THE UNIVERSITY OF MINNESOTA

GRADUATE SCHOOL

Report

of

Committee on Examination

This is to certify that we the undersigned, as a committee of the Graduate School, have given **Walter Fred Hoffman** final oral examination for the degree of Master of **Science** . We recommend that the degree of Master of **Science** be conferred upon the candidate.

Minneapolis, Minnesota

May 25 1921

Chairman

F. H. MacDougall

L. L. Knight

THE UNIVERSITY OF MINNESOTA

GRADUATE SCHOOL

Report

of

Committee on Thesis

The undersigned, acting as a Committee of the Graduate School, have read the accompanying thesis submitted by Walter Fred Hoffman for the degree of Master of Science. They approve it as a thesis meeting the requirements of the Graduate School of the University of Minnesota, and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science.

A. F. Loehr
Chairman

F. H. MacDougall

L. L. Knight

May 28 1921

SULPHUR IN PROTEINS WITH ESPECIAL REFERENCE TO THE BEHAVIOR OF CYSTINE TO ACID HYDROLYSIS.

By Walter Fred Hoffman, A. B.

A THESIS

Submitted to the Graduate School of the University of Minnesota in partial fulfillment of the requirements

For the Degree

of

Master of Science.

St. Paul, Minnesota.

May 1921.

ACKNOWLEDGMENT.

This investigation was carried out in the laboratories of the Division of Agricultural Biochemistry, under the direction of Doctor Ross Aiken Gortner. The author takes this opportunity to express his gratitude and appreciation to him for the help extended in carrying out this work, and for the thoughtful advice and encouragement that was given.

The writer wishes also to thank Dr. George E. Holm for his deep interest and for the valuable aid rendered.

W. F. H.

Agricultural Biochemistry,
University Farm,
University of Minnesota.
May 1921.

TABLE OF CONTENTS.

		page
I.	Introduction	1
II.	Historical	
	The discovery of cystine and the establishment of the structural formula.	2
	The behavior of cystine and cystine sulphur towards chemical reagents and the relation of cystine to the sulphur content of proteins.	5
	The crystal form of cystine as influenced by chemical treatment.	12
	The identity of "stone" cystine and "protein" cystine.	14
	Methods of the preparation of cystine from proteins.	14
	The synthesis of cystine.	17
III.	Experimental	
	The Problem	19
	The Material	20
	The Methods	22
	The Experimental Data	
	A. The decomposition of cystine on boiling with 20 per cent hydrochloric acid for varying lengths of time	
	The hydrolysis.	27
	The carbon dioxide determination.	27
	The sulphur determinations.	28
	The amount of cystine as determined by the potassium bromate method.	32
	The nitrogen precipitated by phosphotungstic acid.	32
	The nitrogen determinations.	34
	The optical activity of the acid solution.	35

page
Chart I. 35

B. The isolation of an "isomeric" cystine from the residual hydrolysate.

Isolation and analysis. 38

Physical properties. 39

Solubility. 40

Precipitability with phosphotungstic acid. 41

C. A comparison of certain derivatives of the "isomeric" cystine and plate cystine.

The hydrochlorides. 43

The benzoyl derivatives. 43

The phenylisocyanate derivatives. 45

The phenyl hydantoins. 46

The cysteic acids. 48

IV. Discussion 50

V. Summary and General Conclusions 52

VI. Literature Cited.

1. INTRODUCTION.

It has been known for a long time that sulphur is a constituent of most proteins. The usual method of identification of unoxidized sulphur is heating the protein with concentrated sodium hydroxide in the presence of lead acetate, the sulphur being split off as a sulfide forming a black precipitate with the lead. The first work on the sulphur in proteins was done about 1800. At the present time only one form of sulphur in proteins is known. It is isolated from the protein hydrolysate in the amino acid cystine but the amount isolated, as such, only accounts for a part of the sulphur.

II. HISTORICAL.

The discovery of cystine and the establishment of the structural formula.- Crawford (1797) made the very interesting and important discovery that during the putrifaction of animal proteins, hydrogen sulfide was given off. A short time after this, Wollaston (1810) discovered cystine in urinary calculus which contains a large per cent of cystine. He reports this as being soluble in alkali but not in water, acetic acid, or alcohol and that when the alkaline solution was neutralized, the precipitate formed contained hexagonal plates. He called this "cystic oxide". Prout* was the forst to analyse cystine. He determined the amounts of nitrogen, hydrogen, oxygen and carbon but overlooked the fact that it contained sulphur.

Baudrimont and Malaguti (1838) had shown that sulphur is present in urine. Thaulow (1838) made the first complete analysis of cystine and gave to it the formula, $C_3H_6NSO_2$. He did not attempt to state the form of the sulphur but said that it was unknown. Gmelin* assigned to cystine the formula, $C_3H_7NSO_2$. Besides these two formulae, Dewar and Gamgee*, on the grounds of their analysis, gave to cystine a third formula which had two less hydrogen atoms than the formula given by Gmelin. They observed that when nitric acid acted on cystine, pyroracemic acid was formed. They gave the first constitutional formula to cystine,

$$\begin{array}{l} CH_2CSCOOH. \\ | \\ NH_2 \end{array}$$

---

* References as cited in articles reviewed are given in "Literature cited". The dates were not obtainable.

10-20 5M

Hoppe-Seyler (1881) made an analysis of cystine and found that his results did not agree with those of Dewar and Gamgee but with the formula, $C_3H_7NSO_2$, assigned by Gmelin. The constitutional formula as given by Dewar and Gamgee should have given methyl amine when heated with alkali. Hoppe-Seyler, the first to test this experimentally, heated cystine with barium hydroxide but obtained no methyl amine and found that all the nitrogen was split off as ammonia. Besides ammonia and barium sulfide, a sulphur and nitrogen free acid was formed but he did not obtain enough for analysis.

Külz (1884) carried out a number of analysis of cystine and came to the conclusion that the Gmelin formula was not correct and substituted in its place the old Thaulow formula. He found that for the correct molecular weight this would have to be doubled and assigned to cystine the formula, $C_6H_{12}N_2S_2O_4$.

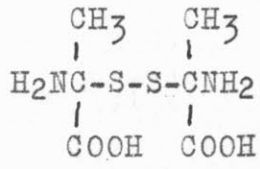
Baumann (1884) by using different methods came to the same conclusions as Külz. He assigned to cystine the same formula and showed that it is a disulfide of the mercaptan like substance, cystein which has the formula, $C_3H_7NSO_2$. He also found that cy cystine is easily changed to cystein by reducing with hydrogen. This is again easily oxidized to cystine by using a weak oxidizing agent. He gave the following formulae to illustrate this;

$C_3H_6NO_2$-SH	$C_3H_6NO_2$-S
Cystein.	$C_3H_6NO_2$-S
	Cystine.

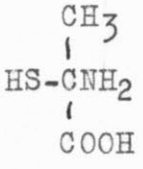
Baumann and Preusse (1881) had shown, in an indirect way, the constitution of cystine and cystein when they fed benzoyl iodide, chloride or bromide to dogs. They were able to isolate a substance from the urine which was levo-rotatory. This was found

to be a substitution product of a mercaptan. From their analytical data, they assigned to it the formula, $C_{11}H_{12}BrNSO_3$. Much work was done on pyroracemic acid and the mercaptan like acids in trying to find a relation between cystine and these compounds. Some of the most important being done by Baumann and Preusse (1879), Jaffe (1879), Baumann and Preusse (1881) and Baumann (1882).

Baumann and Goldmann (1881) pointed out that cystine and mercaptanic acid differed when treated with alkali and heated. The sulphur of cystine is not completely split off even after long boiling but the sulphur of the mercaptanic acid is split off quantitatively in a short time. They explain the former as being due to the "SH" combining through a disulfide linkage. They give the following formulae for cystine and cystein,



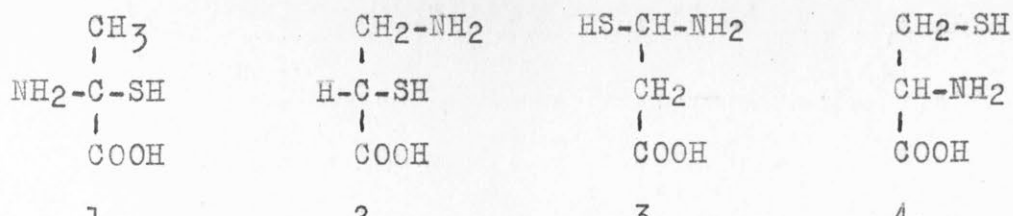
Cystine



Cystein.

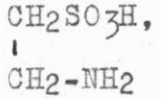
Brenzinger (1892) prepared several derivatives of cystine, among them ethyl cystine and showed that it was the mercaptan "H" that reacted. Suter (1895) states that phenyl cystein is formed in the same way. Brenzinger also prepared the benzoyl derivative and "uramido acid" as further proof of the nitrogen being in as amino group. He further states that the "SH" and the "NH_2" are on the same carbon atom.

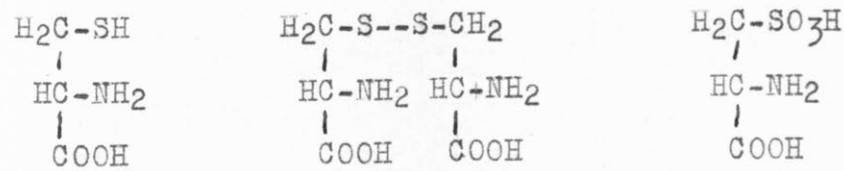
Freidmann (1903) states that from the groups present in cystein, the following formulae are possible, (these hold the same for cystine). From his work with thio-lactic acid, he came to the conclusion that only formulae 3 and 4 were possible for cystein.



1. 2. 3. 4.

To prove the position of the amino group, he oxidised cystein, by means of bromine, to cysteic acid which would give him either;

From the cysteic acid he prepared taurin,  , which showed that the amino group is on the alpha-carbon atom. From this work Friedmann gave the following, now accepted, formula for cystein, cystine and cysteic acid.



Cystein. Cystine. Cysteic acid.

The behavior of cystine and cystine sulphur towards chemical reagents and the relation of cystine to the sulphur content of proteins.- Fleitmann (1847) and (1848) was the first to show that the sulphur in proteins may not all be in one compound or composition. He found that only a part of the sulphur was removed from certain proteins when heated with alkali. On account of their peculair behavior and their similarity to cystine and taurin, the two sulphur compounds then known to be related to animal metabolism, he called the two forms, oxidized and unoxidized sulphur. Danielewski (1869) confirmed Fleitmann's observations and later (1883) called attention to the fact that this peculiar property of

sulphur in proteins had been neglected by those trying to establish formulae for proteins as most of these were made on the bases of one sulphur atom. Kruger (1888) was the first to produce evidence that the terms oxidized and unoxidized sulphur were not correct. He carefully determined the amounts of the two forms of sulphur in ovalbumin and fibrin and showed that mercaptans and thio-ethers behave in a similar manner when heated with alkali. But he also observed that oxidized sulphur compounds as sulfonic and sulfinic acids behave quite differently and were decomposed under the same treatment giving alkali sulfites. For this reason he dropped the terms oxidized and unoxidized sulphur and named the two forms loosely and firmly bound sulphur. He also discusses the possible ways in which the sulphur of the complex that is split off, is bound and how the remaining sulphur could unite in the protein molecule. Goldmann and Baumann (1888) were able to remove 68% of the sulphur of cystine as lead sulfide when cystine was heated with 10% sodium hydroxide in the presence of lead acetate. Suter (1895) also studied the effects of heating proteins with sodium hydroxide and noted the similarity of their behavior to that of cystine when treated in the same manner. When he increased the time of heating he obtained as high as 83% of the sulphur of cystine as lead sulfide Schülz (1898) reviewed the work of these investigators and said the lack of agreement of some of the work was due to the fact that part of the sulphur was oxidized during the long heating. In order to avoid this he added zinc to the sodium hydroxide solution and then was able to account for only 53% of the sulphur in cystine after heating with 30% sodium hydroxide for 25 hours. Osborne (1900) used Schultz's method of determining loosely and firmly bound sulphur in an endeavor to get at the size of the protein molecule.

