omitted,

and the references have been included with the rest at the end of the thesis. A more detailed description of some experimental procedures and results is given in Chapter VIII.

TABLE XI

SULFHYDRYL GROUPS OF DENATURED OVALBUMIN

Investigator	Denaturing Agent	Sulfhydryl Reagent	-SH Groups per 45,000 M.W.	Reference
Greenstein	urea	porphyrindin	3.72	(17)
Greenstein	guanidine hydrochloride	porphyrindin	4.76	(17)
Mirsky	urea	ferricyanide	3.57	(40)
Mirsky	guanidine hydrochloride	ferricyanide	3.57	(40)
Mirsky	Duponol	ferricyanide	3.57	(40)
Rosner	urea	iodoacetate	3.24	(44)
Anson	guanidine hydrochloride	PCMB	4.61	(1)
Anson	Duponol	ferricyanide tetrathionate	4.61	(1)
Hellerman et al.	guanidine hydrochloride	o-iodosobenzoate	4.80	(19)
MacDonnel	guanidine hydrochloride	PCMB	3.98	(36)
Benesch	urea	silver nitrate (amperometric)	4.30	(4)
Shigeki Mori	urea	o-iodosobenzoate	3.79	(41)
Boyer	acetate buffer pH 4.6	PCMB	4.00	(7)
Cunningham	heat	PCMB	4.00	(13)
Lontie & Beckers	urea	PCMB	5.50	(35)

CHAPTER VII

REACTION OF FERRICYANIDE WITH OVALBUMIN

Introduction

Among the functional groups which commonly occur in proteins, the mercapto group shows the greatest and most varied reactivity, and this characteristic can be utilized for analytical purposes as well as to effect chemical modifications in mild conditions. Furthermore, there has been established a relationship between the reactivity of this group and the state of denaturation of the protein, and hence its so-called "tertiary" structure; although the relationship is not well understood at present, its investigation provides one of the few possible chemical means for investigating tertiary structure.

Cysteine can be converted to cystine by a number of oxidizing agents, and it may be expected that the mercapto groups of proteins undergo a similar reaction. However, the reaction requires that a pair of mercapto groups be brought into close juxtaposition, and therefore it may be expected also that the number and relative positions of the cysteine residues in the polypeptide chain, as well as the configuration of the chain, will modify the course of reaction, often hinder it, and sometimes possibly prevent it. For this reason, the reaction of oxidizing reagents with the mercapto groups of proteins is especially interesting.

Unfortunately, the specificity of these reagents is always open to some question. Even if it be established that the reagent reacts with simple mercapto compounds to give disulfides in a perfectly well defined manner, the question remains of what may happen if the normal reaction is hindered or prevented. For this reason, it may be well to avoid reagents like porphyrindin, which has a very high oxidation potential, or iodine, which can oxidize single mercapto groups to sulfenic and sulfonic acid. With these considerations in mind, it was decided to investigate more fully the action of ferricyanide, which is comparatively mild and specific. A further advantage of this reagent is that it can be determined by a sensitive and precise amperometric method which utilizes a pair of polarized electrodes (49).

Ovalbumin has been chosen as the initial subject of study, since it has been investigated more intensively than any other protein with respect to mercapto-group content. Even in this case, however, no satisfactory agreement exists among the results, and another careful determination has, therefore, been made. In addition, some qualitative measurements of the rate of reaction in the presence of different denaturing agents have been conducted, and the results are discussed with reference to the structure of the native and denatured protein.

Experimental

Materials: Ovalbumin, Sample I, was obtained from the Worthington Biochemical Corporation, and was twice crystallized, lyophilized product. Solutions were prepared and filtered to remove the turbidity that developed soon after preparation; only clear solutions were taken for

analysis. The solutions were analyzed for nitrogen by the Kjeldahl procedure, and the concentration of protein calculated on the basis of 15.8% nitrogen (33); this is the same basis as used by most other investigators. Kekwick and Cannan (21) have recommended determining the ash-free weight of dry residue. For Sample I, the dry residue weight was 1.08 times that calculated from nitrogen content. Sample II was obtained by subjecting Sample I to two more crystallizations by the method of Kekwick and Cannan. Sodium dodecyl sulfate (SDS) U.S.P., was obtained from the Fisher Scientific Company. Dodecyltrimethylammonium chloride (DAC) was an especially purified product, obtained from Armour Chemical Company, Research Division. Sodium p-chloromercuribenzoate (PCMB) was obtained from Sigma Chemical Company. All other reagents were of analytical-reagent grade. Potassium ferricyanide solutions were prepared directly from weighed samples, and always protected from direct sunlight; fresh solutions were made every day. Phosphate buffer was 1 M in phosphate and had a pH of 7.1.

