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SULFUR IN PROTEINS. I. THE EFFECT OF ACID HYDROLYSIS UPON CYSTINE.[1]

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Introduction.

The protein molecule has been the subject of many investigations, and a very voluminous literature has resulted. In spite of the enormous amount of work which has been done in this field there are still many problems which remain to be solved. One of these is the question as to whether or not the peptide linkage is the only important grouping joining the individual amino acids. Theoretically there is a possibility of a number of other linkages in which reactive groups other than amino or carboxyl are involved. There is a possibility that ethers or esters are present. The α hydrogen on the indole nucleus is so reactive that it appears almost inconceivable that it does not sometimes become involved in the reactions within the protein molecule. However, none of these modes of union has been demonstrated in protein material. The only form of linkage, aside from the peptide group, which has been demonstrated is the —S—S— linkage in cystine. From a study of the literature it appears that this is the principal form in which sulfur occurs in the protein molecule. The reduced amino acid cystein, $SH—CH_2—CH(NH_2)COOH$, of which cystine is the disulfide, has never been proved to be present, as such, in protein material. Certain physico-chemical data, however, argue for its presence. For example, casein contains about 0.80% of sulfur. On the theory that this sulfur represents one molecule of cystine there must be present two atoms of sulfur with a resulting molecular weight of 8015 for the casein molecule. We have, however, good physico-chemical evidence that the molecular weight of para-casein is approximately 4000, in which case only a single atom of sulfur can be present in the para-casein molecule. Cystine has been isolated from the tryptic digest of casein, but in view of the readiness with which cystein can be oxidized to cystine the presence of cystine instead of cystein in the casein molecule still remains an open question.

In view of the uncertain knowledge regarding the sulfur linkages in protein it appeared wise to plan an extensive investigation in this field. The present paper is the first of a series of papers. Experimental work on subsequent papers is already well advanced.

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[2] Dr. George E. Holm, formerly of this Division, assisted in the plan of the experimental work.

Historical.

The Behavior of Cystine and Cystine Sulfur Towards Chemical Reagents and the Relation of Cystine to the Sulfur Content of Proteins.—Fleitmann[3] was the first to show that the sulfur in proteins may not all be in one compound or composition. He found that only a part of the sulfur was removed from certain proteins when heated with alkali. On account of their peculiar behavior and their similarity to cystine and taurine, the sulfur compounds then known to be related to animal metabolism, he called the two forms, *oxidized* and *unoxidized* sulfur. Danielewski[4] confirmed Fleitmann's observations and later[5] called attention to the fact that this peculiar property of sulfur in proteins had been neglected by those trying to establish formulas for proteins, as most of these were made on the basis of one sulfur atom. Krüger[6] was the first to produce evidence that the terms "oxidized" and "unoxidized" sulfur were not correct. He carefully determined the amounts of the two forms of sulfur in oval bumin and fibrin and showed that mercaptans and thio-ethers behave in a similar manner when heated with alkali. But he also observed that oxidized sulfur 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 sulfur and named the two forms *loosely* and *firmly bound* sulfur. He also discusses the possible ways in which the sulfur of the complex that is split off is bound, and how the remaining sulfur could unite in the protein molecule. Goldman and Baumann[7] were able to remove 68% of the sulfur of cystine as lead sulfide when cystine was heated with 10% sodium hydroxide solution in the presence of lead acetate. Suter[8] 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 sulfur of cystine as lead sulfide. Schulz[9] 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 sulfur 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 sulfur in cystine after heating with 30% sodium hydroxide for 25 hours. Osborne[10] used Schultz's method of determining loosely and firmly bound sulfur in an endeavor to get at the size of the protein molecule. He analyzed a large number of proteins and found that the percentage of loosely bound sulfur varied from 66% in seralbumin to 13% in casein.

The question concerning the possible forms of sulfur in proteins is one of importance. The fact that all the sulfur in proteins does not react in the same manner, when the protein is heated with sodium hydroxide, points towards the sulfur being in two different combinations, but on the other hand pure cystine behaves in so much the same manner that definite proof for more than one form of sulfur is not established.

Mörner[11] attempted to answer the question as to whether the sulfur linkage represented by cystine is the only one to be dealt with in proteins. He found that about the same percentage of sulfur was split off from cystine by heating with alkali as was obtained by treating horn, hair, serum globulin and serum albumin under identical conditions.

