CYSTEINE: IONIZATION CHARACTERISTICS AND ELECTROLYTIC PREPARATION FROM CYSTINE

By

C. W. Clary

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Oklahoma State University

Stillwater, Oklahoma

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Thesis Approved:

George Goring
Thesis Adviser

Paul Arthur

Robert MacVicar
Dean of the Graduate School

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CHAPTER I

INTRODUCTION

The chemistry of thiol compounds has assumed increasing importance in research upon the nature and dynamics of biochemical systems. Especially important is the thiol-disulfide redox system, which is thought to exert considerable control over the physical shape of protein molecules as well as the course of protein reactions in living organisms. (1).

The abundant occurrence of the thiol-amino acid cysteine, structure I, both free in biological fluids and

$$HSCH_2CH(NH_2)COOH$$

I

as a component of proteins, has prompted an intensive study of its properties. Accurate values for the physical properties of cysteine in aqueous solution are necessary for the correct interpretation of its biological role. Such investigations have been hindered by the instability of this substance toward atmospheric oxidation; it is difficult to obtain good samples of the material for investigations requiring a high degree of purity. Indeed, despite the fact that numerous methods for the analytical

determination of cysteine have been developed, e.g., (2) (3) (4), it is not easy to tell how pure a sample may be.

Since cysteine is less easily oxidized in acidic conditions, cysteine hydrochloride hydrate can be obtained fairly pure, and is taken as the starting material in precise measurements. The investigation reported in this thesis began with the assay of commercial samples of cysteine hydrochloride hydrate and the subsequent determination of the second ionization constant, for which somewhat discordant values have been reported in the chemical literature.

Our second and main purpose was to investigate the relative acid strengths of the mercaptide and amino functions, both of which have about the same tendency to transfer a proton to water.

Also included in this work was the preparation of standard cysteine solutions of low concentration by the electrolytic reduction of the disulfide cystine at a large-area mercury cathode.

CHAPTER II

SAMPLE PURITY AND IONIZATION CONSTANTS

Assay of Commercial Cysteine Hydrochloride Hydrate

Experimentally determined values for the second ionization constant of cysteine hydrochloride are not in complete agreement. Some of the more recent investigations and the corresponding values of the pK are listed below.

Investigators	pK_2	Ionic Strength	Temp.
Li and Manning (5)	8.48	0.15	25°
Albert (6)	8.36	?	20°
Gorin (7)	8.27	0.1	25°
Boorsook, et al. (8)	8.34	0.017	25°
Grafius and Neilands (9)	8.30	0.15	25°

In some cases the ionic strengths are different, but the differences in pK_2 are not resolved by reasonable corrections for this. Values have been determined with electronic pH meters and glass electrodes, which do not afford the highest accuracy, but the deviations are outside the accepted limits of experimental error.

In view of the questionable purity of most cysteine samples, it seems reasonable to assume that this might

have been mainly responsible for the variation in pK values.

It was accordingly decided to determine again the value of pK_2, and to assay carefully the sample of cysteine hydrochloride hydrate to be used for this purpose. The results are reported in Table I.

TABLE I

ASSAY OF A COMMERCIAL SAMPLE OF CYSTEINE HYDROCHLORIDE HYDRATE

Method	Percent Purity	Average Deviation
Amperometric Titration of Thiol by Ferricyanide	96.48	0.39
Acidimetric Titration (pH Meter)	99.68	0.43
Chloride Determination by Parr Bomb Fusion and Volhard Titration	97.46	0.51
Kjeldahl Nitrogen Determination	100.38	0.16

The details of these assays are reported in Chapter V.

Measurement of pK_2

Cysteine hydrochloride contains three ionizable protons. If we represent the cysteine cation by $(H_3Cys)^+$, the process with which we are concerned is:

$$H_2Cys \rightleftharpoons HCys^- + H^+$$

The thermodynamic ionization constant for this is then:

$$K^* = \frac{\alpha_{H^+}\alpha_{HCys^-}}{\alpha_{H_2Cys}} = \frac{\alpha_{H^+}C_{HCys^-}\gamma_{HCys^-}}{C_{H_2Cys}\gamma_{H_2Cys}}$$

where α stands for activity and γ the appropriate activity coefficient. For the purpose of this work, we are interested in a practical constant,

$$K = K^* \frac{\gamma_{H_2Cys}}{\gamma_{HCys^-}} = \frac{\alpha_H C_{HCys^-}}{C_{H_2Cys}}$$

In the titration of cysteine hydrochloride with sodium hydroxide, the requirement of electrical neutrality demands that

$$C_{Na^+} + C_{H^+} + C_{H_3Cys^+} = C_{OH^-} + C_{HCys^-} + 2C_{Cys^=} + C_{Cl^-};$$

also,

$$C^o_{Cys} = C_{H_3Cys^+} + C_{H_2Cys} + C_{HCys^-} + C_{Cys^=}$$

where C^o_{Cys} is the initial concentration of cystine hydrochloride.

The determination of pK_2 was accomplished by potentiometric titration of 0.01 M solutions of cysteine hydrochloride in water or in 0.1 M potassium chloride with 0.1 N NaOH. When $1\frac{1}{2}$ moles of base have been added for each mole of cysteine hydrochloride, the pH is about 8.5, and C_{H^+}, C_{OH^-}, $C_{H_3Cys^+}$ can be neglected without appreciable error. If $C_{Cys^=}$ is also neglected, which can be done with an error of 1 - 2%, one finally has;

$$C_{H_2Cys} = C_{HCys}$$

At this point, α_{H^+} is therefore equal to K_2 or the pH to pK.

A typical titration curve is shown in Figure 1. The average value of pK_2 determined from a series of such determinations is 8.32. The details of this work and tabulation of results is found in Chapter V.

