THE DETERMINATION OF CYSTEINE

By

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CHAPTER I

INTRODUCTION

Compounds containing the thiol group are present in most biological systems, and take part in a great number of biological reactions. Knowledge concerning them has been accumulating within the past thirty-five years, and it is now abundantly clear that thiol compounds are very important in the chemistry of living organisms.

Thiols are extremely reactive substances. They are easily oxidized or reduced; they combine with a large number of heavy metals, often reversibly, to give mercaptides; they react with many halogen-containing substances, usually irreversibly; and finally, they have been found to combine with aldehydes, quinones, carbonyl compounds, and olefins.

Thiol groups appear to be necessary for the activity of many enzymes. The possible role of the thiol group and the type of thiols which are responsible for this activity have been studied by Barron and Dickman (4) and Hellerman, et al. (10). It is certain that soluble thiols contribute to the regulation of cellular respiration.

The formation of actomyosin and muscle contraction seem to require fixed thiol groups. This was shown in work by Bailey and Perry (3) and Mendez and Peralta (19).

A definite relationship between cell division and thiol concentration has been demonstrated by Rapkine (25). The thiol content increases during development of the cell and reaches its maximum level just prior

to cell division. Mazia (17) has suggested that formation of the mitotic spindle involves oxidation of thiol groups, giving disulfide cross-linkages between protein fibers.

Baumberger (5) has suggested that the conversion of fibrinogen to fibrin in blood coagulation is due to air oxidation of sulfhydryl groups in fibrinogen.

Many antibiotics have been shown to be inactivated by cysteine, glutathione, or other thiols. These anitbiotics contain aldehyde, carbonyl, or olefin groups, with which thiol groups can react, and the antibiotic activity may be due to the removal of essential thiol groups in this way.

The successful use of glutathione in the treatment of certain cases of diabetes indicates that this thiol-containing tripeptide may be involved in the formation of insulin and in its action.

In the investigation of the functions briefly mentioned above, about which much remains to be learned, and in the discovery of new functions, which will surely take place in the future, the analytical determination of thiol groups is of fundamental importance. In this, as in other problems, accurate analysis is the cornerstone on which sound chemical conclusions must be founded. Thus, new and better methods for the determination of thiol-containing compounds are needed, especially methods capable of application at the low concentrations in which these compounds are usually found.

The purpose of the work reported here was to develop a sensitive and accurate method for the determination of cysteine and to investigate its applicability at low concentrations.

CHAPTER II

METHODS FOR THE DETERMINATION OF CYSTEINE

General Methods

Several excellent surveys of methods for determining cysteine and thiol groups are available. The most complete of these are to be found in the works by Block and Bolling (6) and by Chinard and Hellerman (7).

In general, methods for determining cysteine are based on three reactions of the thiol group; oxidation, mercaptide formation, and thioether formation. Probably the most widely utilized method is the oxidation of cysteine to the disulfide, cystine. The course of reaction may be represented by the equation,

$$2\ R\text{-}SH + \text{oxidant} \longrightarrow R\text{-}SS\text{-}R + \text{reductant} + 2H^{+} + 2e.$$

Many oxidizing agents have been investigated and can be used. Another method is the use of mercaptide-forming agents. These reagents react in the following general manner:

$$R\text{-}SH + R'\text{-}HgX \longrightarrow R\text{-}SHg\text{-}R' + H^{+} + X^{-}.$$

Since, in the reaction, the sulhydryl group is replaced by mercaptide, an external indicator specific for the thiol group may be used to indicate the end point. Finally, alkylating agents may be used; for example, iodoacetic acid reacts in the following manner:

$$R\text{-}SH + ICH_2CO_2 \longrightarrow R\text{-}S\text{-}CH_2CO_2H + HI$$

and the hydriodic acid liberated may be oxidized to iodine and

titrated by conventional means.

Several colorimetric methods have also been reported which employ more specific reactions. In recent years, instrumental methods, applying the above mentioned reactions of the sulfhydryl group, have been reported; especially noteworthy has been the development of amperometric and polarographic methods (13, 14).

Iodine and Ferricyanide as Reagents for the Determination of Cysteine

Many oxidizing agents have been used for the determinations of cysteine and sulfhydryl groups, as has been mentioned in the preceding section. Now we wish to examine more closely the applicability of these methods. In general, the requirements for a satisfactory reagent are that it oxidize cysteine stoichiometrically, preferably to cystine, and that it be specific for the sulfhydryl group.

The most thoroughly investigated and widely applied oxidizing agent has been iodine, but this reagent can oxidize cysteine beyond the disulfide stage. A number of methods have been recommended, but in most of them, excess oxidation occurs, and high results are consequently obtained.

Morner (22) first effected the determination of cysteine by titrating with standard iodine in 10% hydrochloric acid. He obtained results that were only approximate. Okuda (23) placed his cysteine sample in a solution one-tenth normal with iodide ion and six-tenths normal with hydrochloric acid, and titrated with standard potassium iodate. Considerable over-oxidation occurred and the cysteine titer had to be calculated

with an empirical factor, the magnitude of which was dependent on temperature and other conditions. Lucas and King (15) studied the reaction between cysteine and iodine and concluded that the oxidation was of a complex nature and that no simple procedure would give quantitative reaction. They found the best conditions for oxidation to be 0°C. temperature, and the presence of one normal hydrochloric acid and three-hundredths normal iodide ion in the titration medium.