Apparatus and Method: The apparatus used has been described (49). In this work a more sensitive galvanometer was used, Leeds and Northrup lamp-and-scale, approximate sensitivity 0.05 μa/mm. The theory and practice of titrations with polarized electrodes are not very widely known; in order to interpret the results, reference should be made to a general discussion of the subject (9, 15, 25). Briefly, in the case at hand a current flows across the electrodes dipping in the solution when both ferrocyanide and ferricyanide ions are present; at constant ferrocyanide concentration, which obtains after a sample of ovalbumin

has reacted with a small excess of ferricyanide, the current is proportional to the concentration of ferricyanide which remains.

Electrometric Determination of Mercapto-Group Content: At the beginning of each set of measurements, the galvanometer reading corresponding to zero ferricyanide concentration was determined by mixing 5 ml. of ovalbumin solution, 10 ml. of phosphate buffer, 2-3 ml. of 0.001 M potassium iodide, and 10 ml. of detergent solution in a 100-ml. beaker and dipping the electrodes in the mixture. A slight excess of ferricyanide was then added, and the reaction was allowed to proceed in the dark at 37°, for 45 minutes when SDS was used, and for 15 minutes when DAC was used. The electrodes were then dipped in the solution again, and a galvanometer reading was taken; subsequently, small portions of ferricyanide were added, and galvanometer readings were taken after each addition. Figure 5 represents a typical set of results: the original reading was 10, on an arbitrary scale; after ferricyanide was added, the current increased quickly, because ferrocyanide was formed as a result of the reaction and much unreacted ferricyanide was still present; the galvanometer moved off the scale; after standing for the appropriate time, the reading had decreased to 22, and was constant (it may be greater or smaller, depending on the excess amount used); other points were obtained by adding more ferricyanide. The line connecting these points was extrapolated to the original galvanometer reading, and the volume of ferricyanide indicated by the extrapolation was the amount of reagent corresponding to zero excess, i.e., the volume consumed by reaction with the sample.

Spectroscopic Determination of Mercapto-Group Content: A weighed quantity of ovalbumin was dissolved in 5 ml. of phosphate buffer, and to this were added 2 ml. of 0.001 M potassium iodide solution, 5 ml. of approximately 0.004 M ferricyanide solution, and 10 ml. of 10% SDS or DAC. The mixture was allowed to stand for about 45 minutes at 37°, and then was diluted to 25 ml. in a volumetric flask; an aliquot portion was transferred to a spectrophotometer cell, and the absorption at 410 mμ was measured on a Beckman Model DU Spectrophotometer. Blank determinations were run to determine the absorption of unreacted ferricyanide. Dilution of the blank solution demonstrated that Beer's law was applicable; the amount of ferricyanide consumed could therefore be easily calculated from the decrease in absorption.

Rate of Reaction: In order to follow the rate of consumption of ferricyanide, 2 ml. of 0.002 M ferrocyanide was added to the albumin-iodide-detergent mixture; in this case the current reached its maximum value immediately after the ferricyanide was added. In order to get the initial readings on the scale, a shunt which reduced the sensitivity of the galvanometer approximately tenfold was employed. Additional readings were taken after appropriate intervals of time. The reaction was carried out at room temperature (about 28°).

Blocking -SH Groups: To show the specificity of ferricyanide for -SH groups, 10 ml. of an approximately 0.1% PCMB solution was added prior to the addition of ferricyanide. Then the experiment was carried out as described immediately above. It was found that there was no fall in the value of current with lapse of time which showed that no ferricyanide was being consumed, owing to blocking of -SH groups by PCMB.

Results

Table XII shows the results obtained in a few representative determinations, and gives an indication of the precision of the method; it should be noted that the amount of ovalbumin taken for the several determinations was varied within wide limits, to minimize the chance of systematic error. It is seen that the precision is about 1.5%.

TABLE XII

REPRESENTATIVE DETERMINATIONS OF MERCAPTO-GROUP CONTENT

Ovalbumin Taken mg.	-SH Groups per 45,000 M.W.	Deviation from Mean
42.75	3.94	0.05
25.20	3.95	0.04
50.15	3.98	0.01
81.00	3.89	0.10
48.15	4.05	0.06
18.70	4.04	0.05
32.00	3.98	0.01
44.45	4.05	0.06
Mean	3.99	Mean Deviation 0.05

TABLE XIII

MERCAPTO-GROUP CONTENT OF OVALBUMIN

Sample	Method	Denaturant	-SH Groups per 45,000 M.W.	Detns.
I	electrometric	SDS	3.97	24
I	electrometric	DAC	3.98	15
II	electrometric	SDS	4.01	3
I	spectrophotometric	SDS	4.01	7
I	spectrophotometric	DAC	3.95	2

Table XIII gives a summary of the results obtained by the method with two denaturing agents, SDS and DAC. In addition, a few results are reported which were obtained by measuring the consumption of ferricyanide by spectrophotometry.