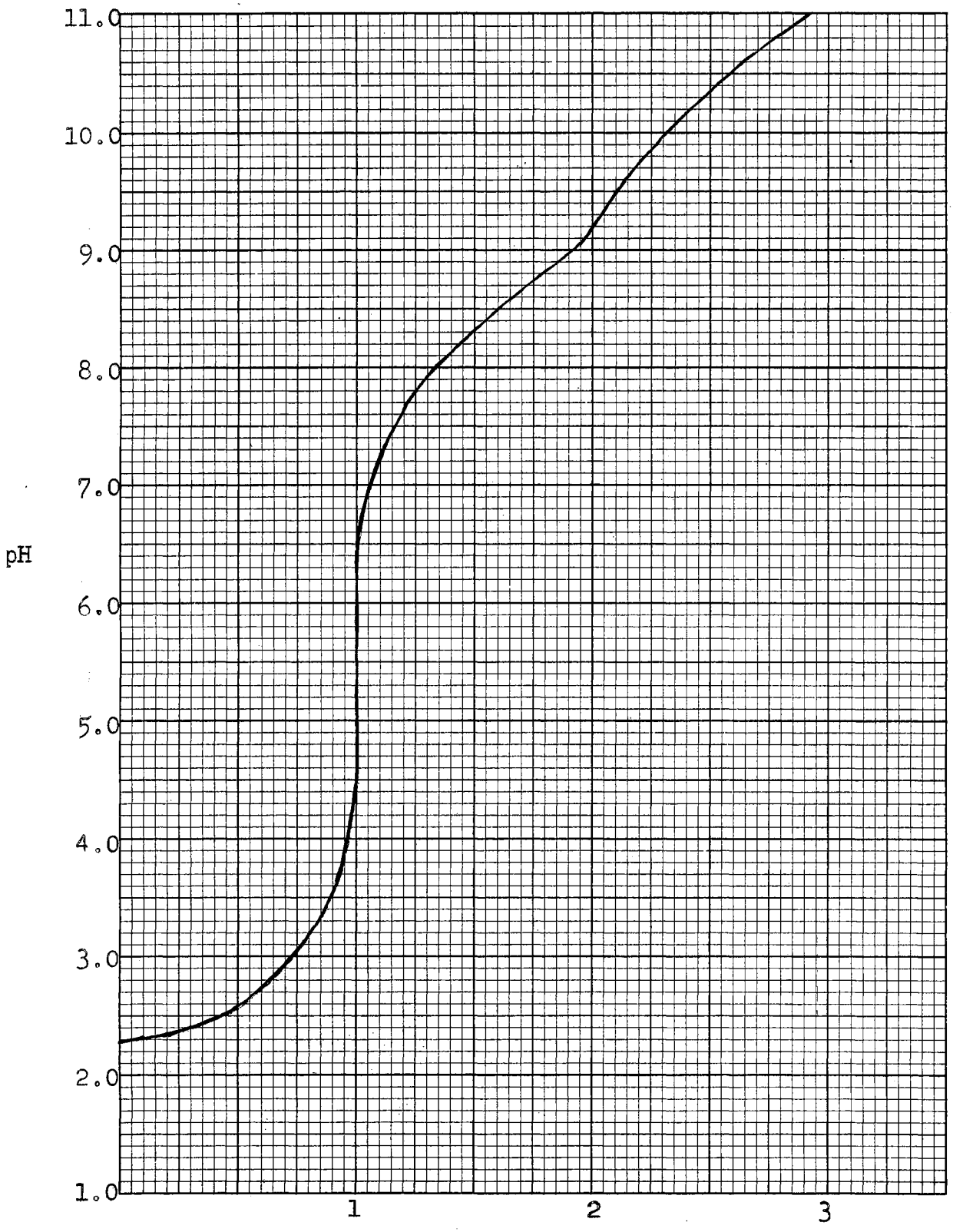


Figure 1. Potentiometric Titration of Cysteine Hydrochloride. 0.01 M solution in 0.1 M KCl

CHAPTER III

THE RELATIVE IONIZATIONS OF THE MERCAPTO AND AMINO GROUPS

Previous Studies

The work of Ryklan and Schmidt (10) Edsall (11), and others (12) (9) (7) has established that the second ionization of cysteine hydrochloride involves protons from both the sulfur and the nitrogen functions. In order to determine the amount of ionization from each of these groups, Grafius and Nielands (9) measured the second ionization constants of cysteine betaine, pK = 8.65, and S-methylcysteine, pK = 8.75; if the same intrinsic ionization constants hold for the ammonium and mercapto groups of cysteine, the measured value pK_2 = 8.30 for cysteine would correspond to a ratio, R, of 1.3 for the concentration of the species involved, I/II.

$$-SCH_2CH(NH_3)^+COO^- \qquad\qquad HSCH_2CH(NH_2)COO^-$$

I II

Benesch (12) calculated a value of 2.1 for R utilizing ultraviolet absorption spectrum measurements, but the assumptions underlying the calculation seem rather precarious. Garfinkel and Edsall (13), using Raman spectroscopy and somewhat more reliable assumptions, placed the value of R at about unity.

The work of Garfinkel and Edsall, which was published shortly before completion of the present work is based on the observation of the lines at 870 and 2575 cm^{-1}, which have been assigned to the S-H bond. Variations of the intensity of these lines should represent a highly specific measure of the ionization of this group as a function of pH. The value for R estimated by these workers, however, was not considered by them to be quantitatively accurate because of difficulties in obtaining quantitative absorption measurements from Raman spectra. A more quantitative experiment has been promised, which would utilize a newly developed photometric instrument of greater sensitivity and accuracy.

Since each approach unavoidably involves certain assumptions and experimental drawbacks, it is felt that the method to be described below would yet be of value in the final solution of this problem.

Theoretical Foundation of Present Work

Reference has been made previously (12) to the ultraviolet absorption which develops during titration of the second proton of cysteine hydrochloride. It has been suggested that this absorption is due, all or in part, to the mercaptide-containing species (I), since a similar absorption has been noted with simple mercaptides. (14). However, it is not possible to determine the concentration of this species, because the molar extinction of this ion

and of its tautomer (II) are not known. No one has, up to this time, attempted to determine these absorptions unequivocally. If the extinction coefficients could be determined for each of these tautomers, the proportion of each could be computed from the absorbancy of the mixture at some point during the titration.

Since one of the forms (I) contains 3 centers of electric charge, it is of a more polar nature than its tautomer (II), and should be favored in solutions of high ionic strength, but suppressed in solutions of low dielectric constant. In other words, the ultraviolet absorption of a solution containing ions (I) and (II) in tautomeric equilibrium should increase with increasing ionic strength, and it may indeed be possible to shift the equilibrium II $\rightleftharpoons$ I to virtual completion in favor of (I). Conversely, the absorbancy should decrease with decreasing dielectric constant of the solution, and all of the cysteinate ion might be converted to (II).