Lavine (12) was the first to develop a quantitative iodimetric method that might be used at room temperature. He reported that the reaction was stoichiometric when an excess of iodine was allowed to react with cysteine in a medium one-molar in both hydrogen and iodide ions. The excess iodine was then back-titrated with standard potassium thiosulfate. A blank was run and the cysteine was calculated from the difference between the blank and the sample titer. Pilmer (24), in an evaluation of Lavine's method, found that both the time of reaction and the amount of excess iodine must be controlled in order to obtain reproducible results. He reported the results to be reproducible to within one and one-half percent. Pilmer developed a modified method, which employs a medium of pH 2 and one-molar in iodine ion. This medium he found to afford adequate protection against over-oxidation. His method did not require back-titration and is therefore more convenient than any of the others.

It is difficult to evaluate the accuracy of the methods referred to, because samples of pure cysteine cannot be easily obtained, and were not available to the investigators mentioned. Therefore, it is open to question, in most cases, whether a stoichiometrically exact relation could be established. It is fair to say, in conclusion, with

respect to iodimetric methods, that reproducibility can be achieved, albeit with difficulty and scrupulous control of conditions; but their accuracy has not been satisfactorily established. Also, many substances interfere with the determination, since they also reduce iodine.

In view of the shortcomings listed for the oxidation of cysteine by iodine, other oxidizing agents were considered, and ferricyanide was the one we would find useful in our work. Mason (16) used potassium ferricyanide first for the assay of cysteine samples. The determination was carried out in a buffer of about pH 7.4 and the end point was signalled by the appearance of the permanent yellow color of potassium ferricyanide. Owing to the relatively pale color which denotes the end point, a large blank is necessary even with rather concentrated ferricyanide solutions, and the method is not applicable at low cysteine concentrations.

Kendall and Holst (11) used ferricyanide to titrate cysteine, in the course of their studies upon certain cobalt complexes of cysteine. Although they did not describe their procedure, apparently it was similar to that of Mason.

Anson (1) was first to establish that the reaction between cysteine and ferricyanide at pH 6.8 is stoichiometrically exact. He also found that at this pH there is little or no interference from other oxidizable amino acids.

Mirsky (21) developed a practical method for determining cysteine with ferricyanide. According to this method, an excess of ferricyanide was added to the cysteine, and the amount of ferrocyanide produced was determined colorimetrically by adding ferric sulfate

to form Prussian blue. He obtained a precision of about ±5%. This method has been used, with some modification, in several studies upon cysteine and the physiological role of thiol groups. However, the results were not very accurate, by chemical standards.

It seemed to us that the use of ferricyanide as an oxidizing agent for the determination of cysteine could be improved in precision and sensitivity. Therefore, a more systematic investigation was undertaken and will be described in Chapter III, Ferricyanide Method. Also, the iodimetric methods, which were used previously to and concurrently with the ferricyanide methods are described in Chapter III, Iodimetry.

CHAPTER III

METHODS EMPLOYED IN PRESENT WORK

Iodimetry

Lavine's method and the modified method developed by Pilmer both were used in an attempt to determine the purity of commercial samples of cysteine and of samples produced by reduction of cystine.

The method of Lavine consists, as mentioned, of treating the cysteine sample, in the presence of one-molar hydrogen and iodide ions, with an excess of iodine. After a period of time, needed to secure completeness of reaction, the excess iodine is back-titrated with standard sodium thiosulfate. A blank is run and the amount of cysteine present is calculated by the equation:

$$N_{CySH} = \frac{(ml_{Blank} - ml_{Sample})\, N_{S_2O_3^=}}{ml_{CySH}} .$$

Pilmer's method requires adjustment of the cysteine solution to a pH of 2 and addition of potassium iodide to give a 1 molar concentration. The titration is carried out with standard iodine solution, prepared from potassium iodate and potassium iodide, of approximately the same concentration as that of the cysteine, and the end point is detected with starch indicator. Since the method employs a direct titration and no blank is necessary, it is more convenient than the method of Lavine, and eliminates one source of

error.

Both methods were applied to commercial cysteine samples and to samples of reduced cystine. The complete results are reported in Chapter IV.

Lavine's method was found to give rather variable results. Usually, samples of cysteine were run in triplicate under identical conditions with a single blank being run for the three. Typical results are shown in the following table:

Table I

Aliquots of Commercial Cysteine Sample Number II
Assayed by Lavine's Method

Aliquot number	1	2	3
Ml of $S_2O_3^=$ for blank	11.62	11.62	11.62
Ml of $S_2O_3^=$ used	7.01	6.95	7.11
Normality cysteine (from assay)	0.0987	0.1000	0.0966
Normality cysteine (theoretical)	0.0993	0.0993	0.0993
Percent purity	99.5	100.8	97.4

With such variations found in samples treated in exactly the same manner and titrated at the same time, it can be seen that an accuracy greater than $\pm 2\%$ cannot be expected.

More consistent results were obtained by using the method of Pilmer, although the precision is not entirely satisfactory; a typical set of determinations is shown in Table II.

It can be seen, that, unfortunately the results of the two methods do not agree. This might be due to the fact that the end point is somewhat indefinite, owing to slow reaction near it, and that there is, consequently, a tendency to undertitrate; this causes high results

by Lavine's method and low results by Pilmer's. In any case, it appears that these methods are not sufficiently reliable for an absolute determination of the purity of cysteine.