[3] Fleitmann, *Ann.*, **61**, 121 (1847); *ibid.*, **66**, 380 (1848).
[4] Danielewski, *Z. Chem.*, **5**, 41 (1869).
[5] Danielewski, *Z. physiol. Chem.*, **7**, 427 (1883).
[6] Krüger, *Pflüger's Arch.*, **43**, 244 (1888).
[7] Goldmann and Baumann, *Z. physiol. Chem.*, **12**, 254 (1888).
[8] Suter, *ibid.*, **20**, 564 (1895).
[9] Schulz, *ibid.*, **25**, 16 (1898).
[10] Osborne, *Report Conn. Agr. Exp. Sta.*, **1900**, p. 443.
[11] Mörner, *Z. physiol. Chem.*, **34**, 207 (1901).

Also, in solutions from which cystine had been separated he found a considerable amount of sulfur which behaved in a manner similar to cystine when treated with alkali, but this substance was not definitely identified. From the experimental results Mörner calculated the percentage of cystine in these proteins and accounted for almost all of the sulfur as being in the form of cystine. As he could account for only a small amount of the sulfur in the membranes of eggs, ovalbumin and fibrinogen as cystine, he concluded that the sulfur of these proteins must be present in other forms. As confirmation of this idea he observed that about $^1/_3$ of the sulfur was lost by the volatilization of a sulfur compound. He did not identify this but said that it is possible that $^1/_3$ of the sulfur was present in the form of a volatile substance and that it is not impossible that sulfur of these proteins is bound in 3 forms.

The fact that the amount of sulfur split off by alkali differs considerably in the proteins examined also points towards different sulfur forms. Osborne[10], as noted above, obtained from 13% to 66% of the sulfur, in various proteins, as the sulfide, by boiling with sodium hydroxide. Suter[8] by heating cystine for 9 hours was able to split off 60% of the sulfur, while by heating hair under the same conditions for 15 hours, 98.8% of the sulfur was split off. Pick[12] made the interesting observation that the albuminoses prepared from fibrin gave up the whole of their sulfur as hydrogen sulfide. He concludes that the sulfur must not be present in the form of cystine. While working with koilin,[13] Hofmann and Pregl[14] observed that when this protein was digested with alkali and lead acetate, lead sulfide was formed. They were unable to isolate cystine from the hydrolytic products. Buchtala,[15] however, later isolated 0.5 g. of pure cystine from 100 g. of koilin.

Johnson[16] criticizes the fact that much stress is still laid on the fact that cystine sulfur and protein sulfur react similarly when heated with alkali and consequently the conclusion is accepted that cystine is the source of the loosely bound sulfur in these natural substances, and also that investigation has been focused almost exclusively on the cystine molecule and no careful consideration has been given to the possibility that there may be other unstable sulfur groupings in some proteins, which on hydrolysis can break down, giving hydrogen sulfide. In regard to this he states, "The fact that cystine behaves like a sulfur protein when heated with alkali, or that other primary sulfur cleavage products have not been isolated, does not preclude the possibility of there being other sulfur 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 inconceivable that in the digestion of proteins with alkalies and acids, there may be atomic displacements involving the removal of unsaturated sulfur 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 sulfur linkages in proteins, he states, "It therefore appears probable from a consideration of the above evidence that there are other sulfur combinations in proteins besides the cystine group which can break down, on hydrolysis, with the formation of hydrogen sulfide."

Van Slyke[17] 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

[12] Pick, *Z. physiol. Chem.*, **28,** 219 (1899).

[13] The horny keratin-like lining of the gizzards of fowls.

[14] Hofmann and Pregl, *Z. physiol. Chem.*, **52,** 448 (1907).

[15] Buchtala, *ibid.*, **69,** 310 (1910).

[16] Johnson, *J. Biol. Chem.*, **9,** 439 (1911).

[17] Van Slyke, *ibid.*, **10,** 15 (1911).

acid for 24 hours only 50% was precipitated by phosphotungstic acid. He states, "It appears possible that the cystine is partially destroyed during the hydrolysis. The results 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[18] 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 conc. hydrochloric acid for 3–4 hours. The yield was very poor when the material is boiled for 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 became yellow or yellow-brown in color. He further states, "Cystine is much more unstable to acid than one is led to expect from the description of its isolation."

Gortner and Holm[19] 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 cystine amino nitrogen has been changed to ammonia nitrogen. They state, "Cystine was not readily deaminized under the conditions of the experiment."

The Crystal Form of Cystine as Influenced by Chemical Treatment.—Mörner[20] noted that the crystals of cystine were different when horn was hydrolyzed 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 2 weeks he obtained very few hexagonal plates, the greater part crystallizing in needles which were optically almost inactive. He describes these as being long needles or long, very thin or narrow, pointed plates which often accumulated 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. Mörner was not able to prepare this substance pure, inasmuch as his preparation 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 was more soluble in hot water than in cold water. Both gave lead sulfide when heated with alkali and lead acetate.