As a preliminary test of this approach to the problem (15), aliquots from a sample of partially neutralized cysteine were diluted, on the one hand with progressivley more concentrated potassium chloride solutions, and on the other with alcohol-water mixtures of progressivley greater alcohol content, and the optical densities were measured at 230 mμ (absorption maximum of the cysteinate ion). Figure 2 shows the spectra in the two types of solutions. The results are expressed in comparison to the optical densities measured

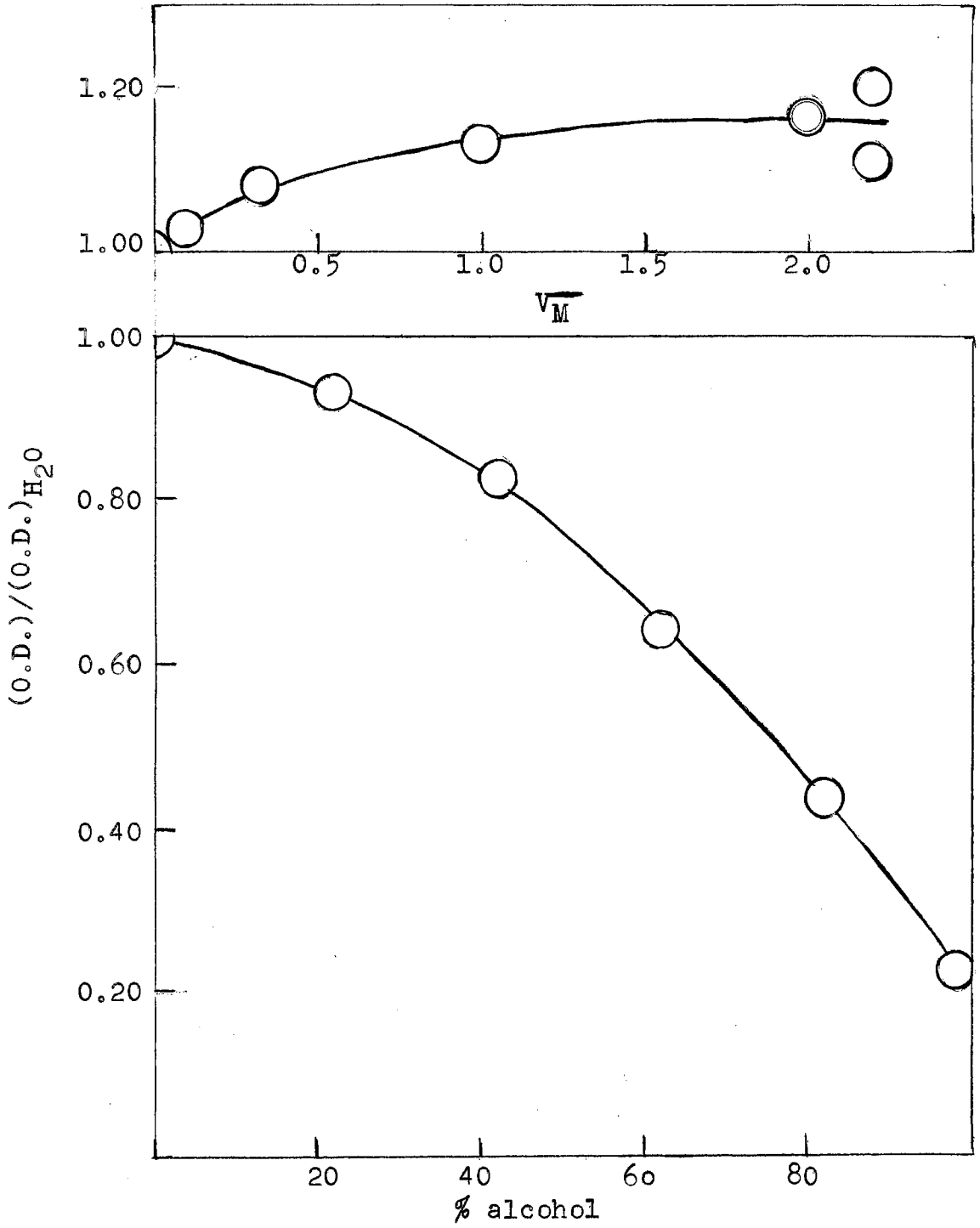


Figure 2. Spectra of Cysteine in Different Media.

Absorption of half-neutralized cysteine relative to water: upper frame, KCl solutions; lower frame, alcohol-water mixtures.

in water (this quantity is given the value of unity). The abscissa in one frame is percent alcohol, and that in the other frame is the square root of the potassium chloride molarity and of the ionic strength. The latter scale is used because it allows a more even spacing of the experimental points.

In another set of measurements, the absorption in water was compared to that in saturated potassium chloride and in 92 percent alcohol. The results of six independent determinations are reported in Table II.

TABLE II

OPTICAL DENSITY OF CYSTEINE IN VARIOUS MEDIA AND VALUES OF R

OD_{KCl}	OD_{H2O}	OD_{EtOH} (92%)	R
0.739	0.661	0.078	7.5
0.731	0.655	0.074	7.6
0.722	0.652	0.068	8.3
0.767	0.692	0.063	8.9
0.764	0.693	0.058	9.0
0.727	0.666	0.076	9.1
			Avg. 8.4 ± 0.6

To ascertain the extent of conversion of cysteinate ion to form (II), the absorption ofasolution in 92 percent alcohol was compared to one of equal concentration in a

ternary mixture of 68% alcohol, 30% isootane and 2% water; addition of isooctane affords a yet lower dielectric constant. No difference in the absorbancy outside of experimental error was found.

Discussion

These results suggest that virtually complete conversion to form (I) takes place in the saturated KCl solution and that conversion to form (II) likewise occurs in 92 percent alcohol. On the basis of these assumptions, and on the further assumption that the different media would not affect the extinction coefficients, we can write:

$$O.D._{H_2O} = [II]\,O.D._{EtOH} + (1 - [II])\,O.D._{satd.KCl}$$

where $[II]$ is the fraction of cysteinate ion in form II. The data of Table II, when subjected to this treatment, give an average value for $\underline{R}$ of 8.4 ± 0.6.