Application of the electrometric method showed that, if the reaction was carried out in the absence of detergent, no current developed in an extended period of time. This can be taken as a sensitive confirmation of the observation already made by several investigators (1, 17) that no reaction takes place between ferricyanide and the native protein, since in the presence of excess ferricyanide, the formation of even small amounts of ferrocyanide would give rise to a substantial current.

Representative measurements of the rate of consumption of ferricyanide in the presence of detergent are shown in Figure 6. Since, in these experiments, an amount of ferrocyanide approximately equal to that expected to develop as a result of the reaction was added at the beginning, a large current flowed as soon as ferricyanide was added. This current was too large for measurement with the galvanometer used for the quantitative determinations at its full sensitivity, and, in these experiments, the sensitivity of the instrument was reduced approximately tenfold by means of a shunt. It can be seen that the current decreased rapidly, and this corresponds approximately to the decrease in ferricyanide concentration; however, the two quantities are not exactly related, because the ferrocyanide concentration increased at the same time. However, the final attainment of a constant galvanometer reading does indicate constant ferri- and ferro-cyanide concentrations, and that the reaction was complete. The reaction carried out with

10 ml. of 1% SDS was complete in about 35 minutes; with 10% SDS, the reaction was only a little faster, and required about 15 minutes for essential completion. In the presence of 1% DAC, on the other hand, the reaction was complete in about 4 minutes.

It may be of some interest to mention that if iodide ion is omitted from the reaction mixture, the reaction of ferricyanide with ovalbumin is sluggish; the galvanometer readings tend to be erratic and decrease slowly with time. Copper (II) ion, which Anson had found to be an effective catalyst for the reaction, and which works well in the titration of cysteine with ferricyanide improves the reaction somewhat, but iodide ion is much more effective.

Another observation of some importance is that in dilute solutions of dodecyltrimethylammonium cation, both ferricyanide and protein are precipitated; the precipitates, however, are soluble in excess detergent, and 10 ml. of 1% detergent as used in the method described above were sufficient to maintain the reaction mixture in a homogeneous state.

Because interesting studies of denaturation have been carried out in urea (46) and in guanidine hydrochloride (45), it was desired to apply the method in the presence of these denaturing agents as well. Unfortunately, this could not be done. In the presence of urea, the response of the electrodes to ferricyanide added in excess was so small that no accurate extrapolation to an end-point was possible. On the other hand, in the presence of guanidine hydrochloride, a very sizeable current flowed even before the end-point so that its use is also prevented. No specific reason can be given for these effects, but it may be surmised that the depressing effect of urea is due to

its action upon the electrode surface, while with guanidine hydrochloride the denaturant itself is oxidized at the anode; whatever the reasons, these observations indicate some practical limitations of the method.

Discussion

A careful and extensive study of the reaction of mercapto groups in ovalbumin with ferricyanide was carried out by Anson (1). The ferricyanide was converted to ferrocyanide, and Anson determined the latter colorimetrically by converting it to Prussian blue. In addition to determining the amount of ferricyanide reduced by this method, Anson also determined the amount of tetrathionate and PCMB needed to react with the mercapto groups, and abolish the reduction of ferricyanide. The results of the several methods were impressively concordant, and the value, 1.21% cysteine, was the most widely accepted one for some time. However, Mirsky, at about the same time, found, with ferricyanide, a very substantially lower value, 0.95% (40). The discrepancy has never been satisfactorily explained; since it is certainly outside the indeterminate error of the method, and the chances for systematic error seem remote, Anson suggested that the materials used by the two investigators had different mercapto-group contents. In his last experimental paper on the subject, Anson reports having encountered samples with a mercapto-group content lower than 1.21%, which could not be increased by recrystallization (1). Unfortunately, it is not now possible to ascertain the nature of the samples used by these investigators.

The results of Longsworth et al. (34), which indicated two electrophoretically separable components in ovalbumin, and which are still generally accepted, support the belief that this protein is heterogeneous. However, there is good evidence to show that the difference between the electrophoretically separable components is their content of phosphoric acid residues, and this would make a negligible difference in the percent cysteine content (42). In the last fifteen years, several groups of investigators, working independently, have found substantially the same result on different ovalbumin samples with the reagent p-chloromercuribenzoate: MacDonnell et al. (36), 3.98; Boyer (7), 4.0; and Cunningham et al. (13), 4.02. It appears that the reproducibility of samples may not be a serious problem, and a lower result than Anson's is definitely indicated, at least as far as this reagent is concerned.