Table II

Aliquots of Commercial Cysteine Sample Number II
Assayed by Pilmer's Method

(Normality of iodine = 0.1063 N)

Aliquot number	1	2	3
Ml of I_2	4.37	4.41	4.35
Normality of cysteine (from assay)	0.0940	0.0940	0.0936
Normality of cysteine (theoretical)	0.0940	0.0984	0.0984
Percent purity	95.5	96.4	95.1

Determination of Cysteine with Ferricyanide With Two Polarized Electrodes

The method utilized for the analysis of cysteine in the work to be described is an adaptation of the so-called "dead-stop" method. This method, first described by Foulk and Bawden (9), uses two electrodes and a low potential difference between them, which is balanced by the back e.m.f. of polarization. With the small potential, no current can flow between the electrodes unless there is present a reversible oxidation-reduction couple, one member of which can be oxidized at the anode while the other is reduced at the cathode. Foulk and Bawden applied the method to the titration of iodine by thiosulfate. Upon addition of the first amount of thiosulfate, the couple, iodine-iodide, is set up. This results in a surge of current that is measured on a galvanometer. With successive additions of thiosulfate, iodine gradually disappears and the flow of current stops,

when all the iodine has been consumed. The galvanometer then abruptly returns to the zero-point; thus, the method was named the "dead-stop" method.

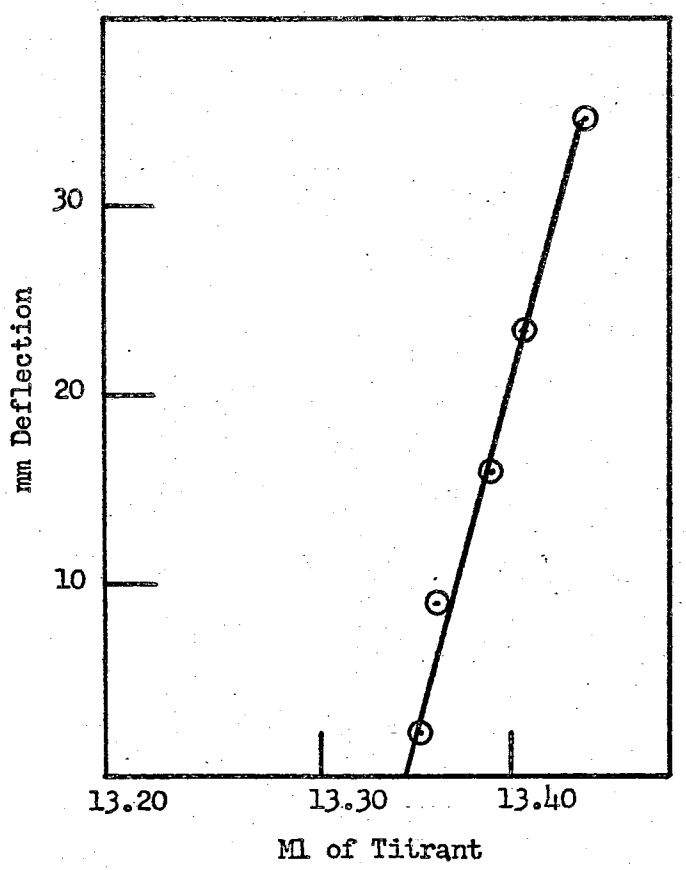
The method employed in this work involves the principle in reverse. Two platinum electrodes and the galvanometer are connected in series to a source of direct current of about fifty millivolts potential. The cysteine sample is added to a phosphate buffer of pH 7 and placed in contact with the electrodes. Potassium ferricyanide is added and oxidizes the cysteine to cystine, while it is itself reduced to ferrocyanide. As long as the ferricyanide is consumed, no current flows through the system, but when the cysteine is completely oxidized, addition of a slight excess of ferricyanide establishes the ferricyanide-ferrocyanide couple, and a surge of current is registered on the galvanometer. A plot of galvanometer deflections versus volume of titrant yields the end point by extrapolation to zero deflection. A typical plot is shown in Figure 1.

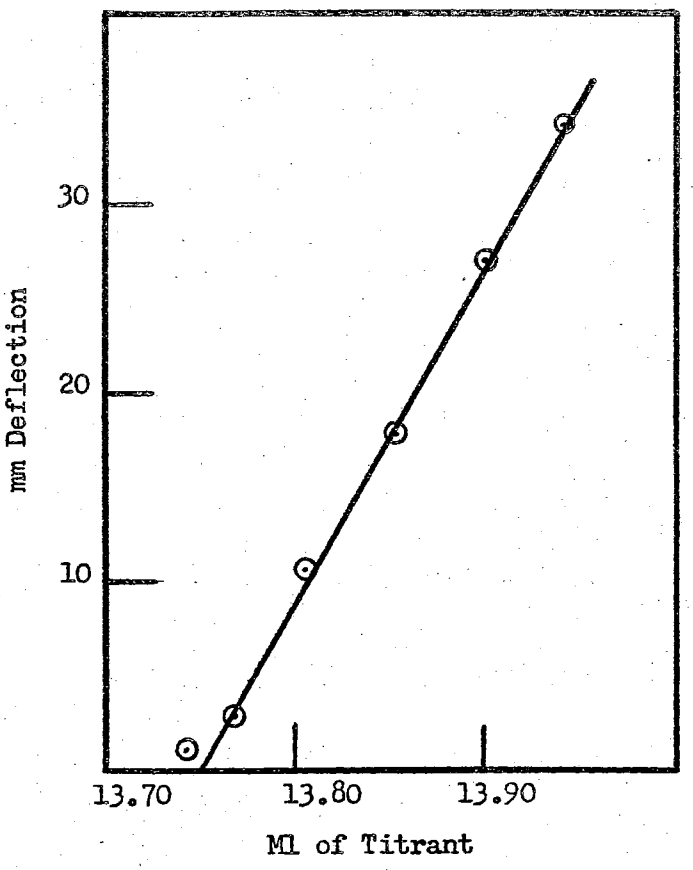
In the first titrations by the ferricyanide method, a relatively long period of time was required for the reaction of each added portion of titrant. This was time-consuming, and also increased the possibility of adventitious air-oxidation. Addition of a trace amount of cupric ion, which Anson (2) had shown to be a catalyst for the oxidation of cysteine by ferricyanide and other oxidizing agents, completely obviated this difficulty. The amount of catalyst was kept below one percent of the cysteine concentration, and this amount apparently did not affect the reaction in any way except by catalysis. A smaller amount of catalyst would probably be sufficient.