In order to check the assumption that the various media did not influence the individual spectra, the absorption of a close analog, cystine, in both acidic and basic solutions was examined for changes as the sample was placed in water, in potassium chloride, and in ethanol. No significant differences were found.

The value of $\underline{R}$ is not much affected by the exact value of $O.D._{EtOH}$, as long as this is small compared to $O.D._{KCl}$. Therefore, any possible error in the value of the former would not affect the final result greatly; this is fortunate,

since the average deviation is larger than one might wish. The variation may be attributed to the presence of varying quantities of natural impurities in the ethanol (e.g., aldehyde from oxidation). The absorbancy ratio $O.D._{H_2O}/O.D._{KCl}$, which is more important in determining the value of R, is fortunately quite consistent: 0.903 ± 0.7%. This reproducibility could be realized despite the fact that the optical density in potassium chloride solutions falls rapidly with time; this is ascribed to oxidation, which probably proceeds by way of form (I). For this reason, the spectra of all samples, and particularly the ones in which form (I) predominated, were measured as quickly and consistently as possible. In some measurements, the rate of decrease in absorption was determined as a function of time and the value of the absorption extrapolated to zero time; this procedure did not yield significant differences in the ratio of absorbancies and hence in the final value of R.

CHAPTER IV

ELECTROLYTIC REDUCTION OF CYSTINE

Introduction

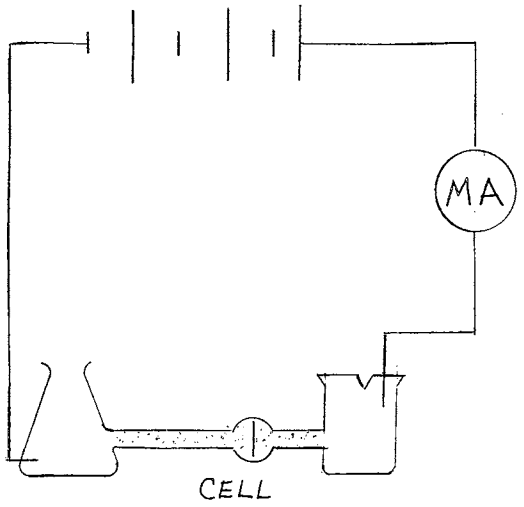
The possibility of a quantitative electrolytic reduction of cystine was investigated with the aim of obtaining cysteine samples of high purity. The attractiveness of this type of reduction is due to three considerations: a) cystine may be obtained commercially in a higher degree of purity than cysteine, and is more stable; b) a singular specificity of reaction may be realized in reductions at a constant cathode potential; and c) quantities of electric current may be measured conveniently and accurately by means of a suitable coulometer. Thus, a reliable starting material might be converted into standard solutions of cysteine, the titer of which might be found by a simple coulometric determination.

The early experiments, unfortunately, were not successful and the intended plan of utilizing the method to prepare samples for the ionization experiments was abandoned. Afterwards, the possibility of applying electrolytic reduction became clearer, as a result of technological advances and the appearance of a recent publication on organic electrode processes. (16). This provided a more precise

approach to the design of both experiment and apparatus, and better success was achieved.

Early Experiments

In brief, the apparatus consisted of the cell and circuit diagrammed in Figure 3.



E = rectified AC variable source

MA = milliammeter

Cell = 250-ml. Erlenmeyer cathode cell, connected by agar bridge to 100 ml. anode cell

Figure 3. Early Design of Apparatus.

The cathode compartment of the cell contained at the start a 0.01 M solution of cystine in 1.0 N sulfuric acid,* over 30 ml. of mercury. This compartment was connected through an agar bridge to the anode compartment, which contained 1.0 N sulfuric acid.

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*Cystine is quite insoluble in neutral solutions. 1.0 N sulfuric acid will allow cystine concentrations up to approximately 0.1 M.

The total potential across the cell was increased in the direction of reduction at the mercury cathode until a suitable current was measured on the milliammeter. Approximately 45 milliamperes were passed, which allowed the reduction to proceed at a reasonable rate. It was assumed that the applied voltage of the cell would approximate sufficiently the cathode voltage. As the reduction proceeded, the applied voltage was maintained constant; the current fell slowly with the passage of time, but no attempt was made at current regulation.

The aim at this time was to achieve total reduction, with or without good electrode efficiency, and it was intended to let the current flow until determination of the thiol content indicated a completed reduction. The method of determination used, amperometric titration with ferricyanide, is described in the experimental chapter. None of several significant attempts resulted in completed reduction. Plots of thiol content in the catholyte versus time showed a rapid increase at first, a tendency to level off at anywhere from 40 to 90 percent of complete reaction, and then usually a slow decline. A strong odor would ensue toward the end of the reaction. The significance of this was not at first appreciated. It now appears likely that, as the process neared completion and the amount of unreacted cystine became small, the cathode voltage increased above a critical value (negatively) and that cystine was reduced beyond the cysteine stage to some volatile compound. The

design of the apparatus was not consistent with the necessity for a highly constant and precise cathode voltage.

Principles of Proper Experimental Design

The most important point brought out by the early experiments is that the cathode voltage is of determining importance. It must be maintained, precisely, at a value more negative than the reduction potential of cystine to cysteine but less negative than the potential at which any further reaction takes place. Maintaining the potential below a certain value may make the allowable current small and the reduction lengthy so it may become desirable to interrupt the reduction at some point short of completion. This, in turn, makes it desirable to realize perfect, or nearly perfect, electrode efficiency. In order to realize such efficiency, the cathode potential must be controlled in such a way that hydrogen evolution or other reaction of the solvent would not occur. If this were realized, standard solutions of cysteine (containing varing amounts of cystine) might be prepared, the thiol titer of which could be determined from the amount of current passed through the circuit.

Reduction at Constant Cathode Potential

Figure 4 shows a schematic diagram of the circuit and cell used for reduction at constant cathode potential. Additional particulars are given in the following chapter.