He analysed a large number of proteins and found the percent of loosely bound sulphur varied from 66% in seralbumin to 13% in casein.

The question concerning the possible forms of sulphur in proteins is one of importance. The fact that all the sulphur in proteins does not behave the same when the protein is heated with sodium hydroxide, points towards sulphur being in two combinations but on the other hand pure cystine behaves in the same manner so this does not seem to be a proof. Since it is known that cystine and proteins behave very similarly when heated with alkali, the question also arises whether the sulphur linkage represented by cystine is the only one to be dealt with in proteins.

Morner (1901) attempted to answer this question. He found that about the same percentage of sulphur was split off from cystine by heating with alkali as was obtained by treating horn, hair, serum globulin and serum albumin under identical conditions. Also in solutions from which cystine had been separated, he found a considerable amount of sulphur which behaved in a manner similar to cystine when treated with alkali but this substance was not definitely identified. From the experimental results, Morner calculated the per cent of cystine in these proteins and accounts for almost all of the sulphur in these proteins as being in the form of cystine. As he could only account for a small amount of the sulphur in the membranes of eggs, ovalbumin and fibrinogen as cystine, he concluded that the sulphur of these proteins must be present in other forms. As confirmation of this idea he observed that about one third of the sulphur was lost by the volatilization of a sulphur compound. He did not identify this but said that it is possible that one third of the sulphur was present in the form of a

volatile substance and it is not impossible that sulphur of these proteins is bound in three forms.

The fact that the amount of sulphur split off by alkali differs considerably in the proteins examined also points towards different sulphur forms. Osborne (1900), as noted above, obtained from 13% to 66% of the sulphur in various proteins, as the sulfide, by boiling with sodium hydroxide. Suter (1895), by heating cystine for 9 hours was able to split off 60% of the sulphur, while heating hair under the same conditions for 15 hours, 98.8% of the sulphur was split off. Pick (1899) made the interesting observation that the albuminases prepared from fibrin gave up the whole of their sulphur as hydrogen sulfide. He concludes that the sulphur must not be present in the form of cystine. While working with koilin*, Hofmann and Pregl (1907) observed that when this was digested with alkali and lead acetate, lead sulfide was formed. They were unable to isolate cystine from the hydrolytic products.

Buchtala (1907) while determining the amounts of cystine in human hair and nails, horses hoofs, cattle hair and hoofs and swine hoofs and bristles, observed that swine hoofs and bristles, when hydrolysed, were the only ones that deposited sulphur on the condenser tube. He later (1900) observed that when hens' claws were hydrolysed with concentrated hydrochloric acid no sulphur separated but when horn shavings were treated the same way, a considerable amount of this element was deposited on the condenser tube. It is possible that this may have some bearing on the forms of sulphur in proteins, however it is equally possible that the concentration of sulphur in the hydrolysate is the determining factor. Cohn (1898) when hydrolysing horn shavings with

---

* The horny keratin-like lining of the gizzards of fowls.

hydrochloric acid reports that the gas given off smelled like hydrogen sulfide and a mercaptan. Buchtala (1908) worked with the egg membranes of three kinds of sharks but was unable to isolate any cystine. He states that it is doubtful if any of the sulphur in these keratins in bound as cystine.

Rettger (1902) observed that milk proteins when heated above 85 C undergo partical decomposition which was indicated by a volatile sulfide being given off. This probably was hydrogen sulfide as it blackened lead acetate paper. Raikow (1905) allowed phosphoric acid to act on unbleached wool and human hair at ordinary temperatures and states that sulphur dioxide was evolved. As sulfonic acids and sulphates do not react in this manner, Raikow concludes that the sulphur is partly linked in keratins in sulfite form. Grandmongin (1907) when allowing phosphoric acid to act on unbleached wool even for a very long time did not get a trace of sulphur dioxide. The negative results of Grandmongin led Raikow (1907) to repeat his former experiments. He, as before, obtained positive results. This, he beleives, confirms the theory that he put forth in his former article that part of the sulphur in keratins is united to oxygen. Raikow used wool from a different source but says that this can hardly account for Grandmongin's results.

Breinl and Baudisch (1907) observed that when cystine was boiled with hydrogen peroxide, carbon dioxide and an aldehyde were given off. The solution contained sulphuric acid. Baudisch (1908) allowed wool to stand, at ordinary temperatures, suspended in neutral hydrogen peroxide and observed that the solution finally became acid from the formation of sulphuric acid. Chlorinated wool, on the other hand, did not give the same results when treated the same way, He therefore concludes that at least a part of the

sulphur of proteins is bound to oxygen.

Neuberg and Meyer (1905) heated stone cystine in a sealed tube with hydrochloric acid for 14 hours at 165 C. They obtained a clear brown solution. The tube was under pressure when opened and a gas of mercaptan or sulfide character was given off. The non-volatile decomposition products remained in the solution. This is very similar to the observations made by Mauthner (1884) when he heated cystine in a sealed tube with water at 140-150 C for many hours. In addition th hydrogen sulfide, mercaptan and ammonia, Mauthner claims to have isolated a sulphur containing acid which was nitrogen free. Analysis of the silver salt indicate a formula of $C_6H_{10}S_2O_4$ or $C_6H_8S_2O_4$. He later (1885) reports that this acid contained nitrogen. Apparently this compound has never been completely identified.

Johnson (1911) criticises the fact that much stress is still laid on the fact that cystine sulphur and protein sulphur react similarly when heated with alkali and consequently the conclusion is accepted that cystine is the source of the loosely bound sulphur in these natural substances and also that investigation has been focused almost exclsively on the cystine molecule and no careful consideration has been given to the possibility that there may be other unstable sulphur groupings in some proteins, which on hydrolysis can break down, giving hydrogen sulfide. In regard to this he states that,"the fact cystine behaves like a sulphur protein when heated with alkali, or that other primary sulphur cleavage products have not been isolated, does not preclude the possibility of there being other sulphur combinations, which can disintegrate to give hydrogen sulfide. This assumption is worthy of some consideration and cannot be excluded from the discussion of this important

question. It is not inconeivable that in the digestion of proteins with alkalies and acids, there may be atomic displacements involving the removal of unsaturated sulphur as in C=S, which can even occur before the cleavage of the protein itself and the liberation of the cystine molecule". In summing up his discussion of the work done on the sulphur linkages in proteins, he states, "It therefore appears probable from a consideration of the above evidence that there are other sulphur combinations in proteins besides the cystine group which can break down, on hydrolysis, with the formation of hydrogen sulfide".

Van Slyke (1911) found that cystine was destroyed or altered during hydrolysis with hydrochloric acid. He determined the amount not destroyed by precipitating it with phosphotungstic acid and found that after cystine was boiled with 20% hydrochloric acid for 24 hours only 50% was precipitated by phosphotungstic acid. He states that, "It appears possible that the cystine is partially destroyed during the hydrolysis. The results (in the following table) show that this is the case,-- the cystine is gradually altered during the hydrolysis, into a substance or substances which are not precipitated by phosphotungstic acid".

Plimmer (1913) finds that cystine is decomposed by prolonged boiling with acid. In preparing cystine from wool or hair he obtained the best yield by boiling the material with concentrated hydrochloric acid for 3-4 hours. The yield was very poor when boiled 5-8 hours. He also found a loss when cystine was purified by boiling with charcoal in an acid solution and if boiled for a long time in dilute acid it becomes yellow or yellow brown in color.

Gortner and Holm (1920) found that cystine, when mixed

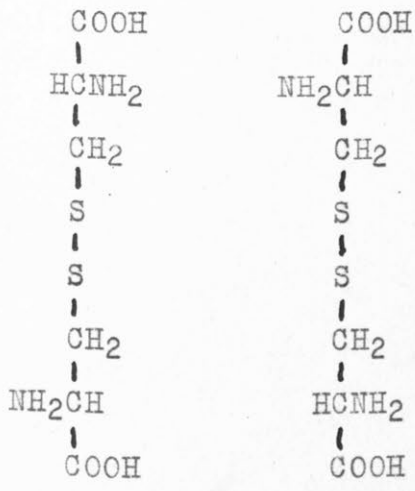
with a number of other amino acids, both with and without the addition of formaldehyde and boiled with 20% hydrochloric acid for 24 hours, was very slightly deaminized. Their results show that if all of the ammonia nitrogen was calculated as being derived from the cystine, only about 2.7% of the amino nitrogen has been changes to ammonia nitrogen. They state that, "Cystine was not readily deaminized under the conditions of the experiment".

The crystal form of cystine as influenced by chemical treatment.- Morner (1899) noted that the crystals of cystine were different when horn was hydrolysed for different lengths of time. When horn was heated for one week with hydrochloric acid, he obtained the typical hexagonal plates which have strong optical rotation. When he heated the horn for two weeks he obtained very few hexagonal plates, the greater part crystallizing in needles which were almost optically inactive. He describes these as being long needles or long, very thin or narrow, pointed plates which often accumlated in groups. Some resemble tyrosine while others are like small dust particles. He also states that some appeared as thin plates of rhombic form, perhaps with the sharp corners cut off. Morner was not able to prepare this substance pure in as much as it always contained some of the hexagonal plates. He found no difference in the reaction of the two forms with acids and alkalies. The needle cystine was more soluble in cold water than the plate cystine and is more soluble in hot water than in cold water. Both give lead sulfide when heated with alkali and lead acetate.

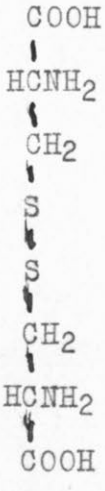
Rothera (1905) when preparing cystine found, as did Morner, that needles were present with the plates and increased with the length of hydrolysis. He found no difference in the yield of cystine if the hydrolysate was boiled 18 or 180 hours. He states,

"The first recrystalization showed tyrosine like crystals but after the second, the crystals resemble lecithin spheres. They give the cystine color tests and the required sulphur and nitrogen content". Neuberg and Mayer (1905a) state that boiling sulphuric acid decomposes cystine much more readily than does hydrochloric acid.

Neuberg and Mayer (1905) heated "stone" cystine with hydrochloric acid in a sealed tube at 165 C for 14 hours. This was optically inactive and formed a white amorphous powder. In another article (1905a) they state that when protein cystine is treated as above, an inactive, needle, crystalline substance is obtained. In this article they compare the active and inactive protein cystine and find them quite different. They give the possibilities of this inactive cystine as being either a meso-cystine or a racemic mixture:



r-cystine



Meso-cystine.

They were able to prepare the inactive cystein from the inactive cystine. This was completely inactive and analysed for cystein. They grew Aspergillus niger on some of the inactive cystine and obtained a cystine that was dextro-rotatory. It was suggested that this method might be used to prepare pure d-cystine. To quote them concerning the crystal form of this preparation,

"Bermerken wollen wir noch, dass unser reinstes d-Cystin ($[\alpha]_D$ = +93.78) mikroskopisch sechseckige Täfelchen neben Nadeln aufwies; die Theorie verlangt ja auch, dass d- und l- Cystin enantiomorph sind".

The identity of "stone" cystine and "protein" cystine.- Neuberg and Mayer (1905) in comparing crystal forms of cystine state, "wir sind nun einem Cystinstein begegnet, der aus einem ausschliesslich in Nadeln kristallisierender, stark optisch aktiven Cystin bestand. Der Einfachheit halber seien im folgenden die beiden Cystin formen als "Protein" und "Steincystin" unterschieden, onhe dass mit dieser Benennung das ausschliessliche Vorkommen am einen oder anderen Orte Behauptet werden soll". They prepared a number of derivatives of "stone" and "protein" cystine, for example, the phenylcyanate derivative of "protein" cystine melts at 160 C and the hydantoin melts at 119 C while the phenylcyanate derivative of "stone" cystine melts at 170-172 C and they were not able to prepare the hydantoin of "stone" cystine, the benzoyl derivative of "protein" cystine melts at 182-184 C and the benzoyl derivative of "stone" cystine melts at 157-159 C, the ethyl derivative of "protein" cystine melts at 228-230 C while the ethyl derivative of "stone" cystine melts at 164-166 C etc. and found none alike. They concluded that the two cystines are not identical. On the other hand Fischer and Suzuki (1905) and Abderhalden (1919) find that the cystine prepared from protiens to be identical with that from "cystine stones" or the so-called "stone cystine". but they present no such detailed study of the two cystines as do Neuberg and Mayer.