Since Anson had determined that the reaction between ferricyanide

Figure 1

Typical Dead-Stop End-Point Plots





and cysteine at pH 6.8 was stoichiometric and subject to little or no interference from other oxidizable amino acids, a phosphate buffer of pH 7 was used in the titrations. Twenty milliliters of buffer were usually used and the addition of as much as five milliliters of cysteine, one normal in hydrochloric acid, was found not to change the pH of the solution appreciably.

Table III summarizes the results obtained with two different commercial samples of cysteine, from which various stock solutions were prepared; several aliquots of different size were, in turn, taken from each stock solution. The precision of the method can be evaluated from the reproducibility of the value of percentage purity calculated for each sample. However, the accuracy cannot be assessed, because the actual purity of the samples is not known. The average of results obtained by iodimetric methods are reported for comparison, but reasons have already been given, why these results are also in doubt. In the next section, the determinations of samples prepared by the reduction of cystine will afford an independent check on the accuracy of the method.

Table III

Summary of Determinations on Cysteine Samples I and II

Sample No. and Method	Approx. Conc. of Stock Solutions	Number of Determinations	Average % Purity	Average Deviation
I-Lavine	0.1-0.2 M	2	96.1	
I-Pilmer	0.1-0.2 M	2	97.5	
I-$Fe(CN)_6^{\equiv}$	0.1-0.2 M	8	96.4	0.18
I-$Fe(CN)_6^{\equiv}$	0.01-0.02 M	6	95.8	0.52
II-Lavine	0.1-0.2 M	2	99.2	
II-Pilmer	0.1-0.2 M	2	96.2	
II-$Fe(CN)_6^{\equiv}$	0.1-0.2 M	15	96.4	0.47
II-$Fe(CN)_6^{\equiv}$	0.01-0.02 M	6	96.9	0.21
II-$Fe(CN)_6^{\equiv}$	0.001-0.002 M	4	96.9	0.15

While reference should be made to Chapter IV, Experimental Methods and Results, for the exact procedure by which these results were obtained, it is perhaps well to mention here, for added emphasis, that reproducible results can be achieved only if air oxidation is avoided, and that this demands much care. Deoxygenated deionized distilled water was used for the making up of the reagents and buffer; purified nitrogen was passed through the buffer solution before addition of the cysteine sample, and over the solution during the titration. In preparing dilute stock solutions, 1.0 normal hydrochloric acid was used as a medium; when stock solutions were made up in water, low results were obtained, even though the water had been deoxygenated as much as possible.

In order to verify the expectation that oxidizable amino acids would not interfere, the method was applied in the presence of a mixture of amino acids (tryptophan, threonine, valine, leucine, isoleucine, lysine, methionine, phenylalanine, glutamic acid, and aspartic acid, the solution being 0.01 molar in each amino acid). Also, since ferricyanide would be particularly liable to oxidize the phenolic groups of tyrosine and serine, each of these amino acids was added individually to samples of cysteine. It can be seen from Table IV that the added amino acids had no effect on the titrations.

Apparently other oxidizable amino acids are attacked only in a more alkaline medium. This is in accordance with the findings of Mirsky (20).

No other substances containing sulfhydryl groups were titrated in conjunction with cysteine. It should be realized that, since

the method is specific for sulfhydryl groups, substances like glutathione, that contain a sulfhydryl group, would interfere with the determination of cysteine.

Table IV

Cysteine Determinations[1] in Presence of Amino Acid Mixtures[2] and with Individual Amino Acids

Amino Acid Present	Percent Purity With Amino Acid	Percent Purity Without Amino Acid
Mixture	96.96	97.14
Mixture	96.35	96.82
Serine	96.96	96.73
Tyrosine	97.70	97.85

[1]Assay by ferricyanide method.

[2]Mixture 0.01 molar in leucine, isoleucine, lysine, methionine, phenylalanine, tryptophan, threonine, valine, aspartic acid, and glutamic acid in 0.1 molar hydrochloric acid.

Potassium ferricyanide in aqueous solution is unstable, and deteriorates on standing. It was necessary to prepare fresh solutions as needed. Care was taken to avoid exposure of the fresh solutions to direct sun light which affects ferricyanide appreciably in a few minutes.

The Reduction of Cystine

It is very difficult to obtain cysteine in pure, solid form. This amino acid, as has previously been mentioned, is susceptible to atmospheric oxidation, and contamination is, accordingly, unavoidable, unless air is totally excluded. The rate of oxidation is diminished in acid solution and oxidation is reduced, but not completely avoided, by maintaining cysteine as its hydrochloride. Shinohara, who investigated the purity of some commercial samples available in 1935, found 5 to 15% impurity, mostly cystine and water (26).

Much purer samples of cysteine are now available commercially, in the form of hydrochloride hydrate; but even these samples are only about 96% pure and cannot be used to test the accuracy of analytical methods. We attempted to purify such commerical samples by crystallization, but obtained a product more impure than the starting material. Since cysteine hydrochloride is exceedingly soluble in water and too sparingly soluble in most organic solvents, purification by ordinary crystallization procedures is almost impossible.

For this reason, the samples of cysteine in this investigation were commerical samples used without further purification, and our method was evaluated mainly be reference to the reproducibility of the thiol content found for a given sample under different conditions and with aliquot portions of variable size.