E = 2,6-volt storage batteries
M = pH meter for S.C.E. and glass electrodes in
C = titration coulometer (silver spiral and platinum cathode in beaker)
MA = milliammeter
A = microammeter
S = double pole-double throw switch
R = decade box
P = potentiometer
C = cell comprising platinum gauze anode, removable agar bridge, and cathode compartment with S.C.E. reference electrode for determining potential of mercury cathode

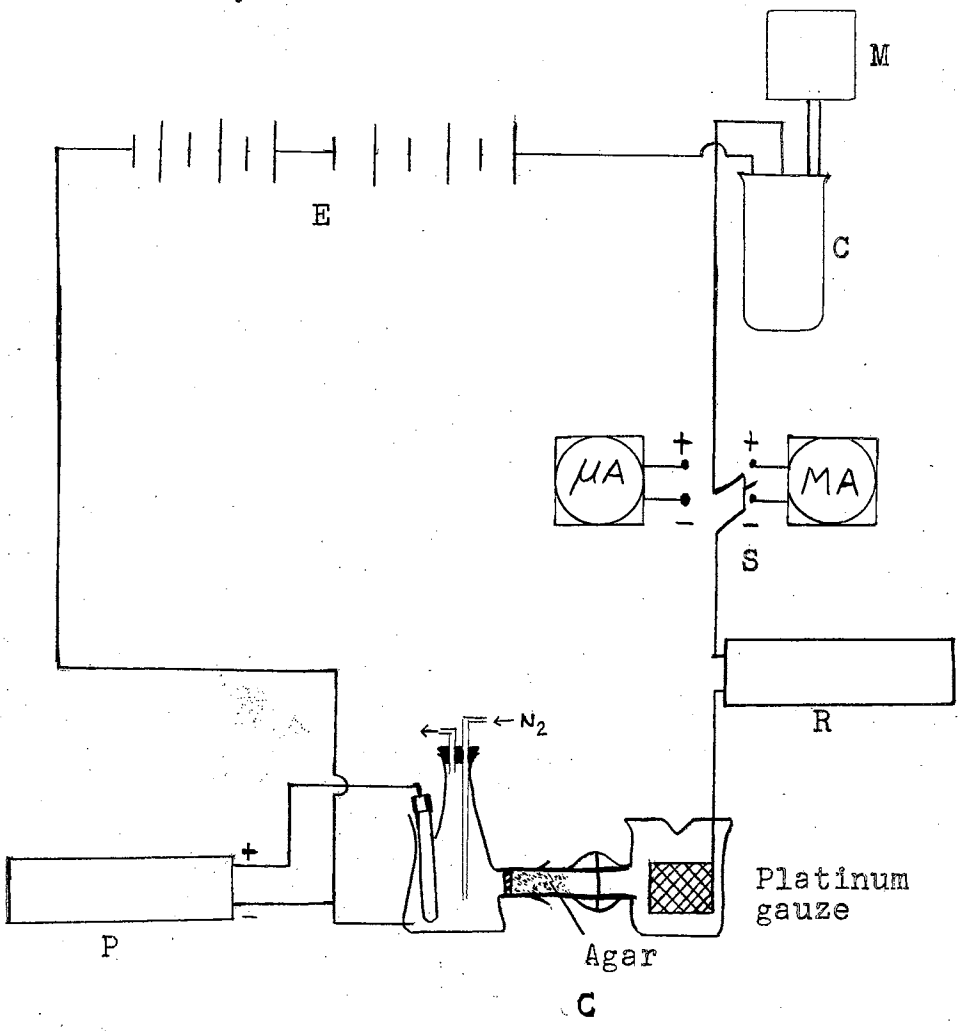

Figure 4.

Reduction at Constant Cathode Potential.

The tangent potential for the reduction of cystine is known from standard tables to be -0.4 volts versus the saturated calomel electrode. (21). In order to ascertain the most negative voltage allowable for the reduction of cystine to cysteine, a voltammetric curve employing the same circuit and cell was made. It is shown in Figure 5. The concentration of cystine was approximately 0.1 M, and the solvent 0.5 N sulfuric acid. The data for the curve were collected by applying increasing cathode voltages to the system and reading the resulting current. The tangent potential associated with this reaction indicates an upper limit of -0.64 volts to the voltage which might be applied without causing other reactions. This value is determined by extrapolating the steeply sloping line to its intersection with the abscissa. The value -0.50 volts was chosen as the voltage at which the reduction would be carried out.

If a potential of this magnitude is applied to the solvent containing no cystine, a substantial current flows at first, which is due to dissolved oxygen and impurities. In a typical experiment at a potential of -0.6 volts with respect to the saturated calomel electrode (slightly higher than the potential to be used in the reduction of cystine) the initial current was about 8 milliamperes, but this fell to less than 100 microamperes in about 30 minutes. Thus the residual current could be reduced to an acceptably low value by pre-electrolysis of the medium combined with