Rothera[21] when preparing cystine found, as did Mörner, that needles were present with the plates and the quantity of needles increased with the length of hydrolysis. He found no difference in the yield of cystine if the hydrolysate was boiled for 18 or 180 hours. He states, "The first recrystallization showed tyrosine-like crystals but after the second, the crystals resemble lecithin spheres. They give the cystine color tests and the required sulfur and nitrogen content." Neuberg and Mayer[22] state that boiling sulfuric acid decomposes cystine much more readily than does hydrochloric acid.

Neuberg and Mayer[23] heated "stone" cystine with hydrochloric acid in a sealed tube at 165° for 14 hours. The resulting product was optically inactive and formed a white amorphous powder. In another article[22] they state that when protein cystine is treated as above, an inactive, needle-like, 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 being either a meso-cystine or a racemic mixture.

[18] Plimmer, *Biochem. J.*, **7**, 311 (1913).

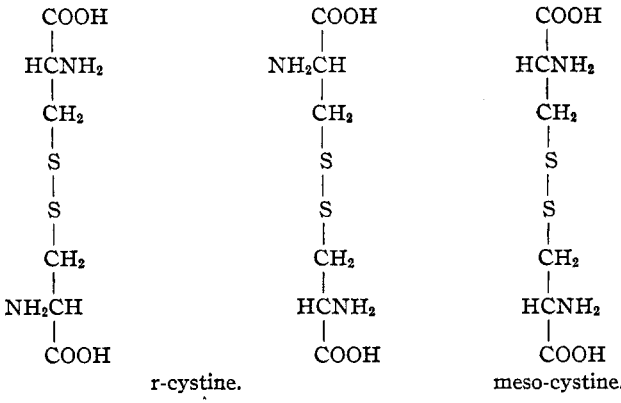
[19] Gortner and Holm, THIS JOURNAL, **42**, 821 (1920).

[20] Mörner, *Z. physiol. Chem.*, **28**, 595 (1899).

[21] Rothera, *J. Physiol.*, **32**, 175 (1905).

[22] Neuberg and Mayer, *Z. physiol. Chem.*, **44**, 98 (1905).

[23] Ref 22, p. 472.



r-cystine. meso-cystine.

They were able to prepare the inactive cystein from the inactive cystine. This was completely inactive and analyzed for cystein. They grew *Aspergillus niger* on some of the inactive cystine and obtained a cystine that was dextrorotatory. It was suggested that this method might be used to prepare pure *d*-cystine. To quote them concerning the crystal form of this preparation: "It is noteworthy that our purest *d*-cystine ($[\alpha]_D = +93.78°$) forms microscopical six-sided plates as well as needles. The theory required that *d* and *l* cystine should be enantiomorphic."

The Synthesis of Cystine.—Erlenmeyer[24] synthesized cystine from benzoyl serin ester. He does not state whether the material was completely inactive nor does he note the crystalline form. Erlenmeyer and Stoop[25] again prepared cystine from benzoyl serin ester and phosphorus pentasulfide. They first obtained cystein which they oxidized to cystine. In regard to the yield and description of the product, they state, "Thus one obtains cystine in a 40% yield as a granular microcrystalline powder. After repeated solution in ammonia and reprecipitation by acetic acid the cystine is pure and forms stellate groups of needles. The only difference between the synthetic and the natural product is that the solution of the former does not show optical rotation. A solution of 0.5 g. of the synthetic cystine in hydrochloric acid in a 2dcm. tube gave no rotation of the polarized light."

Fischer and Raske[26] prepared *d-l*-cystine from *d-l*-α-amino-β-chloro-propionic acid and describe it thus: "After some time the cystine separated as a colorless mass of very tiny crystals, which under the microscope appeared on first glance to be more or less spherical masses but which were resolved into needles or very thin prisms." The analysis of this preparation agreed quite well with that of plate cystine. They also prepared the *l*-cystine from *l*-α-amino-β-chloro-propionic acid. This preparation was optically active $[\alpha]_D^{20} = 209.6° \pm 1°$. Their analyses show this to be identical in chemical composition with plate cystine. Concerning the crystalline form of this preparation, they state: "Our synthetic product has a very different crystal form, the preparation precipitated from an ammoniacal solution with acetic acid forms microscopic tiny, apparently right-angled, prisms or perhaps many-faced crystals."

It is interesting to note that *cystine crystallizing in hexagonal plates has never been synthesized* and in view of Neuberg and Mayer's[23] experiments, 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.

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[24] Erlenmeyer, *Ber.*, **36**, 2720 (1903).

[25] Erlenmeyer and Stoop, *Ann.*, **337**, 236 (1904).

[26] Fischer and Raske, *Ber.*, **41**, 893 (1908).

Experimental.