This procedure is not entirely satisfactory, however, and we sought, therefore, to prepare test samples of accurately known cysteine content by reducing samples of cystine quantitatively. The properties of cystine, notably its sparing solubility in water and its imperviousness to atmospheric oxidation, make it much easier to obtain this compound in pure form; commercial samples of good purity are available. Its unusually high optical rotatory power provides a reliable index of purity, since the common impurities, cysteine and water, have a small and no rotation, respectively.

The literature lists many methods purported to give quantitative reduction of cystine (6), but application of some of these methods failed to give us satisfactory results. Tin and hydrochloric acid were used by Lavine (12) for preparing samples used in testing the iodimetric method devised by him. After reduction, stannous ion was precipitated with hydrogen sulfide; stannous sulfide and excess tin were removed by filtration, and the excess

hydrogen sulfide was removed by passing nitrogen through the solution. We found the method unsatisfactory; it was very difficult to remove the hydrogen sulfide completely. It is significant, by the way, that Lavine reports values in excess of 100% for cysteine samples obtained in this way.

Zinc and hydrochloric acid were also used as reducing agents. Since the zinc ion formed in the reaction does not interfere with analysis by iodimetric methods, treatment with hydrogen sulfide and subsequent manipulations were unnecessary. The results were quite satisfactory. However, the presence of zinc ions in the solution is undesirable, since they would interfere in some uses to which the cysteine solution might be put. For example, zinc ion would interfere with the determination of the absorption spectrum. On the other hand, the difficulties involved in removal of the zinc ion would likely be equal to those encountered in removal of stannous ions. An alternative method of reduction was therefore sought.

Sodium was next considered as a reducing agent. Sodium amalgam had already been used by some investigators (27) for reduction of cystine. Furthermore, it can be expected that the sodium ion will not interfere with most possible uses. A satisfactory procedure was devised, and it is fully described in the experimental section. In brief, 1% sodium amalgam was prepared, best by dilution of 5% amalgam, which is commercially available; the amalgam was added drop by drop to a cystine solution which was sampled periodically and analyzed for cysteine; constant values were obtained, the cystine was considered to be completely reduced. A slight odor of hydrogen sulfide was detected during reduction, but apparently the amount is small and can be minimized by slower addition of amalgam.

Three different reduced samples from one sample of cystine were prepared and tested for thiol content. A total of thirteen titrations were made by

the ferricyanide method, with aliquot samples of varying concentration. The mean of the percent purity was 97.5%. The average deviation from the mean was 0.35. These results agree favorably with the purity indicated by the optical rotation of the cystine sample, which was 97.7%. The individual results are reported in Chapter IV.

CHAPTER IV

EXPERIMENTAL METHODS AND RESULTS

Lavine's Method

Preparation of Solutions

A standard iodine-iodide solution was prepared volumetrically from an exactly weighed amount of Analytical Reagent grade potassium iodate, 86 milliliters of concentrated hydrochloric acid and 166 grams of potassium iodide per liter of solution. The solution was stored in brown glass containers.

Six normal hydrochloric acid was prepared by dilution of 500 milliliters of concentrated acid to one liter.

A solution of 0.1 normal sodium thiosulfate was made and standardized by comparison with the iodine solution previously prepared.

The water used in preparing cysteine solutions was distilled water, first passed through Amberlite MB-1 mixed-bed ion-exchange resin, then boiled; while cooling, nitrogen was bubbled through it. It was stored in a container, from which it could be siphoned, without coming in contact with atmospheric oxygen.

Commercial samples of cysteine hydrochloride monohydrate were obtained from the California Foundation for Biochemical Research, Los Angeles, California (Sample II) and from Schwarz Laboratories, Mount Vernon, New York (Sample I). The samples were stored in a desiccator in a cooler near 0° C.

One-tenth molar solutions were prepared by dissolving a weighed amount of cysteine hydrochloride monohydrate in "oxygen-free" water and diluting to 100 milliliters.

Procedure

Ten milliliters of standard iodine-iodide solution was pipetted into a 250-milliliter Erlenmeyer flask. To this was added a five-milliliter aliquot of 0.1 molar cysteine. Three and one-half grams of potassium iodide were added to each sample to bring the iodide concentration to one molar. After allowing a short time for the iodine to completely oxidize the cysteine, the sample was titrated with standard sodium thiosulfate.

A blank was run omitting the cysteine and the cysteine concentration was calculated from the following equation:

$$(Vol._{Blank} - Vol._{Sample})Conc._{S_2O_3} = = (Vol._{CySH})Conc._{CySH}$$

Pilmer's Method

Preparation of Solutions

A buffer solution was prepared using two-tenths molar phosphoric acid and titrating it with six molar sodium hydroxide to pH 2.

A 0.1 normal standard iodine solution was prepared from a known amount of potassium iodate added to the buffer solution, enough potassium iodide to make the solution one molar in iodide ion, and sufficient pH-2 phosphate buffer to make one liter of solution.

Cysteine samples were prepared in the same manner as described for the Lavine method.

Procedure

A five-milliliter aliquot of cysteine was pipetted into a 250-milliliter Erlenmeyer flask. To this was added twenty milliliters of buffer

solution (pH 2) and four grams of potassium iodide. The sample was then titrated with standard iodine solution to a starch end point.

The percent purity of the cysteine was calculated from the following:

$$\%CySH \cdot HCl \cdot H_2O = \frac{(ml_{I_2})(N_{I_2}) \times \frac{175.61}{1000}}{\text{Sample Wt. of CySH}} \times 100$$

Results

The results of the cysteine determinations by Lavine's and Pilmer's methods are presented in Tables V and VI.

Titration with Ferricyanide

Reagents

Potassium ferricyanide and cupric sulfate were of A.C.S. reagent grade.