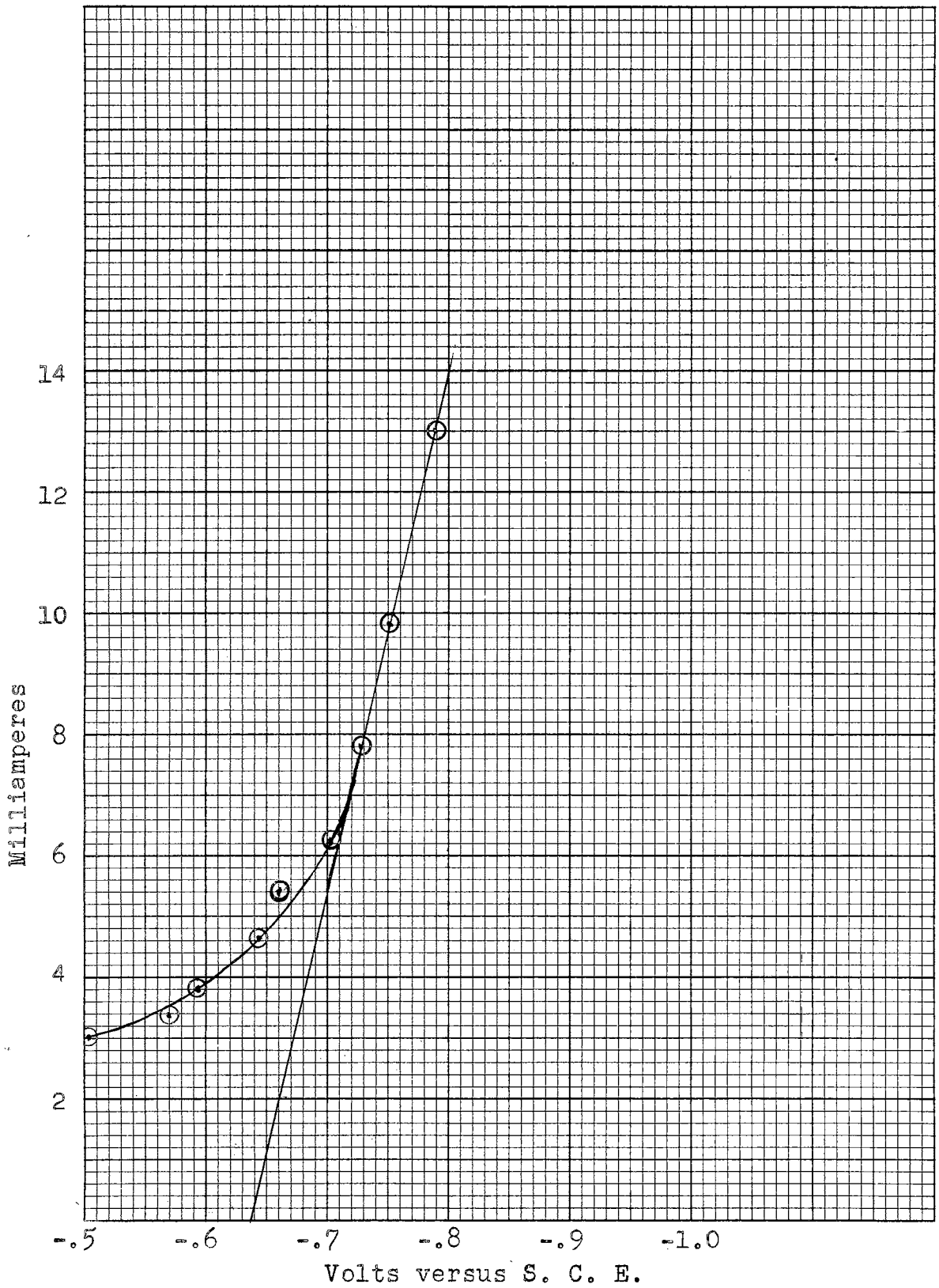

Figure 5. Voltammetric Reduction Curve of Cystine.

0.1 M solution in 0.5 N sulfuric acid
(very slow stirring)

nitrogen degassing. Arrangements then had to be made for introducing the cystine sample without opening the cell. Other factors requiring attention included the rate of stirring and the volume change consequent upon addition of a fixed amount of cystine. The necessary precautions are detailed in the following chapter.

The results are expressed in terms of electrode efficiency. This is calculated by comparing the coulometric titer with the thiol group titer, this being determined, as before, by titration with ferricyanide. Data for two concentrations of cysteine are found in Table III, and show an electrode efficiency of 96.9 percent for solutions

TABLE III

ELECTRODE EFFICIENCIES IN CYSTINE REDUCTION

Conc.	No. of runs	Electrode Efficiency	Average Deviation
0.005 M	4	96.9%	.50
0.01 M	4	99.3%	.88

about 0.005 M and 99.3 percent for solutions about 0.01 M. Inhomogeneous potentials at the mercury surface, "hot spots", could be responsible for the slightly low values. Results indicate an electrode efficiency approaching 100 percent as one increases the percentage of conversion.

CHAPTER V

EXPERIMENTAL

Analysis of Cysteine

Cysteine hydrochloride hydrate (California Corporation for Biochemical Research; $[\alpha]_D = 4.5^\circ$, c = 12 in 1 N HCl) was used in all experiments. The sample was not dried, to avoid possible decomposition.

The Kjeldahl determination of nitrogen was performed according to standard procedure. (22). The results are reported in Table I, page 4.

The determination of chloride presents a somewhat more difficult problem. Cysteine complexes with silver ion and the Volhard determination of chloride cannot be applied without first decomposing the compound. This was done by the Parr bomb method. The sample was mixed thoroughly with sodium peroxide, sodium nitrate, and a small amount of benzoic acid, and fired electrically. (17). The ash was carefully dissolved in water, and the strongly alkaline solution made strongly acid with concentrated nitric acid. A measured excess of standard silver nitrate was added along with ferric alum indicator and nitrobenzene, and back titrated with standard thiocyanate. The result of Table I is the average of three determinations.

The determination of cysteine content by amperometric titration with ferricyanide (4) is particularly important and will be described in detail. Twenty five milliliters of 1.5 M air-free phosphate buffer (pH 7) containing a trace of cupric ion was placed in a 100-ml. Berzelius beaker and to it was added the cysteine sample, containing about 0.25 millimoles (25 ml. X 0.01 M). A microburette with capillary tip was used to dispense standard potassium ferricyanide (0.05 M is convenient). A constant-speed electric moter operated the stirrer. Two platinum electrodes dipped into the solution, and were connected in series to a galvanometer. These electrodes were polarized by a 50-millivolt source of direct current. When a slight excess of titrant was added, a current began abruptly to flow, owing to the establishment of the ferri-ferrocyanide couple. Galvanometer readings were plotted versus milliliters of excess titrant and an extrapolation to zero current indicated the endpoint. The result in Table I is the average of five determinations.

The procedure for the acidimetric titration of cysteine is described in connection with the experiments measuring the pK values. The result of Table I is the average of four titrations.

Ionization Constant Measurements

A Beckman Model G pH meter, calibrated before each use with two standard buffers (pH = 4 and pH = 8 $\pm$ 0.02) was used

for the potentiometric titrations. A Beckman Type "E" (high pH) glass electrode was used because of its greater sensitivity for strongly alkaline solutions.

In all the experiments with cysteine, the greatest precautions were exercised in order to minimize oxidation. The water used was distilled, passed through Amberlite MB-1 mixed ion-exchange resin, boiled, cooled under a stream of nitrogen and stored out of contact with atmospheric oxygen. Nitrogen was of commercial grade and bubbled through a vanadous ion solution to remove oxygen. (18). Purified nitrogen was also passed through or over all easily oxidizable materials, such as the cysteine solutions, whenever their containers were open to the air. Standard sodium hydroxide (0.1 N) was prepared from a 5.00 M concentrated reagent, and its titer checked against potassium acid phthalate. Potassium chloride solutions, 0.1 M, were prepared from A. C. S. reagent grade salt. Stirring for the titrations was provided by bubbling nitrogen through the solution.