Methods of the preparation of cystine from proteins.- Baumann and Preusse (1881) and Baumann and Goldmann (1888) found cystine or a changed cystine compound, in small amounts, in normal

urine and state that cystine appears to be an intermediate product of metabolism. Cloetta (1856) obtained, from ox kidneys, crystalline plates that showed the properties of cystine. Sherer (1857) found the same thing in the human liver. While working with horse liver, Dreschel (1891) obtained hexagonal plates. On analysis, he identified these as cystine. He (1896) also found the same cystine in the liver of a Delphius. He expressed the opinion that cystine is a normal product of metabolism. Külz (1891) when digesting fibrin with pancreas found cystine-like crystals in the residue. He was unable to decide whether this was a constituent of the pancreas or whether it came from the fibrin. Emmerling (1894) while preparing tyrosine from keratin found hexagonal plates that proved to be cystine. He concluded that they must have come from the keratin. Suter (1895) noted that cystine was obtained when horn was heated with acid. Dreschel (1896a) observed the formation of a volatile sulfide when keratin was hydrolysed with hydrochloric acid. He noted that this came from a base which was precipitated by phosphotungstic acid but failed to identify it. He believed it to be identical with ethylsulfide, which Abel (1895) had detected the year before in urine.

Morner (1899) published his important paper entitled, "Cystin, ein Spaltungsprodukt der Hornsubstanz", in which he describes the method of isolating cystine from the acid hydrolysate of the keratins. His method of preparation takes advantage of the insolubility of cystine in a neutral solution. Tyrosine is the only other amino acid that is appreciably insoluble in a neutral solution. He hydrolysed the material (horn) with hydrochloric acid by heating until the biuret test was negative, neutralized the solution and purified the cystine. Embden (1901) not knowing of

Morner's work, published a paper describing a method for the isolation of cystine from the hydrolytic decomposition products of proteins. His method did not differ essentially from Morner'. Morner (1901) next used hair for the preparation of cystine. He found hair more advantageous than horn in that it contains less tyrosine. His yield was about 11.5%. Some of the other methods for the isolation of cystine are noted below. Mathew and Walker (1909) use horn and report a yield of about 4% cystine. Folin (1910) describes a method for the preparation of cystine in which he takes advantage of cystine being insoluble in acetic acid. He uses hair, hydrolysing it with concentrated hydrochloric acid until the biuret test is negative. He then adds solid sodium acetate to the hot solution of amino acids until the test for mineral acids, with congo red, is negative. After standing several hours the precipitate is filtered off and the cystine dissolved with dilute hydrochloric acid, decolorized with bone black and again precipitated with sodium acetate. By this procedure Folin was able to obtain about 5% cystine from hair. Dennis (1911) uses about the same method as Folin except that she evaporates off most of the hydrochloric acid before neutralizing and adds an equal amount of alcohol when the solution is neutralized the last time. She reports about the same yield as Folin. Plimmer (1913) describes a method for preparing cystine which is very similar to the methods of Folin and Dennis. Winterstein (1901) used phosphotungstic acid to precipitate the cystine. Riza (1903) and Patten (1903) separate the cystine by means of mecuric sulphate. Buchtala (1907) following Morner's method, reports a yield of 14.53% of cystine from human hair. Of the literature reviewed, this is the largest yield reported from any protein.

The synthesis of cystine.- Erlenmeyer (1903) synthesised cystine from benzoyl serinester. After precipitating from ammonia solution with acetic acid, he says,"so erhält man nach einiger Stehen einer sandigen Neiderschlag, der nach maliger Reinigung in allen Eigenschaften bis auf die Activität mit dem aus Hornsubstanzen gewonnenen Cystin Übereinstimmt. Bie der Analyse werden die für das Cystin, $C_6H_{12}O_4N_2S_2$, berechneten Werthe Erhalten". He does not state whether the material was completely inactive nor does he note the crystalline form. In 1904, Erlenmeyer (1904) again prepared cystine from benzoyl serinester and phosphorus pentasulfide. He first obtained cystein which he oxidized to cystine. In regard to the yield and description of the product, he says, "Man erhalt so das Cystin in einer Ausbeute von 40 pC. als ein sandiges, mikrokrystallinisches Pulver. Durch nochmaliges Lösen in Ammoniak und wiederamsauren mit Essigsaure erhalt man das Cystin vollig rein in Form von centrisch gruppirten Nadelchen. Der einzige Unterschied des synthetischen und des naturlichen Productes ist, dass die Losung des ersteren die Ebene des Polarisirten Lichtes nicht dreht. Eine Lösung von 0.5 gm. synthetischen Cystin in Sälzsäure gab im 2 dm. Rohre keine Ablenkung des polarisirten Lichtstrahls".

Fischer and Raske (1908) prepared d-l-cystine from d-l-α-amino-β-chlor-propionic acid and describe it as, "Nach einiger Zeit schied sich das Cystin als farblose Masse von sehr kleinen Krystallchen aus, die unter dem Mikroskop zuerst wie Kugeln und spater wie Nadeln oder dünne Prismen aussahen". The analysis of this preparation agreed quite well with that of plate cystine. They also prepared the l-cystine from l-α-amino-β-chlor-propionic acid. This preparation was optically active $[\alpha] = -209.6$. Their analysis show this to be identical in chemical composition with

plate cystine. Concerning the crystalline form of this preparation, they state, "Wir haben aber auch mit unserem synthetischen Produkt ganz andere Formen erhalten z. B. aus der mit Essigsaure versetzen ammoniakalischen Losung mikroskopische, Kurz, scheinbar rechteckige Prismen oder auch flachenreishere Krystalle".

It is interesting to note that cystine crystalizing in hexagonal plates has never been synthesized and in view of Neuberg and Mayer's given above, the question arises as to whether the cystine actually synthesized may not be "stone" cystine, providing that "stone" cystine actually does differ from "protein" cystine.

III. EXPERIMENTAL.

The Problem. In view of the work refered to under "Historical", ir seemed desirable to test the effect of prolonged boiling with hydrochloric acid on cystine with especial reference to the behavior of cystine in a protein hydrolysis. Many investigators seem to think that cystine is easily destroyed during acid hydrolysis. This idea is upheld by the work of Van Slyke and Plimmer while the work of Gortner and Holm shows that in a mixture of pure amino acids, cystine is not easily deaminized during acid hydrolysis. From the work of Morner, Rothera and Neuberg and Mayer it seems probable that cystine may be completely changed to an isomeric cystine during long acid hydrolysis. As most of the work carried out to study the effects of acid hydrolysis has been done on proteins and since the relation of the cystine content to the sulphur content is still uncertain, it seemed desirable to carry out a prolonged acid hydrolysis on cystine alone and follow the possible types of decomposition by analysis after boiling for different lengths of time.

There are several possible changes, one or more of which might take place when cystine is boiled for a long time with hydrochloric acid. Several of the most possible were followed by removing aliquots at definite intervals and performing certain analyses. The possible changes followed during the boiling were:

1. The loss of carbon dioxide which might be formed by decarboxylation.

2. The loss of hydrogen sulphide which might result from a splitting of the -S-S- union and a subsequent evolution of hydrogen sulphide.

3. Some of the sulphur might be oxidized to sulphate.

4. A loss of total sulphur in the hydrolysate.

5. A change in the amount of unoxidized sulphur as determined by the potassium bromate method.

6. A change in the amount of nitrogen precipitated by phosphotungstic acid.

7. A change in the amount of total nitrogen which might result from the evolution of elementary nitrogen or the formation of volatile nitrogen containing substances.

8. The amount of amino nitrogen which might decrease due to deaminization.

9. The formation of ammonia nitrogen as a result of deaminization.

10. A change in the specific rotation of the acid solution of cystine.

11. The isolation of the organic compounds present in the solution after the prolonged boiling and the comparison of their properties with those of cystine in order to determine the extent and direction of the decomposition or alteration of cystine under the conditions of the experiment.

The Material.- For an experiment of this type, a large amount of cystine is required. After trying several methods, a slight modification of Folin's method was used in its preparation.

Human hair or wool (human hair, which can be obtained without difficulty at almost any barber shop, is preferable) is washed cold dilute (1% or 2%) sodium carbonate and dried. This washing is to remove the natural oils from the hair. Two kilos of the dry, washed hair are pushed in a 6-liter pyrex flask (round bottom preferable) and 4 liters of constant boiling (20 per cent) hydrochloric acid are added. In order to get some of the acid quickly to the

bottom of the flask, part of the acid may be added first, then the hair, and finally the remaining acid. It is sometimes convenient to heat the acid and dissolve a part of the hair before all is added. The hair is hydrolyzed by boiling the contents over a free flame or better on a sand bath, or by heating on a steam bath until the biuret reaction is entirely negative. This is negative after boiling for about 4 hours or heating on a steam bath for 5-6 days. A reflux condenser should be used if the hydrolysate is boiled but if the steam bath method is employed an air condenser consisting of a large bore glass tube is equally efficient. The flask is then removed from the steam bath and the solution of amino acids almost neutralized with concentrated sodium hydroxide (commerical 98%) and then sodium acetate added until the congo red test for mineral acids is entirely negative. Care must be taken not to make the solution alkaline with sodium hydroxide. After standing 12-24 hours at room temperature, the liquid is filtered off from the precipitated cystine. This can be best accomplished by filtering on a Büchner funnel, using suction. The crude material, containing in addition to the cystine some "humin", melanin and tyrosine, is dissolved in 3 per cent hydrochloric acid and completely decolorized with good bone black which has been completely freed from calcium phosphate by boiling with hydrochloric acid and washing with cold water. The filtrate after decolorizing should be water clear or at the most only slightly yellow. If it shows much color the treatment with decolorizing carbon should be repeated. The cystine is precipitated from the clear solution as described above, using first sodium hydroxide to weakly acid reaction followed by sodium acetate. After standing 6 hours, the cystine is filtered off and washed with hot water, which will remove the last traces of tyrosine. By this

method, with very little work, the typical, colorless, hexagonal plates of cystine are obtained. With human hair the yield is about 100 grams or 5 per cent.

The Methods.- For carrying out a study as outlined above, an apparatus as shown in figure I, was used.

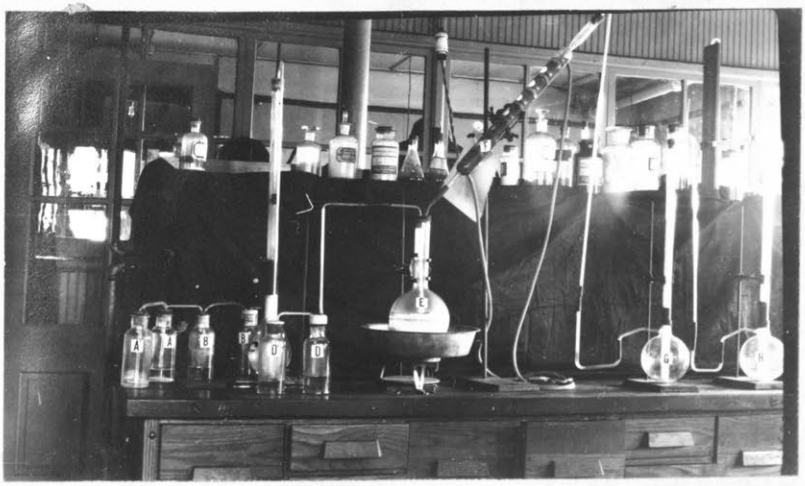


Figure I.

A' is an empty wash bottle connected to A by means of a glass tube reaching from the bottom of A' to the bottom of A. This serves as a trap in case of back pressure.

A is a wash bottle containing lead acetate to remove any traces of hydrogen sulphide in the air.

B' is a trap to B connected as A' and A.

B is a wash bottle containing 50% potassium hydroxide to remove the carbon dioxide from the air.

C is an absorption tower as described by Troug (1915) excepting that a round bottom flask is used. This contained a saturated solution of barium hydroxide and was placed in the series as B alone did not remove all of the carbon dioxide from the air.