Determinations with other reagents, applied in recent past, have given similar results: Mori (41) with o-iodosobenzoate found 3.79 groups; Larson and Jenness (31) by amperometric titration with the same reagent found 4 groups; and Benesch et al. (4) by amperometric titration with silver ion, 4.3. Since Sluyterman (47) has shown that the latter method tends to give high values, the result must be taken as indicating four groups per molecule. On the other hand, Greenstein (17) found, with porphyrindin, 4.6 groups, Hellerman et al. (19) with o-iodosobenzoate, 4.8 groups, and Hess and Sullivan (20) with iodate, 5.2 groups. While these higher results should not be dismissed lightly, it is fair to say that the preponderance of evidence now favors four groups per molecule, and that evidence to the contrary should be

revaluated critically. The writers would undertake this task in part, and cooperation would be welcome.

A further set of results must be considered, obtained by Lontie and Beckers (35) in 1956. These investigators applied the amperometric silver titration of Kolthoff and Harris (26) to native protein and found a value of 5.6 groups per molecule; with p-chloromercuribenzoate and denatured protein, 5.5 groups, and with ferricyanide and denatured protein, 1.7-2.5. The precision achieved by Lontie and Beckers was poor, especially in the case of ferricyanide, but the method employed by them appeared sound in principle. Therefore, it was decided to test it with modifications which would avoid certain undesirable features of the original method: (1) ferricyanide was added prior to the addition of the detergent, in order to avoid the oxidation of the denatured ovalbumin by air; (2) the reaction mixture was diluted for measurement to a smaller volume, so that greater optical densities would be obtained -- this affords more precise data, and Beer's Law is equally well obeyed in this range; (3) the protein was not precipitated before measuring the ferricyanide absorption -- since the protein does not absorb at the wave length in question, this step of precipitation is unnecessary, and involves the risk of absorbing some ferricyanide on the precipitated protein. With these precautions, quite good precision was obtained, much better than that indicated by Lontie and Beckers, and the results, shown in Table XII were found to be in excellent agreement with those of the electrometric method.

The value found in this work is recommended by the following considerations: the precision is good; the completeness of reaction

has been demonstrated by continued measurements of the extent of reaction with the protein itself; the specificity has been established by the fact that no reaction occurs in the native protein or in the denatured protein treated with p-chloromercuribenzoate; and, finally, the number of mercapto groups found corresponds very closely to an integral number per 45,000 M.W., the value indicated by the most reliable analytical results and by the more recent physicochemical measurements (50).

All that have investigated the question agree that p-chloromercuribenzoate can react with two or three mercapto groups in the native protein (7, 13, 36). One may conclude that these groups are accessible to that reagent in aqueous solution, although less easily than when the protein is denatured, and one may surmise that they would also be accessible to ferricyanide. Their failure to react with ferricyanide, accordingly, is likely due to another cause; for instance, it may be that the mercapto groups are located along the polypeptide chain in such a way that a disulfide bond cannot be formed between them, until the chain is "loosened" by denaturation. However, this is an oversimplification, since it has been seen that the reaction proceeds much faster in the case of DAC than of SDS, although both materials denature the protein. Measurements of the rate of denaturation by these reagents are not available, but it cannot be assumed that the slower reaction in the presence of SDS is due solely to slower denaturing action, since increasing the concentration of denaturant ten times increases the rate of reaction with ferricyanide approximately twofold. One must conclude, rather, that each detergent effects unique structural changes in the protein, and that the rate of reaction with ferricyanide is dependent on the resultant configuration of the polypeptide chain.

A more precise investigation of the relation between the development of mercapto-group reactivity and physicochemical measures of denaturation, such as viscosity and optical activity, is now in progress.