Figure 1, page 7, represents a plot of potentiometric titration data. The first inflection corresponds to the titration of hydrochloride, and the second to the titration of amino acid. The second end point cannot be located with great accuracy; an attempt was made to locate it statistically with the aid of the IBM 650 computer and the average deviation of single determinations was found to be 5 percent. The point at which pH would equal pK was taken as that after

addition of 1.5 times the amount of base required to reach the first endpoint.

The values of pK_2 found with 0.01 M cysteine solutions in water and in 0.1 M potassium chloride are given below:

	No. of determinations	pK_2	Average Deviation
water	7	8.30	.03
KCl	7	8.32	.03

Spectrophotometric Determination of R

All spectral measurements were made at 230 mμ (absorption maximum) with the Beckman model DU spectrophometer, using 1.00-cm. silica cells.

Ethanol of reagent grade was used. Different samples of this substance showed appreciable and variable absorption at 230 mμ when measured against a distilled water blank. Freshly distilled ethanol gave a lower absorption reading, but this would increase on standing a few days. Bubbling nitrogen through the ethanol had an effect similar to distilling it. Cysteine solutions prepared with ethanol that had a low absorption had the highest absorptions. It is considered likely that acetaldehyde, formed by autooxidation, is responsible for these effects; this impurity would give a higher blank absorption, and react with the cysteine, lowering the absorption due to it. The following data show that no irreversible reaction with the cysteine occurred in 92 percent alcohol when the latter had been purged with nitrogen immediately before:

Stock solution, 0.2339 g. of cystine hydrochloride hydrate in 100 ml. H_2O, containing 5 ml. of 0.1 N NaOH/each 30 ml.

Stock solution, 5 ml. diluted with 50 ml. H_2O and 55 ml. EtOH; final cysteine concentration 5.00×10^{-4} M, percentage of alcohol 45 — O.D. 0.276

Stock solution, 5 ml. diluted with 50 ml. EtOH final cysteine concentration 10.0×10^{-4} M, percentage of alcohol 91 — O.D. 0.183

Solution in 91% alcohol, diluted with H_2O to 110 ml. — O.D. 0.273

The experiment that involved dilution with isooctane was performed by making an approximately 0.01 M solution of cysteine, adding one and one-half equivalents of base, as usual, and diluting an aliquot portion, on the one hand with alcohol to a final proportion of 95.25% and on the other with the same volume of ternary mixture consisting of 30% isooctane, 68% ethanol, and 2% water (percentages by volume, neglecting volume changes due to mixing). The optical densities obtained were as follows:

Stock solution, 0.2346 g. of cysteine hydrochloride hydrate in 100 ml. of water, containing 5 ml. 1 N NaOH for each 30 ml.

Stock solution, 5 ml., diluted with 100 ml. of ethanol; final concentration of cysteine 5.30×10^{-4} M, proportion of ethanol 92.3% — O.D. 0.271

Stock solution, 5 ml., diluted with 100 ml. of ternary mixture — O.D. 0.277

The experiment done to ascertain the effect of the medium on the spectrum of cystine was performed as follows:

Stock solution, 0.1403 g. of cysteine
in 0.5 N hydrochloric acid

2.00 ml. diluted to 50 ml.	with water	O.D. 0.067
	with KCl	O.D. 0.066
	with EtOH	O.D. 0.068

Stock solution, 0.2782 g. of cysteine
in 0.5 N sodium hydroxide

2.00 ml. dilutedto 50 ml.	with water	O.D. 0.213
	with KCl	O.D. 0.222
	with EtOH	O.D. 0.230

The data given in Table II were obtained in the following way. Immediately before use, nitrogen was bubbled through the three media to be employed, i. e., ethanol, water, and saturated potassium chloride solution. A 0.01 M solution of cysteine was prepared fresh for each experiment by dissolving a weighed sample of cysteine hydrochloride in 100 ml. of water. One and one-half equivalents of sodium hydroxide was added with a 5-ml. pipette. A 2-ml. aliquot was diluted with 50 ml. of water, and a 4-ml. aliquot was diluted with 50 ml. of ethanol. The optical density was determined exactly two minutes from the time of mixing. Distilled water was used as a blank. The absorptions displayed by the media alone against distilled water were subtracted from the measured values. The data are reported in Table IV. The data of Table II were calculated from these by adjusting the concentrations to the same basis, a cysteine concentration of 4.26×10^{-4} M.

TABLE IV

OPTICAL DENSITIES OF CYSTEINE IN THREE DIFFERENT MEDIA

Wt of Cysteine HCl. H_2O, g.	O. D.$_{KCl}$	O. D.$_{H_2O}$	O. D.$_{EtOH}$
0.2339	.739	.661	.150
0.2339	.731	.655	.143
0.2339	.722	.652	.131
0.2339	.767	.692	.121
0.2339	.764	.693	.111
0.2339	.727	.666	.146

The data represented in Figure 2 were obtained in the following way. An approximately 0.01 M solution of cysteine was prepared in water. Aliquot portions were withdrawn, and diluted with potassium chloride solution or alcohol to give several desired concentrations of either component.

The Reduction of Cystine

The cystine used was optically standardized L-cystine, obtained from the Schwarz Laboratories, Mount Vernon, New York. Mercury was of technical grade, and was reused after cleaning it each time it was used. The cleaning was effected by passing the mercury in minute droplets through a 4-ft column of nitric acid, and rinsing it with water.

The apparatus used is sufficiently described by Figure 4, page 19. A magnetic stirrer was used to stir the catholyte. The cathode compartment was charged with 30 ml. of mercury and exactly 49.1 ml. of 0.1 N sulfuric acid; it was determined that this volume would expand to 50.0 ml. when the appropriate amount of cystine was added. Approximately 100 ml. of the same solution were placed in the anode compartment. The sulfuric acid was not degassed because preelectrolysis of the solvent could be conveniently used to reduce impurities, including oxygen.