D' is a trap to D connected as A' and A.

D is a wash bottle containing 20% hydrochloric acid to remove any ammonia from the air and to wash the air before it was drawn into flask E.

E is a round bottomed, two liter flask in which the hydrochloric acid and cystine were placed during the hydrolysis. The flask was placed on a sand bath and this heated by means of an electric hot plate.

F is a water cooled condenser.

G is an absorption tower containing cadmium sulphate. This absorbs the hydrogen sulphide drawn through by the air.

H is another absorption tower containing barium hydroxide. This absorbs the carbon dioxide as it is drawn through by the air.

K is a tube containing soda lime to prevent any carbon dioxide from entering the apparatus.

10-20 5M

To 50 grams of cystine (97.5% pure, the cystine contained 2.5% ash and moisture) enough "constant boiling" hydrochloric acid was added to make a total volumne of 1000 cc. This was placed in flask E and the flask and contents weighed on a large analytical balance (sensitive to 1 mgr. and having a capacity of 1500 grams) to the second decimal place. The flask was connected as shown in figure I, the cadmium sulphate placed in tower G and the apparatus washed free of carbon dioxide by drawing carbon dioxide free air through it for a few minutes. The barium hydroxide was then placed in tower H. Washed air was drawn through the apparatus by means of a water pump. The rate was very slow until just before the aliquot was removed, then was increased so that all of the carbon dioxide and hydrogen sulphide might be washed out in a short time. After the solution had boiled three hours and the carbon dioxide and hydrogen sulphide were washed out of the apparatus, the solutions in towers G and H were titrated as described below. The flask E was disconnected, the contents cooled to room temperature and made up to the original weight with 20% hydrochloric acid. A 50 cc aliquot, containing 2.5 grams of the original cystine or 2.4375 grams of pure cystine, was removed and made up to 250 cc, this being used in subsequent analysis. The flask and contents were again weighed and the process repeated. Aliquots were removed at the end of 3, 6, 12, 24, 48, 96, 144 and 192 hours and the samples numbered 3, 6, 12 etc.

The following methods were used in the analysis of the samples removed at the different intervals:

1. Carbon dioxide; this was determined by placing 25 cc (except the last two determinations, 144 hours and 192 hours, where 50 cc was used) of approximately N/8 barium hydroxide in tower H. After the heating was discontinued and the apparatus washed free of

carbon dioxide, the reamining barium hydroxide was titrated with standard hydrochloric acid, using phenolphtalein as an indicator. As the strength of the barium hydroxide was known in terms of the standard acid, the amount of carbon dioxide was calculated from the difference in the amounts of acid used.

2. The hydrogen sulphide; the principle of this determination is described Scott (1917). It is based on the fact that when hydrogen sulphide comes in contact with a solution of cadmium sulphate, the following reaction takes place;

$$CdSO_4 + H_2S = CdS + H_2SO_4.$$

The sulphuric acid thus formed is titrated with a standard alkali. For this determination a solution of cadmium sulphate, containing 24 grams per liter was neutralized with sodium hydroxide, using sodium alizarine sulphonate as an indicator. 25 cc of this solution (except in the last three determinations, 96 hours, 144 hours and 192 hours, where 50 cc were used) were placed in tower G. This tower was removed as described for tower H and the sulphuric acid formed was titrated. In order to determine the amount of the acidity due to hydrochloric acid which may have been aspirated over into the cadmium sulphate solution from the constant boiling hydrochloric acid, the solution, after neutralization, was titrated with standard silver nitrate, using potassium chromate as an indicator. This was not a very satisfactory method for determining the amount of chlorine but served to check on more then a trace of hydrochloric acid being present in the cadmium sulphate. In no instance was there an appreciable amount of hydrochloric acid present.

3. The sulphur that might be in the sulphate form was determined by adding a small amount of concentrated hydrochloric acid to 20 cc of the solution and heating to boiling, 5 cc of

10 per cent barium chloride was slowly added and the barium sulphate weighed in the usual manner. From the weight of barium sulphate the amount of sulphur was calculated.

4. The total sulphur was determined on 10 cc of the solution by oxidizing to sulphate with 10 cc of the oxidizing reagent described by Denis (1910). This reagent is prepared from 25 grams of copper nitrate (crystalline), 25 grams of sodium chloride and 10 grams of ammonia nitrate in 100 cc of solution. The solution of cystine and oxidizing agent is evaporated to dryness on a steam bath, ignited at red heat for 10 minutes, cooled and the residue dissolved with hot 10 per cent hydrochloric acid. The sulphate was precipitated out of the boiling solution by means of barium chloride and the sulphur determined from the amount of barium sulphate formed.

5. The amount of cystine present as determined by the potassium bromate method was carried out as described by Okuda (1919), according to the formulae:

$$5NaBr + KBrO_3 + 6HCl = 5NaCl + KCl + 3H_2O + 6Br.$$

$$\begin{array}{l} S\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH \\ | \\ S\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH \end{array} + 10Br + 6H_2O = 2(SO_3H\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH) + 10HBr.$$

To .0 cc of the cystine solution, enough hydrochloric acid is added to make a 5-10 per cent solution. To this, 10 cc of 20 per cent sodium bromide is added and the solution titrated with 0.05 normal potassium bromate, the end point being the first faint yellow color (free bromine) that lasts for one minute. 1 cc of the sodium bromate is equivalent to 0.00721 grams of cystine. When this method was used on pure cystine the results checked very well with the

sulphur and nitrogen determinations.

6. The amount of nitrogen not precipitated by phosphotungstic acid was determined by the method previously used by Van Slyke (1911). 10 cc of the solution were placed in a hundred cc volumetric flask, 10 cc of concentrated hydrochloric acid and 5 grams of phosphotungstic acid added and the solution made up to volumne. After the phosphotungstic acid had dissolved the flask was placed in a refrigerator for 24 hours. The supernatant liquid was then carefully decanted through a dry filter and the amount of nitrogen in an aliquot from the filtrate was determined by the Kjeldahl method and as the amount of total nitrogen in 100 cc was known, the difference would represent the amount of nitrogen in the precipitate.

7. The amount of total nitrogen was determined by the Kjeldahl method.

8. The amount of amino nitrogen was determined by using the Van Slyke apparatus for determining amino nitrogen.

9. The ammonia nitrogen was determined on a 20 cc portion of the solution. This was placed in a distilling flask with 100 cc of water and the solution made slightly alkaline with calcium hydroxide. 100 cc of alcohol were added and the ammonia distilled at 40-45 C, under reduced pressure, into standard sulphuric acid, the excess of standard acid titrated with standard alkali. The amount of ammonia nitrogen is then calculated from the amount of acid neutralized.

10. The specific rotation of the acid solution was determined by means of a high grade Schmidt and Hauch polariscope.

The Experimental Data.

A. The decomposition of cystine on boiling with 20 per

cent hydrochloric acid for varying lengths of time. The hydrolysis. When the hydrolysis was started the cystine hydrochloride was not all in solution but at the end of three hours boiling, the cystine hydrochloride was completely dissolved. Very little change in appearance was observed during the first 12 hours except that the color of the solution was yellow and a small amount of sulphur crystallized on the neck of the flask. After 24 hours the solution was dark yellow, a flocculent precipitate began to separate and a considerable amount of sulphur had crystallized on the neck of the flask and in the condenser tube. At 48 hours a very pungent odor was observed, the solution was a yellow brown color and a white substance was deposited on the lower part of the flask or a substance was formed during the hydrolysis that etched the glass. From 48 to 144 hours the solution became darker and murky, the amount of precipitate increased and some of the sulphur in the condenser washed back into the flask. At the end of the hydrolysis the solution was a dirty brown color and gave off a very pungent, irritating odor. The lower part of the flask was covered with a white, insoluble substance or was etched by the products of hydrolysis so as to be perfectly opaque.

It was of interest to note that cystine when hydrolyzed for a long time deposits sulphur on the neck of the flask and in the condenser as does proteins when treated in the same manner. Morner (1899) found that when horn was hydrolyzed with hydrochloric acid for a short time little or no sulphur separared out but when heated for a long time, a not inconsiderable amount of sulphur separated out. Buchtala,(1910) observed that some proteins, when hydrolyzed, deposited sulphur while others did not.

The carbon dioxide determinations.- The analytical data

are shown in Table I. From the results given in this table, it is evident that very little decarboxylization takes place. The carbon dixoide undoubtedly comes from the slow decomposition of cystine caused by the acid hydrolysis and not from a change in the cystine molecule. The large amount given off at the end of the first three hours may be due to the fact that the acid solution of cystine contained dissolved carbon dioxide as it was not boiled before the experiment was started. It is possible that the carbon dioxide results may be somewhat high, for, some volatile acid may have been formed during the hydrolysis and it might not have been absorbed by the cadmium sulphate but neutralized some of the barium hydroxide. Howerer, a considerable amount of barium carbonate was formed during the intervals of hydrolysis showing that carbon dioxide is evolved under the conditions of this experiment.

The sulphur determinations.- The amount of sulphur given off as hydrogen sulphide is shown in Table II. This table shows that only a small amount of the sulphur is evolved as hydrogen sulphide during the prolonged acid hydrolysis. Here, as in the carbon dioxide determination, the only source of error is that a volatile acid may have been formed that would be absorbed by the cadmium sulphate and titrated as sulphuric acid. There is proof of hydrogen sulphide being formed however as the characteristic yellow precipitate of cadmium sulphide was produced in a not inconsiderable quantity. The amount of hydrogen sulphide given off, like the amount of carbon dioxide, does not point toward any change in the cystine molecule but toward a slow decomposition of the cystine. Morner (1899) observed that when keratin was heated with hydrochloric acid, for a long time, hydrogen sulphide was given off.

The results for the sulphur oxidized to the sulphate,

Table I.

Showing decarboxylation of aliquots of cystine boiled for different lengths of time with 20 per cent hydrochloric acid.

Aliquots taken at end of hours boiling.	Original	3	6	12	24	48	96	144	192
Mgs. of carbon dioxide per gram cystine. #	------	1.905	2.337	3.077	3.922	5.784	10.839	15.863	23.505
Per cent of $COOH-CO_2$	------	0.519	0.637	0.839	1.069	1.577	2.956	4.326	6.410

The calculations were made from the amounts of cystine left in the flask at the end of each interval.

Table II.

Showing analyses for the various forms of sulphur in aliquots of cystine boiled for different lengths of time with 20 per cent hydrochloric acid.

Aliquots taken at end of hours boiling.	Original	3	6	12	24	48	96	144	192
Mgs. of hydrogen sulphide per gram cystine. #	------	0.075	0.101	0.145	0.229	0.428	1.319	2.280	4.070
Per cent of sulphur split off as hydrogen sulphide.	------	0.028	0.038	0.054	0.086	0.160	0.494	0.855	1.526
Mgs. sulphate-sulphur per gram cystine.	------	0.84	1.05	1.05	1.26	0.84	0.77	1.40	0.84
Per cent of sulphur as sulphate.	------	0.315	0.394	0.394	0.472	0.315	0.289	0.525	0.315
Mgs. total sulphur per gram cystine.	265.4	259.6	253.6	250.4	255.4	250.4	250.9	258.5	259.1
Per cent total sulphur in original cystine.	26.54	25.96	25.36	25.04	25.54	25.04	24.09	25.85	25.91

The calculations were made from the amounts of cystine left in the flask at the end of each interval.

Table II, show that a very small amount of sulphate is present and that the amount is constant during the hydrolysis. This indicates that no oxidation of sulphur to sulphate has taken place and that the substance weighed as barium sulphate probably is the ash contained in the original cystine. The amount is so small that it can be disregarded.