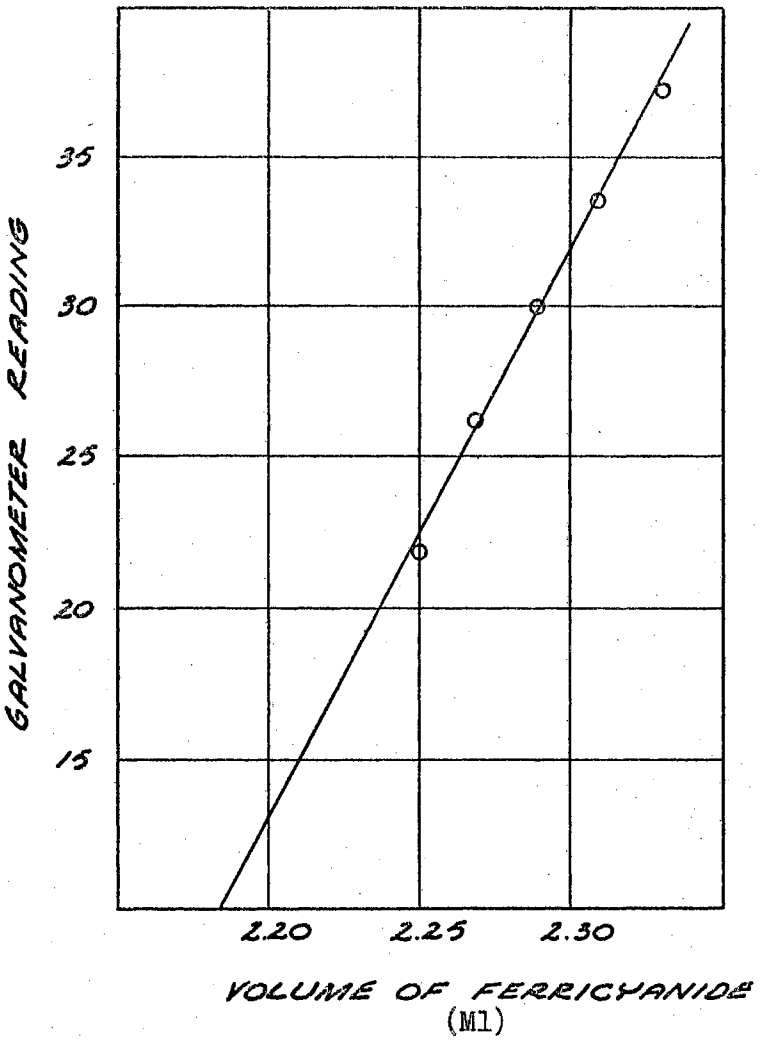

Figure 5

Typical "Dead-Stop" End-Point Titration Plot

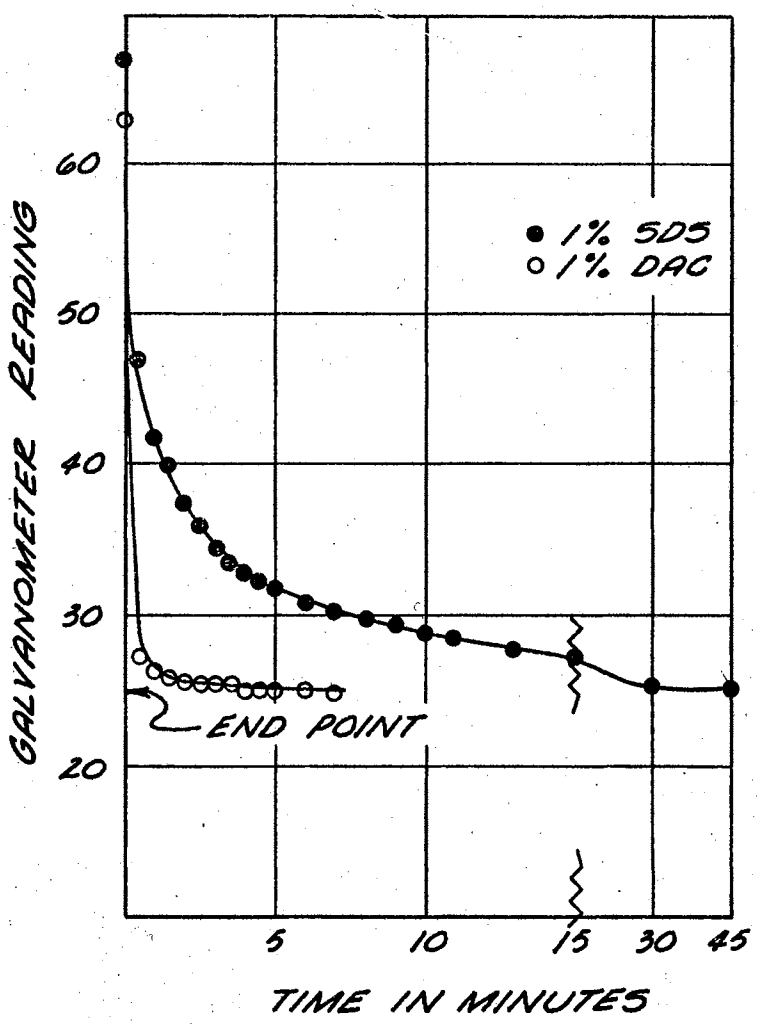

Figure 6

Rate of Consumption of Ferricyanide in Presence of SDS and DAC as Denaturants

CHAPTER VIII

EXPANDED EXPERIMENTAL SECTION AND RESULTS

Purification of Ovalbumin

Two samples of ovalbumin were used in this investigation. Sample I, as already mentioned, was supplied by the Worthington Biochemical Corporation and was a two-times crystallized, lyophilized product. Sample II was obtained by subjecting sample I to two further crystallizations by the method of Kekwick and Cannan (21) as follows.