A voltage of -0.6 volts versus S. C. E., slightly higher than the reduction voltage of the experiment proper, was then applied. The microammeter was connected into the circuit from time to time to determine when the current had fallen to a suitably small value. As long as this value was less than 100 microamperes, it was stable enough to be considered constant. The cystine sample was added without opening the vessel to the air with the aid of an injector made from the sleeve of a hypodermic syringe. The coulometer was connected into the circuit, and the starting time recorded. The cathode potential was held constant at -0.5 $\pm$ .05 volts. The highest rate of stirring physically compatible with cell design allowed a current of approximately 15 milliamperes to pass. A total reduction time of about 1.5 hours was therefore required to generate a 0.01 M cysteine solution, or 45 minutes for 0.005 M. At the end of this time, the current had fallen to around 10 milliamperes.

The circuit was then opened, the time recorded, and a 2 ml. aliquot of the catholyte withdrawn for analysis. The hydroxyl ion generated in the coulometer (.02 M KBr +2 M K_2SO_4) solution was titrated with standard 0.1 M sulfuric acid, (19) and the total quantity of electricity established. The number of coulombs due to the residual current was then subtracted. The resulting value was divided into the number of milliequivalents of thiol (ml. ferricyanide X Normality X 25*) to determine the electrode efficiency. The results are enumerated in Table III, page 22.

*The 2 ml. aliquot withdrawn is 1/25 of the total volume reduced.

CHAPTER VI

SUMMARY AND CONCLUSIONS

A sample of cysteine hydrochloride hydrate used in this work was analyzed by several techniques. The sample was found to be sufficiently pure for the purpose, although it contained small amounts of cystine and other impurities.

The pK_2 of cysteine hydrochloride was determined both in water and 0.1 M potassium chloride. The value in 0.1 M potassium chloride was 8.32, and this, when corrected for activity (20), results in a thermodynamic value of 8.37. The value found in dilute aqueous solution was 8.30. The agreement is within experimental error.

The concentration of the two tautomeric ions formed concomitantly during the second ionization of cysteine hydrochloride (see page 7 for structures) in dilute aqueous solution has been estimated from a comparison of the ultraviolet absorption in water to that exhibited in saturated potassium chloride and 92% ethanol. These media are designed to favor the formation of the tautomers I and II, respectively. The ratio of the concentrations is 8.4 in favor of ionization from the sulfhydryl group. This value is higher than previous estimates; the assumptions upon which the calculations are based may be somewhat in error,

but it is surely indicated that the acid strength of the mercapto group is significantly greater than that of the ammonium group.

An electrolytic method for reducing cystine has been studied. Solutions of cysteine of varying known concentration have been prepared by quantitatively reducing cystine in 0.1 N sulfuric acid at a large-area mercury cathode. The resulting solutions contained varying amounts of unreduced cystine, but this would not interfere in many investigations involving cysteine.

BIBLIOGRAPHY

1. Barron, E. S. G., and Dickman, S., J. Gen. Physio. 32, 595 (1949).

2. Block, R. J., and Bolling, D., The Amino Acid Composition of Proteins and Foods, Charles C. Thomas, Springfield, Ill., 186-223(1951).

3. Chinard, F. P., and Hellerman, L., in Methods of Biochemical Analysis, Vol. I, Interscience Publishers, New York, 1 (1954).

4. Wadill, H. G., and Gorin, G., Anal. Chem., 30, 1069 (1958).

5. Li, N. C., and Manning, R. A., J. Am. Chem. Soc., 77, 5226 (1955).

6. Albert, A., J. Biochem., 50, 693 (1952).

7. Gorin, G., J. Am. Chem. Soc., 78, 767 (1956).

8. Borsook, H., Ellis, E. L., and Huffman, H. M., J. Biol. Chem., 117, 292 (1937).

9. Grafius, M. A., and Neilands, J. B., J. Am. Chem. Soc., 77, 3389-90 (1955).

10. Ryklan, R. L., and Schmidt, C. L. A., Arch. Biochem., 5, 89 (1944).

11. Edsall, J. T., and Wyman, J., Biophysical Chemistry, Vol. 1, Academic Press, New York, N. Y., 496-504 (1958).

12. Benesch, R. E., and Benesch R., J. Am. Chem. Soc., 77, 5877 (1955).

13. Garfinkel, D., and Edsall, J. T., J. Am. Chem. Soc., 80, 3823 (1958).

14. Noda, L. H., Kuby, S. A., and Lardy, H. A., J. Am. Chem. Soc., 75, 913 (1953).

15. Gorin, G., Abstracts, 130th National Meeting, American Chemical Society, Atlantic City, N. J., 17-C (September, 1956).

16. Allen, M. J., Organic Electrode Processes, Reinhold Publishing Corp., New York, N. Y. (1958).

17. Lemp, J. F., and Broderson, H. J., J. Am. Chem. Soc., 39, 2069-74 (1917).

18. Meites, L., Polarographic Techniques, Interscience Publishers, New York, N. Y., 34 (1955).

19. Lingane, J. T., Electroanalytical Chemistry, Interscience Publishers Inc., New York, N. Y., 354 (1953).

20. Kielland, J., J. Am. Chem. Soc., 59, 1675 (1937).

21. Handbook of Chemistry and Physics, 37th Edition, Chemical Rubber Publishing Co., Sandusky, Ohio, p. 1632 (1955).

22. Kolthoff, I. M., and Sandell, E. B., Textbook of Quantitative Inorganic Analysis, The MacMillan Company, New York, N. Y., 536 (1936).

VITA

C. W. Clary

Candidate for the Degree of

Master of Science

Thesis: CYSTEINE: IONIZATION CHARACTERISTICS AND ELECTROLYTIC PREPARATION FROM CYSTINE

Major: Chemistry

Biographical and Other Items:

Born: May 11, 1935, at Oklahoma City, Oklahoma

Undergraduate Study: Attended county grade school in Oklahoma County, graduated from Northeast High School, Oklahoma City, Oklahoma in 1953; attended Eastern Oklahoma A and M College until 1955, received Bachelor of Science degree from Central State College, Edmond, Oklahoma, with major in chemistry, in June 1957.

Graduate Study: Entered Oklahoma State University in September, 1957, pursuing work toward the degree of Master of Science, and completed the requirements in March, 1959.

Organizations: American Chemical Society, Phi Lambda Upsilon, and Phi Theta Kappa.

Date of Final Examination: April, 1959.