The amount of total sulphur serves as a check on the amount of sulphur in the solution but does not show the amount of cystine. Elementry sulphur, which was not obtained in the total sulphur determination, was deposited on the neck of the flask and on the condenser tube. This was then washed down and increased the total sulphur values in later determinations. The results in Table II, show that the amount of total sulphur decreases until 144 hours and then increases. As already stated, the sulphur on the neck of the flask and on the condenser tube washed down so that practically none was left at the end of 192 hours. It is also noted that, at the end of the experiment or 192 hours, 17.5 grams of the cystine had been removed so that part of the sulphur washed back in the tube came from cystine, in the aliquots that had been removed from the flask. There is a possibility that the flocculent precipitate, noted above, might contain some sulphur. This was confirmed by subsequent analysis as a sulphur determination made on the 144 hour aliquot after the precipitate had settled, showed that the sulphur content of the filtrate alone was 0.4% lower than when determined on the solution containing the flocculent precipitate. This shows that some of the sulphur had precipitated out of the solution with the settling of the precipitate. The total sulphur determinations do not show what is happening to the cystine molecule. The hydrogen sulphide determinations give the only index as to what becomes of a

Table III.

Showing the amount of cystine by the bromate method in aliquots of cystine boiled for different lengths of time with 20 per cent hydrochloric acid.

Aliquots taken at end of hours boiling.	Original	3	6	12	24	48	96	144	192
Mgs. of cystine per gram of original cystine.	1000.4	970.9	951.8	948.0	939.9	925.2	953.2	971.1	905.8
Per cent of cystine.	100.04	97.09	95.18	94.80	93.99	92.52	95.32	97.11	90.58

Table IV.

Showing milligrams of nitrogen not precipitated by phosphotungstic acid, per 100 cc of solution, from aliquots of cystine boiled for different lengths of time with 20 per cent hydrochloric acid.

Aliquots taken at end of hours boiling.	Original	3	6	12	24	48	96	144	192
Mgs. of nitrogen not precipitated.	1.20	1.92	2.92	4.16	5.04	6.08	6.12	6.80	6.62
Per cent of nitrogen not precipitated. #	10.29	16.46	25.04	35.68	43.22	52.14	52.49	58.31	56.77

Calculations made from theoretical, 11.66 mgs. nitrogen in 100 cc solution.

small part of the sulphur while the sulphate determinations show that none of the sulphur is oxidized to the sulphate.

The amount of cystine as determined by the potassium bromate method.- The results given in Table III, show that the sulphur in cystine is not readily oxidized by boiling with acid under the conditions of this experiment, at any rate, most of the sulphur still reacts with bromine. The end points were very indistinct after 24, when the color of the solution was almost that of the end point therefore making it very difficult to obtain accurate titrations. It is readily seen that for colored solutions the bromate method is of little or no value as a quantitative method but serves as a qualitative method showing that the sulphur in the cystine is still largely in an unoxidized form.

The nitrogen precipitated by phosphotungstic acid.- The amounts of nitrogen not precipitated by phosphotungstic acid, per 100 cc of solution, are given in Table IV. The results are given in this form rather than substracting from the total nitrogen which would give the amounts precipitated by phosphotungstic acid. The amount of nitrogen not precipitated increases until the cystine had been boiled for 48 hours and then the amounts remain about constant for the longer periods of boiling. After 48 hours, no more of the typical cystine phosphotungstate precipitate as formed with protein (plate) cystine was obtained but a more granular one. The precipitates of 96, 144 and 192 hours all looked alike and contained about the same quantity of the precipitate, while from the original to 48 hours, the amount of precipitate decreased and contained less of the typical plate cystine precipitate. This shows that the cystine has been changed in some respect making the phosphotungstic acid precipitate more soluble or changing it into a form that is not

Table V.

Showing analyses for the various forms of nitrogen in aliquots of cystine boiled for different lengths of time with 20 per cent hydrochloric acid.

Aliquots taken at end of hours boiling.	Original	3	6	12	24	48	96	144	192
Mgs. total nitrogen per gram cystine.	114.8	112.3	110.5	111.6	111.7	111.5	111.5	110.9	114.6
Per cent nitrogen in original cystine.	11.48	11.23	11.05	11.16	11.17	11.15	11.15	11.09	11.46
Mgs. amino nitrogen per gram cystine.	129.0	137.7	131.0	131.5	129.8	129.3	127.6	127.6	126.9
Per cent amino nitrogen in original cystine.	12.90	13.77	13.10	13.15	12.98	12.93	12.76	12.76	12.69
Mgs. ammonia nitrogen per gram cystine.	-----	2.35	2.56	2.66	3.28	3.28	3.69	7.69	11.16
Per cent ammonia nitrogen from original cystine. #	-----	2.02	2.19	2.28	2.81	2.81	3.16	6.59	9.57

Calculations made on the basis of pure cystine, nitrogen = 11.66%.

Table VI.

Showing changes in the specific rotation of aliquots of cystine boiled for different lengths of time with 20 per cent hydrochloric acid.

Aliquots taken at end of hours boiling.	Original	3	6	12	24	48	96	144	192
Specific rotation of acid solution.	-201.7	-180.2	-152.5	-124.7	-48.5	-20.8	00.0	-----	-----
Per cent decrease in specific rotation.	00.00	10.66	24.39	38.17	75.95	89.69	100.0	-----	-----

precipitated by phosphotungstic acid. Van Slyke (1911) found that under my conditions of precipitation, 1.3 mgs. of nitrogen per 100 cc was not precipitated when pure cystine was used but with cystine which had been boiled with 20 per cent hydrochloric acid for 24 hours, he found that about 7 mgs. of nitrogen per 100 cc of the solution was not precipitated by phosphotungstic acid.

The nitrogen determinations.- The total nitrogen TableV, merely serves as a check on the amount of nitrogen in the solution. It shows that very little, if any, nitrogen was lost in the form of a volatile substance during the long boiling with acid. The nitrogen determinations do not show any change in the form of the nitrogen.

The amino nitrogen figures, Table V, are not very consistent. Even when they are multiplied by the factor 0.926, used for cystine nitrogen when determined in the Van Slyke apparatus (Van Slyke (1911a)), they give a higher result than the total nitrogen figures. There is a possibility that the amount of substance causing the high result when an acid solution of cystine is determined in the Van Slyke apparatus, is increased during the hydrolysis thus making the results too high. It is probable that the amount of amino nitrogen decreases slightly during the hydrolysis.

The figures in Table V, show that the amino nitrogen is slowly deaminized, forming ammonia. With the exception of one or two samples, the ammonia nitrogen increases almost proportionally to the decrease of amino nitrogen. Gortner and Holm (1920) found that when a mixture of amino acids and formaldehyde was boiled for 24 hours with 20 per cent hydrochloric acid very little ammonia was formed. Assuming that all of the ammonia nitrogen in their experiment number II, came from cystine, only 2.74 per cent of the

nitrogen was changed. This agrees very well with the results obtained in this experiment where only 2.79 per cent of the nitrogen is in the form of ammonia at the end of 24 hours.

The optical activity of the acid solution.- The specific rotations, Table VI, show that cystine is gradually and completely changed into an inactive substance or substances by prolonged acid hydrolysis. The greatest change takes place during the first 48 hours and at 96 hours the solution is completely inactive. Morner (1901) observed that, when cystine was heated on a steam bath for 109 hours with 10 per cent hydrochloric acid, the specific rotation changed from -223 to -134 and the cystine was changed to a more soluble form. As to my original rotation of -201.7 as compared with that of -223 usually given for cystine, one can easily see from the specific rotations that cystine is very easily changed, by acid hydrolysis, to an inactive form and that it is almost impossible to prepare the pure plate cystine by this method. Morner, in his first paper on the preparation of cystine, realized the fact that it is almost impossible to obtain two preparations with identical optical rotations.

Chart I. In this chart the percentages are plotted against the time of boiling. Only the results of five of the determinations are plotted as the total nitrogen, the amino nitrogen, the sulphur oxidized to sulphate, the amount of cystine as determined by the potassium bromate method and the total sulphur figures show no significant differences. The curve showing the percent of carbon dioxide split off from the carboxyl group is practically a straight line. The hydrogen sulfide curve is very similar to the carbon dioxide curve. The curve showing the percent of ammonia nitrogen is almost a straight line as in the case of the carbon dioxide and

the hydrogen sulphide. The curve for optical ortation shows the per cent decrease during the first 48 hours to be very rapid indicating that the greatest change takes place during that time. From 48 to 96 hours, the decrease is very slight and it is probable that it reaches 100 per cent before 96 hours. The amount of nitrogen not precipitated by phosphotungstic acid shows a very similar curve to that of the specific rotation. The curves fall in two groups; one, consisting of the carbon dioxide, hydrogen sulphide and ammonia nitrogen show slow decomposition, the other, consisting of the specific rotation and the amount of nitrogen not precipitated by phosphotungstic acid indicate a marked change in the cystine during the first 48 hours of hydrolysis.

From these results, one concludes that only a small amount of the cystine is <u>decomposed</u> during prolonged acid hydrolysis. The major portion of the cystine is <u>changed</u> into a form that is optically inactive and that is not as completely precipitated with phosphotungstic acid as is the plate cystine. It is evident then, that some other solution of the problem aside from a study of decomposition products is necessary.

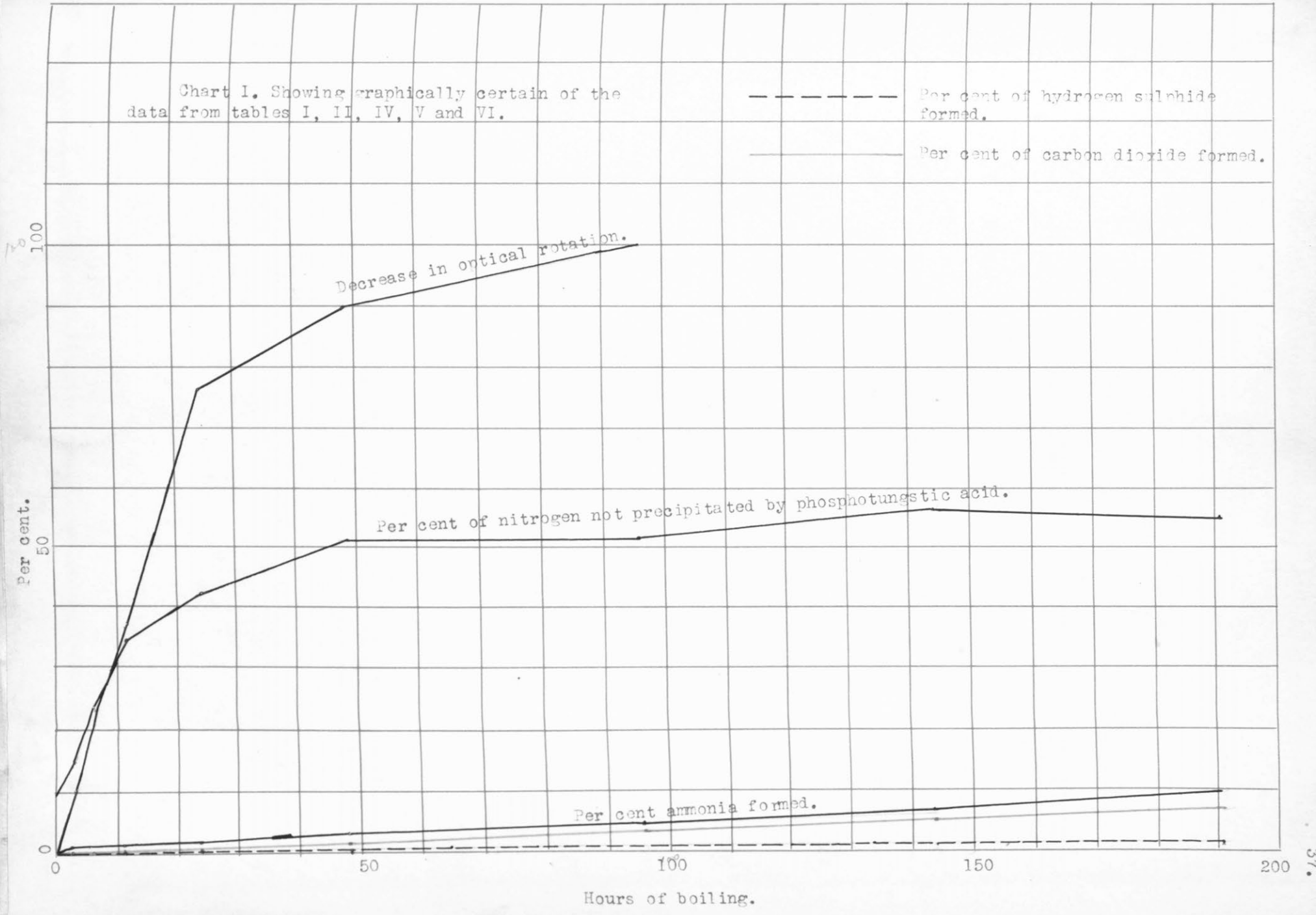


Chart I. Showing graphically certain of the data from tables I, II, IV, V and VI.
Per cent of hydrogen sulphide formed.
Per cent of carbon dioxide formed.
Decrease in optical rotation.
Per cent of nitrogen not precipitated by phosphotungstic acid.
Per cent ammonia formed.
Per cent.
0
50
100
0
50
100
150
200
Hours of boiling.