About 1 g. of ovalbumin (sample I) was dissolved in 50 ml. of deionized distilled water. The solution was allowed to stand for a few minutes, and then filtered through a Whatman #1 filter paper. To the filtrate was added slowly, and with constant stirring, a solution of 0.2 N sulfuric acid, to bring the pH to about 4.7. Addition of the acid was followed by that of small quantities of anhydrous sodium sulfate, until an opalescence developed. About 10 to 11 g. of the salt were required for this purpose. The supernatant liquid was decanted to remove any undissolved sodium sulfate and kept for a couple of days to crystallize the protein. The crystalline material was removed by filtration and redissolved in the same quantity of water. Recrystallization was again effected by the procedure described above. The product was dried in air at room temperature by spreading it into a thin layer over a big sheet of Whatman lintless filter paper.

Detailed Analytical Results

Tables XIV and XV show the detailed results of determination of the mercapto-group content of samples I and II.

TABLE XIV

MERCAPTO-GROUP CONTENT OF OVALBUMIN (SAMPLE I) BY AMPEROMETRIC TITRATION WITH 0.002 M FERRICYANIDE USING PLATINUM ELECTRODES

Denaturant	Amount of Ovalbumin Taken (mg)	-SH Groups per 45,000 M. W.		Deviation from Mean
SDS	58.5	3.97		0.00
	58.5	3.98		0.01
	23.4	3.95		0.02
	23.4	3.94		0.03
	46.8	3.95		0.02
	46.8	3.97		0.00
	33.2	4.01		0.04
	33.2	3.98		0.01
	24.9	3.95		0.02
	24.9	3.97		0.00
	76.5	3.95		0.02
	76.5	3.96		0.01
	30.6	3.97		0.00
	30.6	3.95		0.02
	61.2	4.03		0.06
	61.2	4.01		0.04
	50.5	3.96		0.01
	20.2	3.95		0.02
	20.2	3.94		0.03
		Mean 3.97	Mean Deviation	0.02
DAC	50.2	3.97		0.01
	52.3	4.05		0.07
	50.2	3.92		0.06
	22.0	4.04		0.06
	40.0	3.98		0.00
	39.8	3.96		0.02
	49.1	3.91		0.07
	50.0	3.93		0.05
	48.5	3.99		0.01
	34.8	4.01		0.03
	31.5	3.99		0.01
	50.0	3.98		0.00
		Mean 3.98	Mean Deviation	0.03

TABLE XV

MERCAPTO-GROUP CONTENT OF OVALBUMIN (SAMPLE II) BY AMPEROMETRIC TITRATION WITH 0.002 M FERRICYANIDE USING PLATINUM ELECTRODES

Denaturant	Amount of Ovalbumin Taken (mg)	-SH Groups per 45,000 M.W.	Deviation from Mean
SDS	64.75	4.02	0.01
	64.75	4.01	0.00
	25.90	3.99	0.02
	Mean	4.01 Mean Deviation	0.01

Spectroscopic Determination of Mercapto Groups

Details of the spectroscopic method for determining mercapto-group content of ovalbumin have already been given in the previous chapter. The standard curve for the experiment was drawn as follows:

An exactly weighed quantity (0.1338 g.) of reagent-grade potassium ferricyanide was dissolved in deionized distilled water and the volume made to 100 ml. Known volumes (5 ml., 4 ml., and 2 ml.) of this solution were pipetted into four 25-ml. graduated flasks. To each flask was added 5 ml. of 1 M phosphate buffer, 2 ml. of 0.001 M potassium iodide, and 10 ml. of 10% SDS solution. The flasks were allowed to stand in a thermostat at 37°C for about 45 minutes. The volume was then made up to the graduation mark with distilled water, and the optical densities of the solutions determined with a Beckman DU spectrophotometer at 4100 Å. Table XVI shows the results obtained in these determinations.

TABLE XVI

OPTICAL DENSITIES OF POTASSIUM FERRICYANIDE SOLUTIONS (1.338 g. per 1) AT 410C Å WITH BECKMAN DU SPECTROPHOTOMETER

No. of Determ.	Volume of Ferricyanide soln. (ml)	Amount of Ferricyanide Present (mg)	Optical Density (average)
3	5	6.690	0.810
2	4	5.352	0.660
3	3	4.014	0.500
2	2	2.676	0.328

The data were plotted and gave a linear graph (Figure 7), showing that Beer's Law is obeyed in this range of concentrations. The results of analysis of ovalbumin (sample I) by this method are shown in Table XVII.