The buffer was prepared by titrating 0.2 molar phosphoric acid to pH 7 with one molar sodium hydroxide.

The nitrogen used was a commercial, water-pumped grade, purified before use by bubbling through a vanadous ion solution to remove oxygen (18).

Water used in preparing cysteine and ferricyanide solutions was purified in the same manner as previously described for the iodimetric methods.

The commercial cysteine samples were the same as those employed in the iodimetric methods.

Apparatus

The electrical circuit is shown diagramatically in Figure 2. The source of current was two one- and-one-half volt dry cell batteries connected in series, shorted through a 100,000-ohm fixed resistance and through a voltage divider by which the desired potential was impressed upon the electrodes. A potential of fifty millivolts was employed; the value, however, is not

Table V

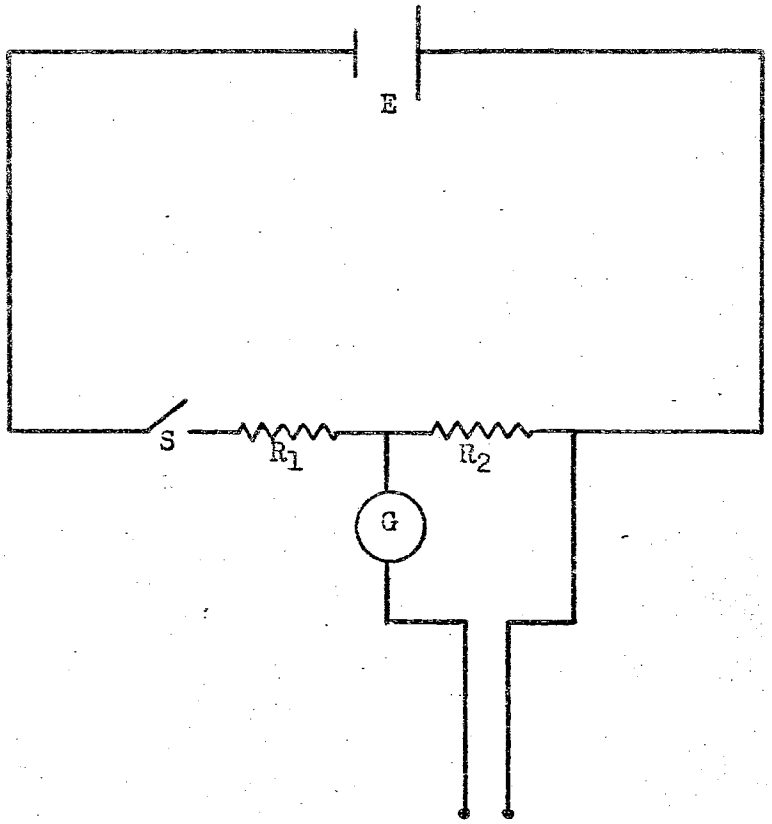
Cysteine Determinations by Lavine's Method

Sample Description	Amount Taken (moles × 10^5)	Amount Found (moles × 10^5)	Percent Purity	Deviation from Mean % Purity
Commercial Sample III	49.60	49.4	99.5	+0.3
	49.60	50.0	100.8	+1.6
	49.60	48.3	97.4	-1.8
	49.20	47.8	97.0	-2.2
	49.20	48.3	98.2	-1.0
	49.20	49.1	99.8	+0.6
	49.20	48.6	98.9	-0.3
	49.20	48.6	98.7	-0.5
	45.65	45.3	99.2	0.0
	45.65	45.2	99.0	-0.2
	45.65	44.8	98.2	-1.0
	49.45	50.6	102.2	+3.0
	49.45	49.4	99.8	+0.6
	49.45	49.8	100.7	+1.5
		Mean	99.2	1.0
		Standard Deviation	1.4	
Commercial Sample II	48.60	47.8	98.2	+1.3
	48.60	47.6	98.0	+1.1
	52.30	49.9	95.5	-1.4
	52.30	48.8	93.4	-3.5
	52.30	51.9	99.2	+2.3
		Mean	96.9	1.9
		Standard Deviation	2.4	

Table VI

Cysteine Determinations by Pilmer's Method

Sample Description	Amount Taken (moles x 10^5)	Amount Found (moles x 10^5)	Percent Purity	Deviation from Mean % Purity
Commercial Sample III	49.20	47.5	96.5	+0.3
	49.20	48.4	98.3	+2.1
	49.20	47.5	96.5	+0.3
	49.20	47.4	96.4	+0.2
	49.20	47.0	95.5	-0.7
	49.20	46.8	95.1	-1.1
	45.65	43.6	95.5	-0.7
	45.65	43.6	95.5	-0.7
		Mean	96.2	0.7
		Standard Deviation	1.0	
Commercial Sample II	52.30	50.0	95.5	-2.0
	52.30	48.8	93.5	-4.0
	52.30	51.9	99.2	+1.7
	46.30	46.0	99.2	+1.7
	46.30	46.4	100.1	+2.6
		Mean	97.5	2.4
		Standard Deviation	2.9	



E = 1.5 V Dry Cell
S = Knife Switch
R_1 = 100,000 Ohm Resistance
R_2 = Decade Box
G = Galvanometer

Figure 2

Dead-Stop End-Point Circuit

critical. The galvanometer used was of the pointer-type (G.M. Laboratories, Catalot no. 570-211). The damping resistance for the galvanometer was somewhat less than the critical value. The circuit was disconnected only when the apparatus was not to be used for prolonged periods of time, since the batteries discharge only very slowly through a 100,000 ohm resistance.