B. The isolation of an "isomeric" cystine from the residual hydrolysate.

Isolation and analysis.- After the residual hydrolysate had stood for 28 days, the flocculent precipitate, noted in the previous section, was filtered off, washed with 20 per cent hydrochloric acid and dried. It was grayish black in color. The black portion was soluble in hot water and after the water was evaporated, a jet black residue remained. A part of the precipitate was extracted with carbon disulphide to remove any elementary sulphur. When the carbon disulphide was evaporated the characteristic yellow crystals of sulphur remained. After the residue was extracted with hot water, the insoluble portion contained some inorganic matter besides a small amount of organic matter. An analysis of the black, water soluble portion of the original precipitate gave the following results:

Amino nitrogen = 7.73%

Total nitrogen = 15.89%

Total sulphur = 8.73%

This analysis shows that this residue is not cystine but probably some decomposition product. Only a small quantity was obtained, not sufficient in amount for further experimental work.

The filtrate was evaporated to dryness, in vaccuo, on a boiling water bath and then held at 100 C, in vaccuo, until the excess of hydrochloric acid was driven off. Flaky, needle like crystals were formed. This was then dissolved in water and a portion of the solution was removed for analysis. The amounts of total nitrogen, amino nitrogen and total sulphur were determined. The ratio of nitrogen to sulphur was almost identical with the ratio for cystine.

Amino nitrogen = 12.96 mg. in 10 cc. (corr. for cystine).

Total nitrogen = 12.96 mg. in 10 cc.

Total sulphur = 30.00 mg. in 10 cc.

In the hope of precipitating cystine from the solution, sodium acetate was added to the solution until no more free hydrochloric acid was present. A copious precipitate settled out. The filtrate was again analyzed for total nitrogen, amino nitrogen and total sulphur.

Amino nitrogen = 4.92 mg. in 10 cc. (corr. for cystine).

Total nitrogen = 5.03 mg. in 10 cc.

Total sulphur = 10.60 mg. in 10 cc.

This data show that there was probably only one substance present in the original hydrolysate in any appreciable amount, for the ratio of the amino nitrogen to the total nitrogen and to the total sulphur in the filtrate before precipitating with sodium acetate and after precipitating is about the same.

The precipitate was filtered off, dissolved in dilute hydrochloric acid, decolorized with acid extracted bone black and the substance precipitated from the colorless solution with sodium acetate. The precipitate was dried at 100 C and analyzed.

	Calculated for cystine.	Found.
Amino nitrogen	11.66%	11.65% (corr)
Total nitrogen	11.66%	11.56%
Total sulphur	26.69%	26.83%

These figures show that during the long boiling, the composition of cystine has not been changed.

- Physical properties.- This "isomeric" cystine consists of white, powdery crystals which, at first, look like solid spheres

but after a time the crystal form appears to be very fine, short needles. Under a high power microscope (preferably oil immersion). these do not prove to be needles but long, blunt prisms while the plate cystine consists of large, hexagonal plates as shown in Figure II. This is undoubtedly the same crystal form that Morner (1899) and Rothera (1905) observed in their preparations of plate

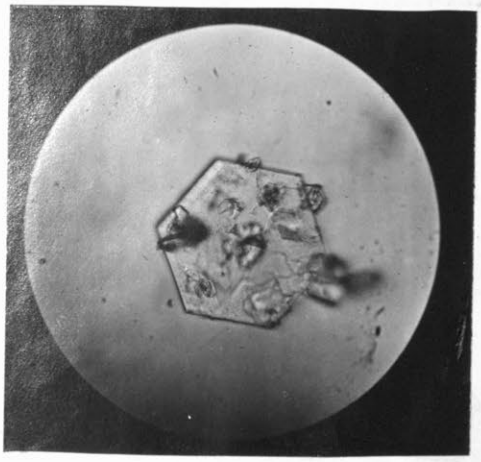
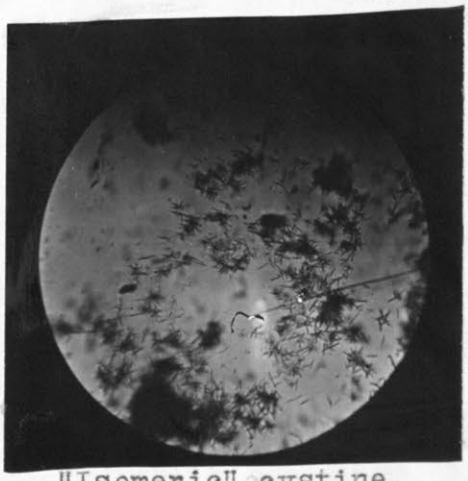


Plate cystine.



"Isomeric" cystine.

Figure II.

cystine prepared from keratin hydrolyzed for a long period of time. These crystals compare very well with the discription which Erlenmeyer (1904) and Fischer and Raske (1908) gave for their synthetic preparations of inactive cystine and also for the l-cystine prepared by Fischer and Raske (1908).

This "isomeric" cystine is completely inactive as 0.1000 grams dissolved in 25 cc of dilute hydrochloric acid and placed in a 2 decimeter tube, gave no optical rotation. It is insoluble in hot or cold alcohol, appreciably soluble in hot water and more soluble in cold water than the plate cystine. When this "isomeric" cystine is boiled with concentrated sodium hydroxide and lead acetate, lead sulphide is formed.

Solubility.- The relative solubility of the two forms of

cystine was determined as follows; 200 cc of carbon dioxide and ammonia free water and 1 gram of cystine were placed in a glass container. This was agitated in a shaking machine for 7 hours. After standing several days, the solution was filtered, by gravity, through dry S & S hardened filter paper. 50 cc of the filtrate was placed in a weighed platinum dish, evaporated on a steam bath, dried at 100 C and weighed. The solubility of the glass from the containers was also determined by using water alone and carrying out the procedure described above. This "blank" was then substracted from the result obtained when cystine was used. The solubility in pure water at 20 C is:

Plate cystine = 1 : 5263.

"Isomeric" cystine = 1 : 2059.

These results show that the "isomeric" cystine is 2.556 times as soluble as the plate cystine. Neuberg and Mayer (1905a) give the solubilities of plate cystine and their inactive cystine as:

Plate cystine = 1 : 8840.

Inactive cystine = 1 : 3070.

These results show that the inactive cystine is 2.879 times as soluble as the plate cystine. The difference in the amount dissolved is probably due to the method of obtaining a saturated solution. They do not state as to how they obtained a saturated solution of their cystines or their method of determining the amount of cystine in solution.

Precipitability with phosphotungstic acid.- The amount of the "isomeric" cystine precipitated by phosphotungstic acid was compared with the amount of plate cystine precipitated under identical conditions. 0.2500 grams of the cystine was placed in a 250 cc flask, 12.5 grams of phosphotungstic acid and 25 cc of

concentrated hydrochloric acid were added. This was diluted to 250 cc and placed in an ice box for 24 hours. Total nitrogen was then determined on aliquots of the filtrate.

The analysis of the filtrate from the plate cystine precipitate shows that 1.2 mgs. of nitrogen per 100 cc of the solution was not precipitated by phosphotungstic acid under these conditions, while the analysis of the filtrate from the "isomeric" cystine precipitate shows 4.82 mgs. of nitrogen not precipitated, under the same conditions. The phosphotungstic acid precipitate of the "isomeric" cystine is 4 times as soluble as that of the plate cystine. Another difference was noted in the form of the precipitates. The "isomeric" cystine precipitate did not contain the typical plate cystine phosphotungstate precipitate but a heavy, granular like precipitate. The precipitate of the "isomeric" cystine did not form for several hours while that of the plate cystine formed immediately.

C. A comparison of certain derivatives of the "isomeric" cystine and plate cystine.

The hydrochlorides.- The difference in the hydrochlorides was shown by making use of the microscopic method described by Doniges (1920). A small quantity of the fine material is placed on a glass slide and a drop of concentrated hydrochloric acid is added. This is examined, without a cover glass, under a microscope. Crystals of the hydrochloride soon form. The crystals of the plate cystine hydrochloride are long, prismatic needles while those of the "isomeric" cystine hydrochloride are diamond or rectangular shaped prisms or masses of plates. The hydrochlorides of the two forms of cystine are decidedly different, thus forming an easy method of detecting small amounts of either form.

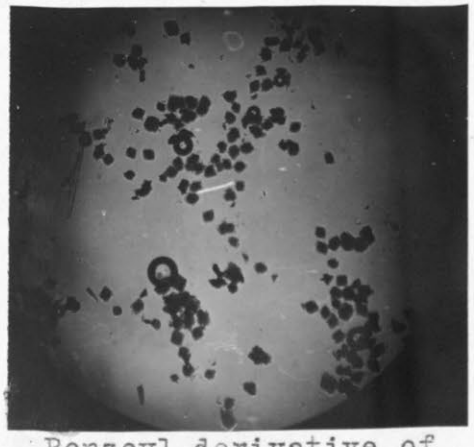
The benzoyl derivatives.- These were prepared as described by Brenzinger (1892). 2 grams of cystine were dissolved in a little sodium hydroxide and 10 grams of benzoyl chloride added, then enough sodium hydroxide to make the total amount added 6 grams. This was shaken and after the odor of benzoyl chloride disappeared, the solution was neutralized with hydrochloric acid.

The plate cystine, when treated as above, formed a solid gel when the solution was neutralized with hydrochloric acid. This was filtered, washed and dissolved in alcohol. On dilution, the gel again formed. The gel was filtered and dried at 100 C. This preparation melted at 180-181 C (uncor.). Brenzinger gives the melting point as 180-181 C while Goldmann and Baumann (1888) give the melting point as 156-158 C. Brenzinger states that when only 2 grams of cystine were used, he was able to dilute the alkaline solution of this derivative to 3 liters, acidify it and obtain a solid gel. I have found it possible to prepare a 0.2% gel that was

very rigid and a 0.1% gel which "set" but was not as rigid as the 0.2% gel. The preparation, when purified from alcohol, appeared very much as described by Brenzinger, "Die jetzt benzoësäurefrei, jedoch wieder gallertartig sich abscheidende Substanz wurde schliesslich aus mässig verdünnten Alkohol umkrystallisirt, wobei sich blumenkohlartige massen auscheiden, die aus feinen biegsamen Nadeln bestehn, welche geringer Seidenglanz besitzen".

The benzoyl derivative of the "isomeric" cystine was prepared by the method described above. When the solution was neutralized with hydrochloric acid, no gel was formed as in the case of the plate cystine but the substance precipitated out in crystal form. These crystals were diamond shaped plates and a few appeared to be broken fragments of plates. The crystal forms of the plate

Benzoyl derivative of plate cystine.



Benzoyl derivative of "isomeric" cystine.

Figure III.

cystine and "isomeric" cystine derivatives are shown in Figure III. The melting point of this derivative is 168 C (uncor.) but one sample that precipitated out of the mother liquor melted at 170 C (uncor.). The melting point is not very sharp as the substance seems to soften at 122 C and is not completely melted below 168 C. These melting points were carried out with the aid of a microscope focused on the melting point tube. A typical example of the melting point

behavior is; the sample softened at 113-114 C, some gas was given off at 117 C, melted at 122 C with gas evolution. The melt then solidified slowly and after being held at 145 C for one hour, this was completely solid and the sample finally melted at 168 C (uncor.) with the evolution of gas. On cooling this set to a glass like substance which melted at 91 C (uncor.). It is possible that there was water of crystallization present but samples dried at 100 C, before the determination was showed the same series of melting points. Neuberg and Mayer (1905) prepared the benzoyl derivative of "stone" cystine which melted at 157-159 C. There is, of course, the possibility of internal rearrangement during the melting point determination. This phase of the work needs farther investigation but could not be completed at the present time. The preparation of benzoyl "isomeric" cystine was analyzed as follows;

	Calculated for benzoyl cystine.	Found.
Nitrogen	6.25%	6.15%
Sulphur	14.28%	14.16%.