The Problem.—In view of the varied opinions referred to under "Historical," it seemed desirable to test the effect of prolonged boiling with hydrochloric acid on cystine with special reference to the behavior of cystine in a protein hydrolysis. Many investigators appear to believe that cystine is destroyed either in whole or in part during acid hydrolysis. This idea is upheld by the work of Van Slyke[17] and Plimmer.[18] Gortner and Holm[19] show, however, that in a mixture of pure amino acids, cystine is not easily de-aminized during acid hydrolysis. From the work of Mörner,[20] Rothera[21] and Neuberg and Mayer[22] 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 sulfur 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 probable 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 sulfide which might result from a splitting of the —S—S— union and a subsequent evolution of hydrogen sulfide; (3) some of the sulfur might be oxidized to sulfate; (4) a loss of total sulfur in the hydrolysate due to the formation of volatile sulfur compounds; (5) the amount of unoxidized sulfur as determined by the potassium bromate method of Okuda;[31] (6) the amount of nitrogen precipitated by phosphotungstic acid; (7) the amount of total nitrogen, which might be altered by 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) 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 several methods had been tried, a slight modification of Folin's method[27] was used.

Human hair or wool (human hair, which can be obtained without difficulty at almost any barber shop, is preferable) is washed in *cold* dilute (1% or 2%) sodium

[27] Folin, *J. Biol. Chem.*, **8**, 9 (1910).

carbonate solution and dried. This washing is to remove the natural oils from the hair. Two kg. of the dry, washed hair is pushed into a 6-liter Pyrex flask (round-bottom preferable) and 4 liters of constant boiling (20%) hydrochloric acid is 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 of the flask 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 is equally efficient. The flask is then removed from the steam-bath and the solution of amino acids *almost neutralized* with conc. sodium hydroxide (commercial 98%) solution and then sodium acetate solution is 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 for 48 to 72 hours at room temperature, the liquid is filtered from the precipitated cystine. This can be best accomplished on a Büchner funnel, using suction. The crude material, containing in addition to the cystine some "humin," melanin and tyrosine, is dissolved in 3% hydrochloric acid and completely decolorized with a vegetable decolorizing carbon or 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 a faint straw color. If it shows much color the treatment with decolorizing carbon should be repeated. The cystine is precipitated from this clear solution using first sodium hydroxide to weakly acid reaction followed by sodium acetate. After standing for at least 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 g., or 5%.

The Methods.—For carrying out a study as outlined above, an apparatus as shown in Fig. 1, was used.

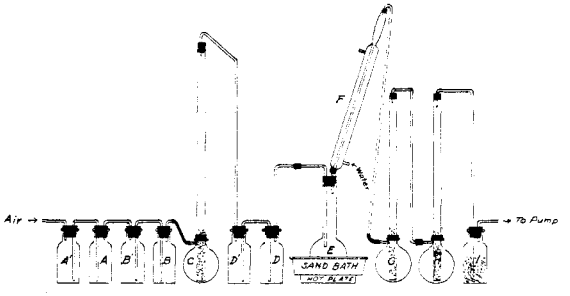

Fig. 1.

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 sulfide in the air.

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

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

C is an absorption tower as described by Truog[28] 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-bottom, 2-liter flask in which the hydrochloric acid and cystine were placed during the boiling. The flask was placed on a sand-bath and heated by means of an electric hot-plate.

F is a water-cooled condenser.

G is an absorption tower containing cadmium sulfate. This absorbs the hydrogen sulfide 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 carbon dioxide from entering the apparatus.

To 50 g. of cystine (97.5% pure, containing 2.5% of ash and moisture) enough constant-boiling hydrochloric acid was added to make a total volume of 1000 cc. This was placed in flask E and the flask and contents weighed to 0.01 g. on a large analytical balance (sensitive to 1 mg. and having a capacity of 1500 g.). The flask was connected as shown in Fig. 1, the cadmium sulfate placed in Tower G and the apparatus washed free from 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 the flow of air was increased so that all of the carbon dioxide and hydrogen sulfide might be washed out in a short time. After the solution had boiled for 3 hours and the carbon dioxide and hydrogen sulfide had been 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 50cc. aliquot, containing 2.5 g. of the original cystine or 2.4375 g. of pure cystine, was removed and made up to 250 cc., this being used in subsequent analyses. 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. (or 50 cc. in the last two determinations, 144 and 192 hours) of approximately 0.125 *N* barium hydroxide in Tower H. After the heating was discontinued and the apparatus washed free from carbon dioxide, the remaining barium hydroxide was titrated with hydrochloric acid, using phenolphthalein as an indicator. As the strength of the barium

[28] Truog, *J. Ind. Eng. Chem.*, **7**, 1045 (1915).