The electrodes were pieces of twenty-two guage platinum wire about 0.5 centimeters in length, sealed through soft glass tubing. Connection was made with the rest of the circuit by means of mercury inside the tubing. No special care was required in the preparation and maintenance of these electrodes.

Vessels of two sizes were used in the titrations; one vessel, used for titrations of 1.0 to 0.005 millimoles of cysteine, was a Berzelius beaker closed with a rubber stopper. The rubber stopper was fitted with the electrodes, a gas bubbling tube, the burette tip, and the shaft of a glass stirrer. The stirrer was propelled by an ordinary electric stirring motor. A ten milliliter burette was used.

The vessel used for titrations of 10^{-3} to 10^{-4} millimoles of cysteine was an 18 X 50 mm. Pyrex test tube. Platinum electrodes were sealed through the side of the test tube. No cap was used, and into the open mouth of the tube was placed the shaft of the stirrer, a gas-bubbling tube and the tip of the burette. The burette used in these titrations was a Kirk horizontal capillary burette of 0.10 milliliter capacity. A uniform rate of stirring was found to be important in the small-scale titrations. Satisfactory results were obtained with a glass stirrer with a small paddle, actuated by a stirrer motor of variable speed.

Analytical Procedure

The same general method was applied to both the large and small

titrations. The titrations differ only in the size of sample and amount of buffer solution used.

The titration medium was prepared in the following manner; in a Berzelius beaker was placed an adequate amount, usually twenty milliliters, of phosphate buffer solution. To this was added two milliliters of 10^{-4} molar cupric sulfate. Cupric sulfate of 10^{-5} molarity was added to the more dilute samples. The stopper was placed on the beaker and deoxygenated nitrogen was passed through the solution for about ten minutes while the solution was being stirred. After this time, the bubbling tube was raised above the solution and nitrogen was allowed to flow over the surface of the solution. The cysteine sample, in the range of 10^{-1} to 10^{-3} molar, was carefully pipetted into the titration beaker. Care was taken, in transferring the sample, to minimize contact with the air. After this was done, the burette tip was placed below the surface of the liquid. A capillary tip of the burette allowed no diffusion of ferricyanide into the beaker while the stopcock was closed. The potassium ferricyanide was allowed to enter the solution slowly. Nearness to the end point could be detected by fluctuations by the galvanometer needle and finally by drift of the needle from the zero point. The galvanometer readings and corresponding burette readings were recorded and later plotted.

Results

The results of the titration with ferricyanide are given in Tables VII and VIII.

The Reduction of Cystine

Reagents

Mercury, used in preparation of sodium amalgam, was A.C.S. reagent grade.

Table VII

Cysteine Determinations at Variable Concentrations
Of Commercial Sample III by the
Ferricyanide Method

Cysteine Concentration	Amount Taken	Amount Found	Percent Purity	Deviation from Mean % Purity
Range	(moles $\times 10^5$)	(moles $\times 10^5$)		
one-tenth	51.45	49.55	96.31	-0.05
to	51.45	49.70	96.60	+0.24
two-tenths	48.05	45.90	95.52	-0.84
	48.05	46.15	96.04	-0.32
	42.35	41.05	96.95	+0.59
	42.35	40.90	96.58	+0.22
	30.90	29.50	95.53	-0.83
	30.90	29.70	96.12	-0.24
	79.40	77.45	97.54	+1.18
	59.65	57.80	96.90	-0.54
	44.30	42.70	96.39	+0.03
	35.44	33.96	95.85	-0.51
	62.02	59.43	95.85	-0.51
	45.00	43.74	96.20	-0.16
	45.00	43.69	97.08	+0.72
		Mean	96.36	0.47
		Standard Deviation	0.59	
one-hundredth	5.400	5.241	97.06	+0.16
to	3.600	3.495	97.07	+0.17
two-hundredths	4.685	4.543	96.96	+0.06
in HCl	4.685	4.551	97.14	+0.24
	3.748	3.611	96.35	-0.55
	3.748	3.629	96.82	-0.08
		Mean	96.90	0.21
		Standard Deviation	0.29	
one-hundredth	4.805	4.420	91.99	-2.00
to	4.805	4.430	92.20	-1.79
two-hundredths;	4.235	4.045	95.06	+1.07
no extra HCl	3.090	2.935	94.98	+0.99
added	1.236	1.142	92.33	-1.66
	7.940	7.655	96.41	+2.42
	5.965	5.660	94.89	+0.90
	2.386	2.244	94.05	+0.06
		Mean	93.99	1.36
		Standard Deviation	1.64	
one-thousandth	0.216	0.208	96.30	-0.29
to	0.144	0.139	96.74	+0.15
two-thousandth;	0.180	0.174	96.57	-0.02
in one molar HCl	0.252	0.244	96.73	+0.14
		Mean	96.59	0.15
		Standard Deviation	0.21	

Table VIII

Cysteine Determinations at Variable Concentrations of Commercial Sample II by the Ferricyanide Method