This derivative shows the required amount of nitrogen and sulphur for the plate cystine derivative but is different in physical properties, crystal form and melting point.

The phenylisocyanate derivatives.- These derivatives were prepared by dissolving 2 grams of the cystine in 24 cc of water by the addition of 18 cc of normal sodium hydroxide and adding 2 grams of phenylisocyanate. This mixture was agitated or shaken until the odor of the phenylisocyanate disappeared, filtered and acidified the filtrate with hydrochloric acid.

The plate cystine solution when treated in this manner set to a solid crystal gel. This was filtered and purified by

recrystallization from alcohol. This preparation melted at 148-149 C (uncor.). The analysis of this preparation showed:

	Calculated for cystine phenyl-isocyanate.	Found.
Nitrogen	11.72%	11.38% (Dumas method)
Sulphur	13.39%	13.48%.

This substance has already been prepared by Hueberg and Mayer (1905) who gave the melting point as 160 C (cor.). 1.5 grams of this pure substance in 150 cc of dilute alcohol set to a rigid gel when the crystals were fully formed. These were very fine needles or tyrosine like crystals.

The phenylisocyanate derivative of the "isomeric" cystine was prepared as described above. When the solution was neutralized with hydrochloric acid, a white gelatinous precipitate formed but soon changed to a crystalline form. This was filtered and purified from alcohol. This preparation consisted of very fine, long, silky, colorless needles which melted at 181 C (uncor.). This was not easily soluble in alcohol as compared with the plate cystine derivative which was very soluble in alcohol. The preparation analyzed for nitrogen (by Dumas method) and sulphur gave:

	Calculated for cystine phenyl-isocyanate.	Found.
Nitrogen	11.72%	12.88%
Sulphur	13.39%	13.66%.

The phenyl hydantoins.- These were prepared by boiling the phenylisocyanate derivatives with 10 per cent hydrochloric acid. 2 grams of the plate cystine phenylisocyanate was boiled with 150 cc of 10 per cent hydrochloric acid. This formed a clear solution and on cooling a white precipitate formed. This was filtered off and

purified by recrystallization from dilute alcohol. The pure substance consisted of fine needles which melted at 122-123 C (uncor.) This compound was prepared by Patten (1903) who noted a melting point of 117 C (uncor.).

2 grams of the "isomeric" cystine phenylisocyanate were boiled with 150 cc of 10 per cent hydrochloric acid. This did not dissolve completely as did the phenylisocyanate of the plate cystine. Only a small amount separated out on cooling and the solution stood for a long time. This precipitate was filtered off and purified by recrystallization from dilute alcohol. The preparation consisted of fine, silky needles that melted at 181 C (uncor.). The portion that did not dissolve in the hydrochloric acid was recrystallized from alcohol and the melting point and crystal form found to be the same as that which dissolved in the acid. This was analyzed for nitrogen by the Dumas method.

	Calculated for cystine phenyl hydantoin.	Found.
Nitrogen	12.67%	12.69%.

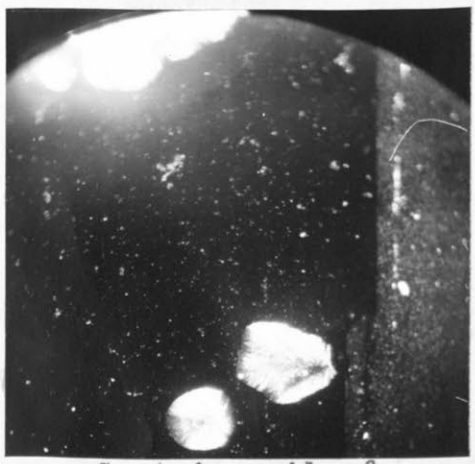
This preparation has the same melting point as before boiling with hydrochloric acid. When some of the crystals were mixed with crystals of the phenylisocyanate derivative noted above, the mixture melted at 181 C (uncor.). The phenyl hydantoin of the "isomeric" cystine is probably formed immediately when the phenylisocyanate is prepared as the melting point is the same and the phenylisocyanate of the "isomeric" cystine has the same nitrogen content as the phenyl hydantoin.

Neuberg and Mayer (1905) prepared the phenylisocyanate of "stone" cystine which melted at 170-172 C (cor.). They were <u>not</u> able to prepare the hydantoin from this derivative as they did from

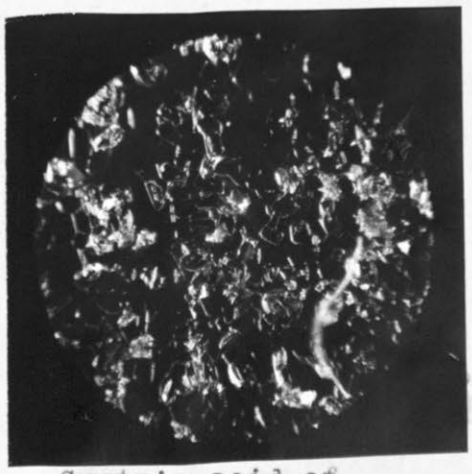
the phenylisocyanate derivative of "protein" cystine. Their statement concerning this is,"Durch Kochen kit Salzsäure haben wir keine Anhydrisierung dieser Hydäntoinsaure zum Phenylhydantoin erzielen können".

The cysteic acids.- The cysteic acids were prepared according to the method used by Friedmann (1903). 10 grams of plate cystine was suspended in 75 cc of water and bromine was added until the oxidation was almost complete and then strong bromine was slowly added until a light yellow color was permanent. This This solution was then evaporated to dryness, in vaccuo, and washed by grinding with successive portions of alcohol until the cysteic acid was free of hydrobromic acid. The product was then recrystallized from water. The preparation consisted of a white powdery like mass of crystals.

The cysteic acid of the "isomeric" cystine was prepared in the same way as the cysteic acid of the plate cystine. This



Cysteic acid of plate cystine.



Cysteic acid of "isomeric" cystine.

Figure IV.

preparation was pure white and consisted of plates, some being square, some rectangular and others were more or less irregular. In some cases these appeared to be prisms or stacks of plates. The

crystalline form of the two cysteic acids is very different as shown in figure IV. The cysteic acid of the "isomeric" cystine did not melt below 245 C. It is optically inactive as a solution of this cystine containing 2.5 grams per 100 cc in a 2 decimeter tube gave no optical rotation. The analyses of the cysteic acid of the "isomeric" cystine gave:

	Calculated for cysteic acid.	Found.
Total nitrogen	8.30%	8.96%
Amino nitrogen	8.30%	7.73%
Total sulphur	18.87%	18.96%

The sulphur content is theoretical for cysteic acid while the nitrogen is slightly higher. The strange behavior of the amino nitrogen may be due to the fact that this is a strong acid, thus causing the amino group to behave differently in the Van Slyke apparatus.

IV. DISCUSSION.

It was necessary to present a discussion of each topic at the time the analytical data were presented. There is little need of further extensive discussion.

The data in Tables I-VI show that very little change occured in aliquots of a cystine solution boiled for varying lengths of time with hydrochloric acid. The analyses show that there is but little decomposition that can be measured by the methods used. Three of the possible changes i.e., ammonia nitrogen formation, loss of carbon dioxide and hydrogen sulphide evolution show a slow progressive decomposition while the precipitability with phosphotungstic acid and the optical activity show that the cystine undergoes some change that makes the cystine or the products resulting from the long boiling optically inactive and the phosphotungstate more soluble. The greatest change takes place during the first 48 hours. At the most, only about 10 per cent of the cystine was decomposed by boiling for 192 hours.

An "isomeric" cystine was isolated from the residual hydrolysate. This "isomeric" cystine proved to be the principle constituent of the hydrolysate. It was found to be optically inactive and its phosphotungstate much more soluble than that of the plate cystine. The derivatives of this cystine were compared with those of plate cystine and these, as do the other comparisons made, show the two forms of cystine to be different. This tends to prove the conclusions one might draw from the first part of the experiment, i.e, cystine is not appreciably decomposed or destroyed when boiled for a long time with 20 per cent hydrochloric acid but is changed into another compound that is optically inactive and its phosphotungstate is more soluble. This "isomeric" cystine showed

the required amount of amino nitrogen, total nitrogen and total sulphur for cystine. It differs from plate cystine in every comparison made between the two forms of cystine.

Whether or not the "isomeric" cystine is simply a racemic form of the original l-cystine has not yet been definitely proven but in view of the fact that the cystine synthesized by Fischer and Raske (1908) and Erlenmeyer (1904) crystallized in the form of needles and the synthetic l-cystine prepared by Fischer and Raske (1908) likewise crystallized in the form of needles, it appears possible that the natural l-cystine, crystallizing in hexagonal plates, has never been synthesized and that I have merely succeeded in changing the natural hexagonal cystine into the needle form. The structural relationships involved in such a change must remain unknown until further experimental work can be carried out. Likewise the nutritional utilization of the "isomeric" cystine is a problem for future study.

V. SUMMARY AND GENERAL CONCLUSIONS.

Cystine was boiled for varying lengths of time with 20 per cent hydrochloric acid and analyses made on aliquots at each time interval. A study was also made on the residual hydrolysate, after 192 hours boiling, and an "isomeric" cystine isolated. The "isomeric" cystine and its derivatives were compared with the original cystine..

The data seem to warrent the following conclusions:

1. Cystine is only slowly decomposed or destroyed during long boiling with 20 per cent hydrochloric acid. This would not be appreciable during the time of ordinary protein hydrolysis (12-24 hours).

2. The carboxyl group of cystine is not appreciably destroyed when cystine is boiled for 192 hours with 20 per cent hydrochloric acid. Carbon dioxide is slowly evolved during the boiling.

3. The sulphur of cystine is not broken off to any appreciable extent by boiling with 20 per cent hydrochloric acid for 192 hours. A small amount of hydrogen sulphide is evolved, some elementary sulphur separates, no sulphates are formed but about 90 per cent of the original sulphur is still in an unchanged and unoxidized condition at the end of the boiling period.

4. The amount of cystine precipitated by phosphotungstic acid decreases rapidly during the first 48 hours of boiling and the amount precipitated from 48 hours until the end of the experiment remained constant.

5. The nitrogen of cystine is not appreciably changed during this experiment. The amount of total nitrogen remains constant, the amount of amino slowly decreases and there is a slow

progressive increase in the amount of ammonia nitrogen.

6. The optical rotation of the cystine solution rapidly falls during the boiling period, from -201.7 to complete inactivity at the end of 96 hours.

7. An "isomeric" cystine was separated from the residual hydrolysate. This "isomeric" cystine crystallizes in small, microscopic prisms and showed markedly different behavior to the original cystine. It was approximately 2.5 times as soluble in water and the phosphotungstate was about 4 times more soluble. It showed no optical activity. The yield indicates that this compound is probably the only substance present in appreciable amounts, in the residual hydrolysate.

8. This "isomeric" cystine was analysed for its nitrogen and sulphur content and was found to be isomeric with the original cystine.

9. Several derivatives of this "isomeric" cystine were prepared and compared with those of the original cystine. The hydrochlorides showed a marked difference in crystal form. The benzoyl derivative of the "isomeric" cystine melted at 168 C (uncor.) and the plate cystine derivative melted at 180-181 C(uncor.). The phenylisocyanate of the "isomeric" cystine melted at 181 C(uncor.) and that of the plate cystine melted at 148-149 C(uncor.). The phenyl hydantoin of the "isomeric" cystine melted at 181 C(uncor) and that of the original cystine melted at 122-123 C(uncor.). The cysteic acids showed a marked difference in crystal form.