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. Hydrogen sulfide. The principle of this determination is described by Scott.[29] It is based on the fact that when hydrogen sulfide comes in contact with a solution of cadmium sulfate, the following reaction takes place,

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

The sulfuric acid thus formed is titrated with a standard alkali. For this determination a solution of cadmium sulfate containing 24 g. per liter was neutralized with sodium hydroxide, using sodium alizarine sulfonate as an indicator Twenty-five cc. of this solution (or 50 cc. in the last three determinations, 96, 144 and 192 hours), was placed in Tower G. This tower was removed as described for Tower H and the sulfuric 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 sulfate solution from the constant-boiling hydrochloric acid, the solution after neutralization was titrated with standard silver nitrate solution, using potassium chromate as an indicator. In no instance was there an appreciable amount of hydrochloric acid present.

3. The sulfur that might be present in the sulfate form was determined by adding a small amount of conc. hydrochloric acid to 20 cc. of the solution and heating to boiling. Five cc. of 10% barium chloride was slowly added and the barium sulfate weighed in the usual manner. From the weight of barium sulfate the amount of sulfate sulfur was calculated.

4. Total sulfur was determined in 10 cc. of the solution by oxidizing to sulfate with 10 cc. of the oxidizing reagent described by Denis.[30] 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% hydrochloric acid. The sulfate was precipitated out of the boiling solution by means of barium chloride and the sulfur calculated from the amount of barium sulfate formed.

5. The amount of cystine present as determined by the potassium bromate method was carried out as described by Okuda,[31] according to the equations

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

$$HOOC—CH\ (NH_2)—CH_2—S—S—CH_2—CH(NH_2)—COOH + 10Br + 6H_2O = 2(SO_3H—CH_2CH\ (NH_2)—COOH) + 10HBr.$$

To 10 cc. of the cystine solution, enough hydrochloric acid is added to make a 5 to 10% solution. To this, 10 cc. of 20% sodium bromide solution is added and the solution titrated with 0.05 *N* potassium bromate solution, the end-point being the first faint yellow color (free bromine) that persists for one minute. One cc. of the sodium bromate solution is equivalent to 0.00721 g. of cystine. When this method was used on pure cystine the results checked with the sulfur and nitrogen determinations.

6. The amount of nitrogen not precipitated by phosphotungstic acid was determined by the method previously used by Van Slyke.[17] Ten cc. of the solution was placed in a 100cc. volumetric flask, 10 cc. of conc. hydrochloric acid and 5 g. of phosphotungstic acid were added and the solution was made up to volume. 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

[29] Scott, "Standard Methods of Chemical Analysis," D. Van Nostrand Co., New York, 2nd Ed., pp. 398–403.

[30] Denis, *J. Biol. Chem.*, **8**, 401 (1910).

[31] Okuda, *J. Coll. Agr. Imp. Univ. Tokyo*, **7**, No. 1, 69 (1919).

of total nitrogen in 100 cc. was known, the difference represented the amount of nitrogen in the precipitate.

7. Total nitrogen was determined by the Kjeldahl method.

8. Amino nitrogen was determined by the Van Slyke apparatus[32] for determining amino nitrogen.

9. Ammonia nitrogen was determined on a 20cc. 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 was added and the ammonia distilled at 40–45°, under reduced pressure, into standard sulfuric acid.

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

The Experimental Data.

A. The Decomposition of Cystine on Boiling with 20% Hydrochloric Acid for Various Lengths of Time. *The Hydrolysis.*—When heating began the cystine hydrochloride was not all in solution, but at the end of 3 hours' boiling the cystine hydrochloride had completely dissolved. Very little change in appearance was observed during the first 12 hours except that the color of the solution became somewhat yellow and a small amount of sulfur crystallized on the neck of the flask. After 24 hours the solution was deep yellow, a flocculent precipitate began to separate and a considerable amount of sulfur had crystallized on the neck of the flask and in the condenser tube. At 48 hours a very pungent odor was noticed when the flask was opened: the solution was yellow-brown in color and a white substance was deposited on the lower part of the flask or else a substance was formed during the boiling that had etched the glass. During 48 to 144 hours the solution became darker and murky, the amount of flocculent precipitate increased and some of the sulfur in the condenser washed back into the flask. At the end of the boiling 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 so as to be perfectly opaque.

It was of interest to note that cystine when boiled with acid for a long time deposits sulfur on the neck of the flask and in the condenser, as do proteins when treated in a similar manner. Mörner[20] found that when horn was hydrolyzed with hydrochloric acid for a short time little or no free sulfur was formed, but when the heating was continued for a long time, a not inconsiderable amount of sulfur separated. Buchtala[15] observed that some proteins when hydrolyzed deposited sulfur, 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 decarboxylation takes place. The carbon dioxide undoubtedly comes from the slow decomposition of cystine and not from a change in the

[32] Van Slyke, *J. Biol. Chem.*, 9, 185 (1911).

cystine molecule. The large amount given off at the end of the first 3 hours may be due to the acid solution of cystine containing dissolved carbon dioxide, since it was not boiled before the experiment was started.