Cysteine Concentration Range	Amount Taken (moles×10^5)	Amount Found (moles×10^5)	Percent Purity	Deviation from Mean % Deviation
one-tenth to two-tenths	53.85	51.97	96.47	-0.37
	53.85	52.08	96.75	-0.09
	53.85	52.36	97.21	+0.37
	43.08	41.68	96.75	-0.09
	96.93	93.87	96.84	0.00
	49.01	47.38	96.67	-0.07
	39.21	37.95	96.79	-0.05
	68.61	66.73	97.26	+0.42
		Mean	96.84	0.18
		Standard Deviation	0.27	
one-hundredth to two-hundredths; in one molar HCl	1.960	1.875	95.61	-0.23
	1.568	1.518	96.79	+0.95
	4.308	4.107	95.35	-0.49
	5.385	5.197	96.47	+0.63
	3.921	3.731	95.15	-0.69
	4.901	4.690	95.69	-0.15
		Mean	95.84	0.52
		Standard Deviation	0.65	
one-hundredth to two-hundredth; no extra HCl added	5.249	5.249	87.06	-2.21
	4.283	4.283	88.73	-0.54
	4.362	4.362	90.39	+1.12
	6.454	6.454	89.15	-0.12
	1.823	1.823	90.52	+1.25
	1.432	1.432	88.90	-0.37
	2.179	2.179	90.17	+0.90
		Mean	89.27	0.93
		Standard Deviation	1.22	
one-thousandth to two-thousandth; no extra HCl added	0.4828	0.4005	82.93	-1.38
	0.6035	0.5203	86.25	-1.94
	0.7242	0.6064	83.76	-0.55
		Mean	84.31	1.29

Sodium amalgam, assaying approximately 5% sodium, was obtained from the General Chemical Division, Allied Chemical and Dye Corporation.

The cystine was chemically pure L-cystine, obtained from Schwarz Laboratories, Mount Vernon, New York. The optical rotation of the commercial sample was -208°, which compared with the value given for pure cystine, -213° (8); indicates a purity of 97.7%.

Preparation of Amalgam

At first, sodium amalgam was prepared by direct reaction between sodium and mercury under an organic solvent. This was done by weighing out freshly cut sodium under the solvent, usually xylene. The sodium and solvent were placed on a hot plate and heated until the sodium reached the melting point. At this time, enough mercury, previously weighed out, to form a one percent amalgam was quickly added to the sodium. After the vigorous reaction had subsided, the resulting amalgam was purified by filtering.

This method of making the amalgam involved considerable hazard, because the reaction was quite vigorous; also some mercury was wasted owing to the formation of amalgam of approximately 6% sodium content which was solid and could not be used for reduction.

A more convenient method of making one percent amalgam was subsequently found. It simply involved dilution of commerically available 5% sodium amalgam to one percent by grinding with the appropriate amount of mercury in a mortar.

Reduction Procedure

A cystine solution, tenth molar in cystine and one molar in hydrochloric acid, was prepared and placed in a dry centrifuge bottle of 250-milliliter capacity. Sodium amalgam under a layer of isooctane was placed in a separatory funnel to which a one-millimeter capillary was

attached. The centrifuge bottle was closed with a rubber stopper, through which was fitted the capillary so that its tip dipped into the solution; the amalgam was allowed to drop slowly into the solution for about two and one-half hours. Samples were taken and analyzed for cysteine at different times during the reaction. Both the ferricyanide and the iodimetric methods were used. Aliquot samples were analyzed until constant values of cysteine content were obtained; it was then assumed that reduction was complete and the reaction was stopped.

Results

The results of the determinations of reduced samples are given in Table IX.

Table IX

Determinations of Reduced Samples

Method	Amount Taken	Amount Found	Percent Purity	Deviation from Mean % Purity
Ferri-cyanide	(moles $\times 10^5$)	(moles $\times 10^5$)		
	60.000	58.726	97.88	+0.38
	50.000	48.700	97.40	-0.10
	40.000	38.810	97.02	-0.48
	6.000	5.855	97.58	+0.08
	5.000	4.884	97.68	+0.16
	4.000	3.862	96.56	-0.94
	0.280	0.273	97.42	-0.08
	0.200	0.196	97.81	+0.31
	0.160	0.156	97.33	-0.23
	50.350	50.506	98.35	+0.85
	39.660	38.784	97.79	+0.29
	39.660	38.485	97.03	-0.47
	39.660	38.737	97.67	+0.17
		Mean	97.50	0.35
		Standard Deviation	0.45	
Lavine's	50.00	48.0	96.0	+0.7
	50.00	47.3	94.6	-0.7
		Mean	95.3	0.7
Pilmer's	49.67	48.7	98.1	-0.4
	48.40	47.0	97.2	+0.5
		Mean	97.7	0.5

SUMMARY AND CONCLUSIONS

A method of analysis for cysteine and sulfhydryl groups has been developed which utilizes titration with standard potassium ferricyanide solution, and the "dead-stop" method for determining the end point. The method has many advantages over other procedures tested by the writer.

The apparatus used in the titration procedure is of simple design and consists of materials that can be found in every modern laboratory. The electrodes used are easily constructed and need no special care. This is in contrast with the requirements for electrodes used in amperometric methods.

The results indicate that this method is more sensitive than the iodimetric methods of Lavine and of Pilmer. At much lower cysteine concentrations than can be determined by iodimetric methods, ferricyanide titration gives precise results if precautions are taken to minimize atmospheric oxidation.

There is no absolute independent method of analysis for cysteine, to which the results obtained by the ferricyanide method may be compared. It should be noted, however, that the results obtained with ferricyanide were consistent over a wide variation of concentrations. The precision affords some confidence about the reliability of the method.

Since ferricyanide is a milder oxidizing agent than iodine, the ferricyanide method should be less subject to interference from other oxidizable

substances. Indeed, none of the essential amino acids were found to have a detectable effect on cysteine titrations. For this reason, the method developed in this work will be useful for the determination of sulfhydryl content in protein hydrolysates and in body fluids. It may even be possible, with appropriate electrodes, to measure the sulfhydryl content of intact cells.

A simple method has been perfected for preparing pure samples of cysteine through the quantitative reduction of cystine. Although the reducing agent employed has been used for cystine reduction before, no description was available of the procedure needed to obtain quantitative results. The procedure described in this work is simple, fast, and the resulting solution contains only sodium and chloride ions, which are not likely to interfere with any purpose to which the cysteine may be put.

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