TABLE XVII

DETERMINATION OF MERCAPTO-GROUP CONTENT OF OVALBUMIN (SAMPLE I) BY STANDARD (1.338 g./liter) FERRICYANIDE WITH BECKMAN DU SPECTROPHOTOMETER AT 4100 Å

Amount of Ovalbumin mg.	Vol. of Ferricyanide Added	Average Optical Density of the Reacted Sol.	Vol. of Unreacted Ferricyanide	-SH Groups per 45,000 M.W.
56.8	5	0.644	3.96	3.97
87.8	5	0.552	3.38	3.97
100.8	5	0.508	3.10	4.06
86.4	5	0.555	3.40	4.01
54.0	5	0.655	4.00	3.99
39.8	5	0.698	4.25	4.06
16.7	5	0.770	4.69	4.00
			Mean	4.01

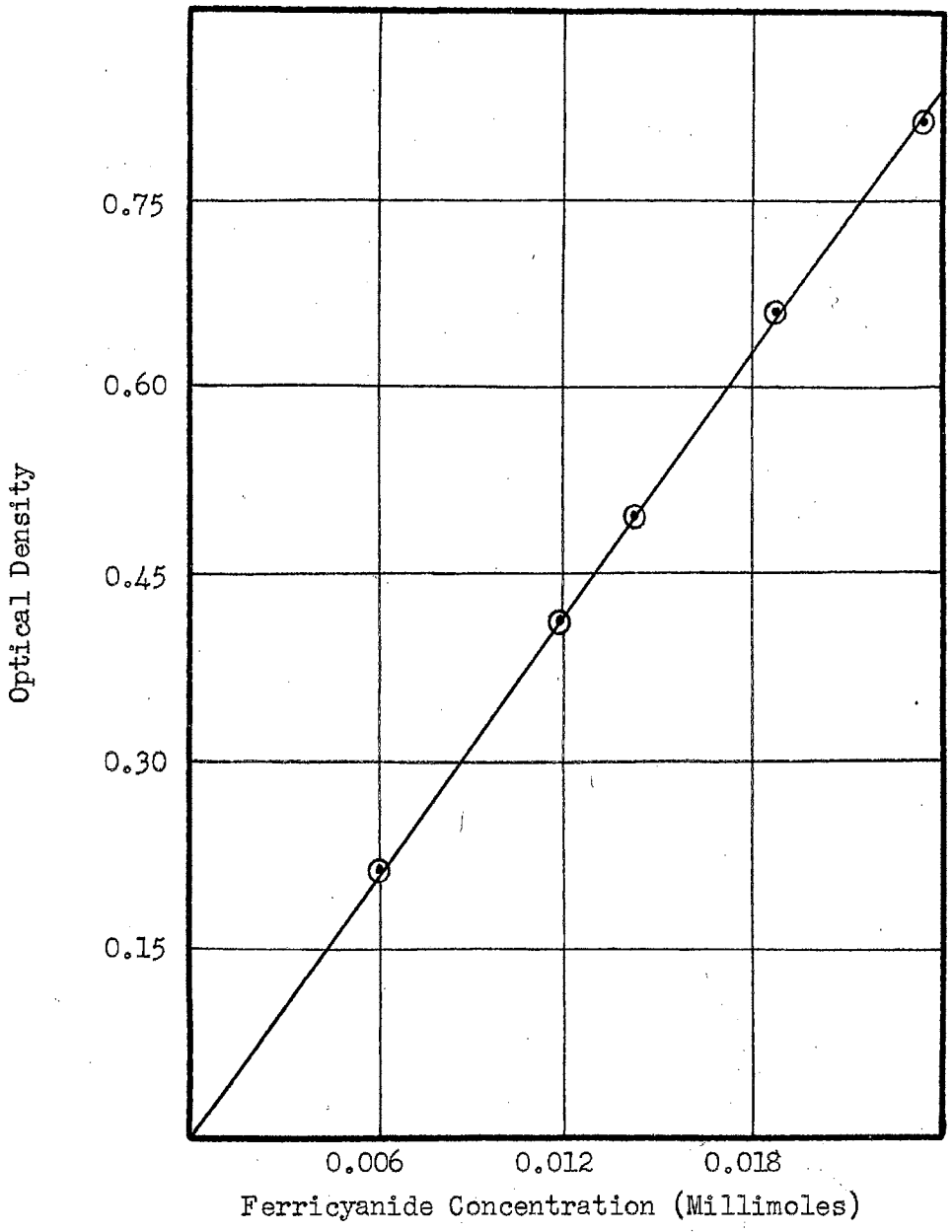

Figure 7

Optical Densities of Ferricyanide Solutions at 4100 Å
With Beckman DU Spectrophotometer