The Sulfur Determinations.—The amount of sulfur given off as hydrogen sulfide is shown in Table II. This table shows that only a small amount of the sulfur is evolved as hydrogen sulfide during the prolonged boiling with acid. 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 sulfate and titrated as sulfuric acid. There is proof, however, that hydrogen sulfide was formed, for the characteristic yellow precipitate of cadmium sulfide was produced in a not inconsiderable quantity. The amount of hydrogen sulfide given off, like the amount of carbon dioxide, does not point toward any changes in the cystine molecule but toward a slow decomposition of the cystine. Mörner[20] observed that when keratin was heated with hydrochloric acid, for a long time, hydrogen sulfide was evolved.

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

The amount of total sulfur serves as a check on the amount of sulfur in the solution but does not show the amount of cystine. Some elementary sulfur was deposited on the neck of the flask and in the condenser tube. In the earlier aliquots this would not be accounted for in the "total sulfur" figures. This sulfur later washed down and increased the total sulfur values in later determinations. The results in Table II show that the amount of total sulfur decreases until 144 hours and then increases. As already stated, the sulfur 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 g. of the cystine had been removed, so that part of the sulfur which washed back into the flask came from the cystine in the aliquots that had been removed from the flask. There was a possibility that the flocculent precipitate, noted above, might contain some sulfur. This was confirmed by subsequent analysis, as a sulfur determination made on the 144-hour aliquot after the precipitate had settled, showed that the sulfur 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 sulfur had precipitated out of the solution with the settling of the precipitate. The total sulfur determinations do not show what is happening to the cystine molecule. The hydrogen sulfide determinations give the only index

TABLE I

DECARBOXYLATION OF ALIQUOTS OF CYSTINE BOILED FOR DIFFERENT LENGTHS OF TIME WITH 20% HYDROCHLORIC ACID

	Hours' Boiling							
	3	6	12	24	48	96	144	192
Carbon dioxide per g. of cystine,[a]...mg	1.905	2.337	3.077	3.922	5.784	10.839	15.863	23.505
COOH CO_2,.....%	0.519	0.637	0.839	1.069	1.577	2.956	4.326	6.410

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

TABLE II

ANALYSES FOR THE VARIOUS FORMS OF SULFUR IN ALIQUOTS OF CYSTINE BOILED FOR DIFFERENT LENGTHS OF TIME WITH 20% HYDROCHLORIC ACID

		Hours' Boiling							
	Original	3	6	12	24	48	96	144	192
Hydrogen sulfide per g. of cystine,[a]...%	.....	0.075	0.101	0.145	0.229	0.428	1.319	2.280	4.070
Sulfur split off as hydrogen sulfide,....%	.....	0.028	0.038	0.054	0.086	0.160	0.494	0.855	1.526
Sulfate-sulfur per g. of cystine,...mg	.....	0.840	1.050	1.050	1.260	0.840	0.770	1.400	0.840
Sulfur as sulfate,.....%	.....	0.315	0.394	0.394	0.472	0.315	0.289	0.525	0.315
Total sulfur per g. of cystine,.....mg	265.4	259.6	253.6	250.4	255.4	250.4	250.9	258.5	259.1
Total sulfur in original cystine,.....%	26.54	25.96	25.36	25.04	25.54	25.04	25.09	25.85	25.91

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

TABLE III

AMOUNT OF CYSTINE BY THE BROMATE METHOD IN ALIQUOTS BOILED FOR DIFFERENT LENGTHS OF TIME WITH 20% HYDROCHLORIC ACID[a]

		Hours' Boiling							
	Original	3	6	12	24	48	96	144	192
Cystine per g. of original cystine,...mg	1000.40	970.90	951.80	948.00	939.90	925.20	953.20	971.10	905.80
Cystine,.....%	100.04	97.09	95.18	94.80	93.99	92.52	95.32	97.11	90.58

[a] See text for a possible explanation of the discordant results for 96 and 144 hours.

as to what becomes of a small part of the sulfur while the sulfate determinations show that none of the sulfur is oxidized to the sulfate.