10. The comparison of the several derivatives of the two cystines shows that the derivatives are in every instance different, therefore acid hydrolysis has apparently converted one cystine into an isomeric compound. This appears to be the only important change

to cystine brought about by acid hydrolysis.

VI. LITERATURE CITED.

Abderhalden, E.
1919, "Weiter Beitrag zur Kenntnis des im Harn und in Harnsteinen vorkommenden Cystins". Zeit. f. physiol. Chemie, 104, p.129-132.

Abel, J. J.
1895, "Ueber das Vorkommem von Aethylsulfid im Hundeharn, über das Verhalten seiner Lösung in concentrirter Schwefelsäure gegen Oxydationmittel und über einige Reactionen zur Auffindung der Alkylsulfide". Zeit. f. physiol. Chemie, 20, p.253-279.

Baudisch, O.
1908, "Zur Kenntnis der Bindung des Schwefels im Keratinmolekül". Chemiker Zeitung, 32, p.620.

Baudrimont and Malaguti.
1838, "Observations sur la note de M. Thaulow". Journ. d. Pharm., 24, p.633-634.

Baumann, E.
1882, "Zur Kenntniss der Phenylmercaptursaure, des Cystins und des Serins". Berichte der deutsch. chem. Gesellschaft, 15, p.1731-1735.

1884, "Ueber Cystin und Cystein". Zeit. f. physiol. Chemie, 8, p.299-305.

Baumann, E. and Preusse, C.
1879, "Ueber Bromphenylmercaptursäure". Berichte der deutsch. chem. Gesellschaft, 12, p.806-810.

1881, "Zur Kenntniss der synthetischen Prozesse im Thierkörper". Zeit. f. physiol. Chemie, 5, p. 309-343.

Breinl, F. and Baudisch, O.
1907, "Beiträge zur Kenntnis des oxydativen Abbaues der Keratine mit Wasserstoffsuperoxyd". Zeit. f. physiol. Chemie, 52, p.159-169.

Brenzinger, K.
1892, "Zur Kenntniss des Cystins und des Cysteins". Zeit. f. physiol. Chemie, 16, p.552-588.

Buchtala, H.
1907, "Uber das Mengenverhältnis des Cystins im verschiedenen Hornsubstanzen". Zeit. f. physiol. Chemie, 52, p.474-481.

1908, "Elementaranalyse der Eihäute von Scyllium stellare, Pristiurus melanostomis und Scullium canicula und Verteilung des Stickstoffes in denselben". Zeit. f. physiol. Chemie, 56, p.11-17.

Buchtala, H.
1910, "Uber den Schwefel- und Cystingehalt der Keratine von Geflügelarten". Zeit. f. physiol. Chemie, 69, p.310-312.

Cloëtta, A.
1859, "Ueber das Vorkommen von Inosit, Harnsäure, etc. im thierischen Körper". Annalen d. Chemie u. Pharmacie, 99, p.289-305.

Cohn, R.
1898, "Ueber eine quantitative Eiweissspaltung durch Sälzsaure". Zeit. f. physiol. Chemie, 26, p.395-410.

Crawford, A.
1797, "Fortsetzung der Versuche und Beobachtungen uber die Kerbsmaterie und uber einige luftige Flussigkeiten, die vermittlest der Destillation und Faulniss aus thierischen Substanzen entwickelt werden". Crell's Chemische Annalen, 1797,1, p.335-364.

Danilevsky, A.
1869, "Studien uber die Eiweisskorper". Zeit. f. Chemie,. 5, p.41-43.

1883, "Zur vorläufigen Abwehr". Zeit. f. physiol. Chemie, 7, p.427-449.

Denis, W.
1910, "The determination of total sulphur in urine". J. Biol. Chem., 8, p.401-403.

1911, "The oxidation of the amino acids, 1. Glycocoll and cystine". J. Biol. Chem. 9, p.365-374.

Dewar and Gamgee.
Journ. of Anat. and Physiol. 7, p.142. Cited by Friedmann in Beitrage zur chem. Physiol. u. Path. 3, p.2. (Original was not obtainable at Minnesota).

Doniges, G.
1920, "A new microchemical reaction of cystine applicable to its detection in urinary calculi". J. Soc. Pharm. Bordeaux, 58, p.8. (Chem. Abs. 14, p.1689).

Drechsel, E.
1891, "Ueber des Vorkommen von Cystin und Xanthin in der Pferdleber". Archiv. f. Anat. u. Physiol., Physiol. Abth., 1891, p.243-247.

1896, "Beiträge zur Chemie einiger Seethiere". Zeit. f. Biologie, 33, p.85-107.

1896a, "Ueber die Bindung des Schwefels im Eiweissmolecul". Centralblatt f. Physiologie, 10, p.529-530.

Embden, G.
1901, "Ueber den Nachweis von Cystin und Cystein unter den Spaltungsprodukten der Eiweisskörper". Zeit. f. physiol. Chemie, 32, p.94-103.

Emmerling.
1894, "Cystin unter der Spaltungsproducten des Horns". Chemiker Zeitung, 18, p.1539.

Erlenmeyer, E.
1903, "Synthese des Cystins". Berichte der deutsch. chem. Gesellschaft, 36, p.2720-2722.

1904, "Zur Kenntniss der α-Amidosauren". Annalen d. Chemie u. Pharmacie, 337, p.205-263.

Fischer, E. and Raske, K.
1908, "Verwandlung des l-Serins in aktives naturliches Cystin". Berichte der deutsh. chem. Gesellschaft, 41, p.893-897.

Fischer, E. and Suzuki, U.
1905, "Zur Kenntnis des Cystins". Zeit. f. physiol. Chemie, 45, p.405-411.

Fleitmann, Th.
1847, "Ueber die Existenz eines schwefelfreien Proteins". Annalen d. Chemie u. Pharmacie, 61, p.121-126.

1848, "Bestimmungen des Verhaltniss, in welchem der Schwefel in seinem zwei verscheiden Formen in der schwefel- und stickstoffhaltigen organischen Verbindungen erhalten ist". Annalen d. Chemie u. Pharmacie, 66, p.380-381.

Folin, O.
1910, "On the preparation of cystine". J. Biol. Chem., 8, p.9-10.

Friedmann, E.
1903, "Beiträge zur Kenntnis der physiologischen Beziehungen der schwefelhaltigen Eiweissabkömmlinge". Beitrage zur chem. Physiol. u. Path., 3, p.1-46.

Gmelin.
Gmelen, Handbuch, IV. Aufl.5, p.133. Cited by Friedmann, Beitrage zur chem. Physiol. u. Path., 3, p.1. (Original was not obtainable at Minnesota).

Goldmann, E. and Baumann, E.
1888, "Zur Kenntniss der schwefelhaltigen Verbindugen des Harns". Zeit. f. physiol. Chemie, 12, p.257-261.

Gortner, R. A. and Holm, G. E.
1920, "The origin of the humin formed by the hydrolysis of proteins". J. Amer. Chem. So., 42, p.821-827.

Grandmougin, E.
1907, "Ist in der Wolle der Schwefel an Sauerstoff gebunden?". Chemiker Zeitung, 31, p.174.

Hofmann, K. B. and Pregl, F.
1907, "Über Koilin". Zeit. f. physiol. Chemie, 52, p.448-471.

Hoppe-Seyler, F.
1881, Published in an article by Baumann and Preusse, Zeit. f. physiol. Chemie, 5, p.330.

Jaffe, M.
1879, "Ueber die nach Einführung von Brombenzol und Chlorbenzol im Organismus entstehenden schwefelhaltigen Saüren". Berichte der deutsh. chem. Gesellschaft, 12, p.1092-1098.

Johnson, T. B.
1911, "Sulfur linkages in proteins". J. Biol. Chem., 9, p.439-448.

Külz, E.
1884, "Zur Kenntnis des Cystins". Zeit. f. Biologie, 20, p.1-10.

1890, "Zur Kenntniss des Cystins". Zeit. f. Biologie, 27, p.415-417.

Krüger, A.
1888, "Uber der Schwefel der Eiweissstoffe". Pflüger's Archiv. 43, p.244-264.

Mathews, A. P. and Walker, S.
1909, "The spontaneous oxidation of cystein". J. Biol. Chem. 6, p.21-28.

Mauthner, J.
1884, "Zur Kenntnis des Cystins". Berichte der deutsch. Chem. Gesellschaft, 17, p.293-295.

1885, "Berichtigung betreffend das Cystin". Berichte der deutsch. Chem. Gesellschaft, 18, p.451.

Morner, K. A. H.
1899, "Cystin, ein Spaltungsprodukt der Hornsubstanz". Zeit. f. physiol. Chemie, 28, p.595-615.

1901, "Zur Kenntniss der Bindung des Schwefels in den Proteinstoffen". Zeit. f. physiol. Chemie, 34, p.207-338.

Neuberg, C. and Mayer, P.
1905, "Uber Cystein". Zeit. f. physiol. Chemie, 44, p.473-497.

Neuberg, C. and Mayer, P.
1905a, "Uber d-, l-, und r-Proteincystin". Zeit. f. physiol. Chemie, 44, p.498-510.

Okuda, Y.
1919, "A method for the determination of cystine". Journ. of the Coll. of Agr., Imp. Uni. of Tokyo, 7, No. 1, p.69-76.

Osborne, T. B.
1900, "Sulfur in protein bodies". Conn. Exp. St. Report, 1900, p.443-471.

Patten, A. J.
1903, "Einige Bemerkungen über das Cystin". Zeit. f. physiol. Chemie, 39, p.350-355.

Pick, E. P.
1899, "Zur Kenntniss der peptischen Spaltungs produkte des Fibrins". Zeit. f. physiol. Chemie, 28, p.219-287.

Plimmer, R. H. A.
1913, "The separation of cystine and tyrosine". Bio-Chem. Journal, 7, p.311-317.

Prout.
Gmelin, Handbuch der org. Chemie, Bd. 5, S.133. Cited Brenzinger, zeit. f. physiol. Chemie, 16, p.552. (Original not obtainable at Minnesota).

Raikow, P. N.
1905, "Uber den Zustand des Schwefels in den Eiweisskörper" Chemiker Zeitung, 29, p.900-901.

1907, "Uber die Existenz von an Sauerstoff gebundenem Schwefel in der Wolle". Chemiker Zeitung 31, p.539-540.

Rettger, L. F.
1902, "The liberation of volatile from milk on heating". Amer. Journ. of Physiol. 6, p.450-457.

Riza, A.
1903, "Nouveau reatif de la cystine". Bull. Soc. Chim. (3) 29, p.249-250.

Rothera.
1905, "Experiments on cystin and its relation to sulfur metabolism". Journ. of Physiol. 32, p.175-182.

Schulz, Fr. N.
1898, "Die Bindungsweise des Schwefels im Eiweiss". Zeit. f. physiol. Chemie, 25, p.16-35.

Scott, W. W.
1917, Standard methods of chemical analysis - 2nd edition, p.398-403. D. Van Nostrand Co., New York.

Sherer.
Jahresber. u. d. Fortschr. d. Chemie, 1857, p.561.

Suter, F.
1895, "Ueber die Bindung des Schwefels im Eiweiss". Zeit. f. physiol. Chemie, 20, p.564-582.

Thaulow, M. C. J.
1838, "Ueber die Ausammensetzung des Blasenoxyds". Annalen d. Chemie u. Pharmacie, 27, p.197-201.

Truog, E.
1915, "Methods for the determination of carbon dioxide and a new form of absorption tower adapted to titrimetric method". Journ. Ind. and Eng. Chem. 7, p.1045-1049.

Van Slyke, D. D.
1911, "The analysis of proteins by determination of the chemical groups characteristic of the different amino acids". J. Biol. Chem. 10, p.15-55.

1911a, "A method for quantitative determination of aliphatic amino groups". J. Biol. Chem. 9, p.185-204.

Winterstein, E.
1901, "Ueber eine Methode zur Abscheidung der organischen Basen aus den Phosphorwolframsäureniederschlägen und über das Verhalten des Cystins gegen Phosphorwolframsäure". Zeit. f. physiol. Chemie, 34, p. 153-156.

Wollaston.
1810, "On cystic oxide, a new species of Urinary Calculus" Philosophical Transactions, 1810, p.223-230.