SUMMARY AND CONCLUSIONS

The first part of this thesis has dealt with the application of polarized electrodes to detecting the end point in titrations. The response of platinum electrodes, i.e., the magnitude of the current which flows across these electrodes in solution containing iodine-iodide ion, was studied for the purpose of establishing factors that are important in determining the response. It was found that:

1. thick wire electrodes are more sensitive than thin wire electrodes.
2. the longer the wire protruding from the glass, the more sensitive the electrodes. The effect was quite pronounced.
3. more vigorous stirring and greater distance between the electrodes does not have any pronounced effects. However, if the stirring is stopped altogether just before taking each reading, the response of the electrodes becomes very poor; this shows that the current is diffusion-controlled.

The above information indicates that the sensitivity of the method can be varied within wide limits by adjusting these factors.

Secondly, the response of various metal electrodes to silver, mercury (II), and copper (II) ions was investigated. It has been ascertained that:

1. platinum electrodes respond to silver ions in the presence of sulfite or nitrite, but only the latter gives an approximately linear dependence of current on silver ion concentration.

2. silver electrodes give a greater and nearly linear response in the above conditions as well as in the presence of ammonia-ammonium nitrate buffer.
3. one platinum and one silver electrodes can be used in conjunction.
4. platinum electrodes have been seen to respond to cupric ions at high potentials; they do not respond usefully to mercuric ions.

These results have been employed in developing a convenient and simple method for the estimation of cysteine and cystine, which is described in the second part of the thesis. The determination is done by titration with standard copper (II) sulfate in an ammonia-ammonium nitrate-sodium sulfite medium. The end point is detected with two dissimilar polarized electrodes; the cathode is made of platinum wire, the anode of silver. A potential of about 50 mv. is applied. No current flows before the end point, but after it the current increases linearly with the amount of the reagent added up to a considerable excess; currents up to 10 μa are developed. The end point is determined by extrapolation. The stoichiometry is 2 gram-ions of copper per mole of cysteine or cystine. The method has been applied to quantities as small as 1 mg. with a precision of 1 per cent. Accuracy of the method has been established by getting consistent results which agree fairly well with those obtained by the already known amperometric methods.

The use of a pair of platinum or copper electrodes has been found to be unsatisfactory in the above method as the end point can not be located correctly in these cases. Silver ions with platinum electrodes

in ammoniacal solution have been tried but the plots of current versus volume of reagent are not linear; with silver electrodes current flows throughout the titration, with a minimum near the end point, but this minimum can not be located with precision. Mercury (II) ions with platinum electrodes give no response under the same conditions.

In the third part of the investigation, the mercapto groups in ovalbumin have been determined by titration with ferricyanide, using a pair of platinum electrodes to detect the end point. The method is faster, more accurate, and more convenient than that involving the colorimetric determination of ferrocyanide produced. There is no reaction with native ovalbumin. In the presence of sodium dodecyl sulfate as denaturant, 3.98 mercapto groups have been found per 45,000 M.W. unit; the precision is better than 3 per cent. Para-chloromercuribenzoate added in slight excess completely blocks the reaction, showing that it is specific in the conditions employed. The rate of uptake of ferricyanide has been followed continuously with time. The determination has also been carried out in the presence of another denaturant, dodecyltrimethylammonium chloride. The uptake of ferricyanide is found to be much faster in the presence of this denaturant though the number of mercapto groups agrees with that found in the presence of sodium dodecyl sulfate.

The results of the above method agree very well with those of a spectrophotometric method, which has been modified to get good precision.

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Education: Attended D. A. V. High School, Lyallpur, Punjab; passed the Matriculation Examination from D. A. V High School, Lahore in 1939; Intermediate Examination from Government College, Lahore in 1941; received the Bachelor of Science (Honours in Chemistry) degree from the Punjab University in May, 1944; passed the Bachelor of Arts (Economics only) Examination of the Punjab University in September 1944; received the Master of Science degree from the Punjab University, with a major in Industrial Chemistry, in 1945.

Professional experience: Entered the Punjab Education Department in 1946, and is now a senior lecturer in Chemistry; joined the National Cadet Corps, Senior Division, India, in 1949 and is a Lieutenant in the 3rd. Punjab Battalion attached to Government College, Ludhiana, India.