The Amount of Cystine as Determined by the Potassium Bromate Method.—The results given in Table III show that the sulfur in cystine is not readily oxidized by boiling with acid under the conditions of this experiment; at any rate, most of the sulfur still reacts with bromine. The end-points were very indistinct after 24 hours, 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 sulfur 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 are given in Table IV. The data are given in this form rather than subtracting from the total nitrogen which would give the amounts precipitated by phosphotungstic acid. The amount of nitrogen not precipitated increases until the cystine has been boiled for 48 hours and then the values remain practically constant for the longer periods of boiling. After 48 hours no more of the typical crystalline cystine phosphotungstate precipitate as formed with protein (plate) cystine was obtained, but instead a more granular and amorphous precipitate formed. 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 crystalline plate cystine precipitate. This indicates 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 precipitated by phosphotungstic acid. Van Slyke[17] found that under our conditions of precipitation, 1.5 mg. of nitrogen per 100 cc. was not precipitated when pure cystine was used, but with cystine which had been boiled with 20% hydrochloric acid for 24 hours, he found that about 7 mg. of nitrogen per 100 cc. of the solution was not precipitated by phosphotungstic acid.

The Nitrogen Determinations.—The total nitrogen, Table V, 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 total 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,[32] they give a higher result than the total nitrogen values. There is a possibility that the amount of substance, causing the high amino nitrogen values when cystine is de-

TABLE IV

NITROGEN NOT PRECIPITATED BY PHOSPHOTUNGSTIC ACID, PER 100 CC. OF SOLUTION, FROM ALIQUOTS OF CYSTINE BOILED FOR DIFFERENT LENGTHS OF TIME WITH 20% HYDROCHLORIC ACID

		Hours' Boiling							
	Original	3	6	12	24	48	96	144	192
Nitrogen not precipitated, ... mg.	1.20	1.92	2.92	4.16	5.04	6.08	6.12	6.80	6.62
Nitrogen not precipitated,[a] ..... %	10.29	16.46	25.04	35.68	43.22	52.14	52.49	58.31	56.77

[a] Calculated, 11.66 mg. of nitrogen in 100 cc. of solution.

TABLE V

ANALYSES FOR THE VARIOUS FORMS OF NITROGEN IN ALIQUOTS OF CYSTINE BOILED FOR DIFFERENT LENGTHS OF TIME WITH 20% HYDROCHLORIC ACID

		Hours' Boiling							
	Original	3	6	12	24	48	96	144	192
Total nitrogen per g. of original cystine, ... mg.	114.8	112.30	110.50	111.60	111.70	111.50	111.50	110.90	114.60
Amino nitrogen per g. of original cystine.....	129.0	137.70	131.00	131.50	129.80	129.30	127.60	127.60	126.90
Ammonia nitrogen per g. of original cystine.....	.....	2.35	2.56	2.66	3.28	3.28	3.69	7.69	11.16
Total nitrogen as ammonia nitrogen,[a] ..... %	.....	2.02	2.19	2.28	2.81	2.81	3.16	6.50	9.57

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

TABLE VI

CHANGES IN THE SPECIFIC ROTATION OF ALIQUOTS OF CYSTINE BOILED FOR DIFFERENT LENGTHS OF TIME WITH 20% HYDROCHLORIC ACID

		Hours' Boiling							
	Original	3	6	12	24	48	96	144	192
Specific rotation of acid solution.....	−201.70°	−180.20°	−152.50°	−124.70°	−48.50°	−20.80°	00.00°	....	....
Decrease in specific rotation, ... %	0.00	10.66	24.39	38.17	75.95	89.69	100.00	....	....

aminized in the Van Slyke apparatus, is increased during the hydrolysis, thus making the error still greater. It is probable that the amount of amino nitrogen actually decreases slightly during the hydrolysis, due to the formation of ammonia.

With the exception of one or two samples, the ammonia nitrogen, Table V, increases almost proportionally to the decrease of amino nitrogen. Gortner and Holm[19] found that when a mixture of amino acids and formaldehyde was boiled for 24 hours with 20% hydrochloric acid very little ammonia was formed. Assuming that all of the ammonia nitrogen in their Expt. No. II came from cystine, only 2.74% of the nitrogen was split off as ammonia. This agrees very well with the results obtained in this experiment where only 2.81% 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.* Mörner[11] observed that when cystine was heated on a steam-bath for 109 hours with 10% hydrochloric acid, the specific rotation changed from $-223°$ to $-134°$ and the cystine was changed to a more soluble form. As to our 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 we believe that it is almost impossible to prepare pure plate cystine with maximum optical rotation by any method which involves heating for any considerable period of time in a strong acid solution. Mörner, 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.

From these results, one concludes that only a small amount of the cystine is *decomposed* during prolonged boiling with acid, as measured by production of ammonia, carbon dioxide, or hydrogen sulfide or by changes in unoxidized sulfur. The major portion of the cystine is apparently *changed* into a form that is optically inactive and is not as completely precipitated with phosphotungstic acid as is plate cystine. We therefore attempted to isolate the organic compound which remained in the solution after 196 hours of boiling.

B. The Isolation of an "Isomeric" Cystine from the Residual Solution.

Isolation and Analysis.—After the residual solution had stood for 28 days, the flocculent precipitate noted in the previous section was filtered off, washed with 20% 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 disulfide to remove any elementary sulfur. When the carbon disulfide was evaporated the characteristic yellow crystals of sulfur remained. The residue from the carbon disulfide extraction was extracted with hot water. The insoluble portion contained some inorganic matter besides a very small amount of organic matter. An analysis of the black water-soluble portion of the original precipitate gave amino nitrogen, 7.73%; total nitrogen, 15.89%; and total sulfur, 8.73%. This analysis shows that this residue is not cystine but probably some decomposition product. Only a very small quantity was obtained, not sufficient in amount for further experimental work.

The filtrate from the flocculent precipitate was evaporated to dryness *in vacuo* on a boiling water-bath, and then held at 100° *in vacuo* until the excess of hydrochloric acid was driven off. Flaky, needle-like crystals were formed. These were then dissolved in water and a portion of the solution was removed for analysis. The amounts of total nitrogen, amino nitrogen and total sulfur were determined. The ratio of nitrogen to sulfur was almost identical with the ratio for cystine.

Mg. in 10 cc.	Mg. in 10 cc.
Amino nitrogen = 12.96 (corr. for cystine according to Van Slyke[32])	Total sulfur = 30.00
Total nitrogen = 12.96	Ratio N:S = 1:2.31 (ratio for cystine = 1:2.29).

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

Mg. in 10 cc.	Mg. in 10 cc.
Amino nitrogen = 4.92 (corr. for cystine)	Total sulfur = 10.60
Total nitrogen = 5.03	Ratio N:S = 1:2.11.

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

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

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

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 somewhat resembling the

crystal balls of tyrosine, 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 thin, blunt prisms, while the plate cystine consists of large, hexagonal plates. This is undoubtedly the same crystal form that Mörner[20] and Rothera[21] observed in their preparations of plate cystine prepared from keratin by prolonged acid hydrolysis. The average length of the prism crystals is probably about 1/20 of the average diameter of the plate crystals. The cross section of the prism crystals is extremely minute. *These crystals compare very well with the description which Erlenmeyer[25] and Fischer and Raske[26] gave for their synthetic preparations of inactive cystine and also for the l-cystine prepared by Fischer and Raske.*

This "isomeric" cystine is optically inactive; a 1.0 g. dissolved in 25 cc. of dil. hydrochloric acid and placed in a 2dcm. 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 is the plate cystine. When this "isomeric" cystine is boiled with conc. sodium hydroxide and lead acetate, lead sulfide 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 g. of cystine were placed in a glass container. This was agitated in a shaking machine for 7 hours. After standing for several days, the solution was filtered by gravity through dry hardened filter paper. Fifty cc. of the filtrate was placed in a weighed platinum dish, evaporated on a steam-bath, dried at 100° 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 subtracted from the result obtained when cystine was used. The solubility in pure water at 20° is 1 g. in 5263 parts of water for plate cystine and 1 g. in 2059 parts of water for "isomeric" cystine. These results show that the "isomeric" cystine is 2.556 times as soluble as is the plate cystine. Neuberg and Mayer[22] give the solubilities of plate cystine and their "inactive" cystine as 1 : 8840 for the former, and 1 : 3070 for the latter. Their results show that the "inactive" cystine is 2.879 times as soluble as is the plate cystine. The difference in the amount dissolved is probably due to the method of obtaining a saturated solution. They do not state how they obtained a saturated solution of their preparations or how they determined 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. One-quarter of a gram of the cystine was placed in a 250cc. flask, 25 cc. of conc. hydrochloric acid and 12.5 g. of phosphotungstic acid were added, and the

mixture 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 showed that under these conditions 1.2 mg. of nitrogen per 100 cc. of the solution was not precipitated by phosphotungstic acid, while the analysis of the filtrate from the "isomeric" cystine precipitate showed 4.82 mg. of nitrogen not precipitated. The phosphotungstate of the "isomeric" cystine is therefore 4 times as soluble as that of the plate cystine. Another difference was noted in the physical state 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.

The Hydrochlorides.—The difference in the hydrochlorides was shown by making use of the microscopic method described by Doniges.[33] A small quantity of the fine material is placed on a glass slide and a drop of conc. 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.

Organic Derivatives of the Two Compounds.—We have prepared a number of derivatives of the "isomeric" cystine and plate cystine and have found them to possess entirely different properties. Inasmuch, however, as this comparative study is not complete, a detailed description of the derivatives will be the subject of a subsequent paper.

Discussion.

It was necessary to discuss 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 occurred in aliquots of a cystine solution boiled for various 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 formation, loss of carbon dioxide, and hydrogen sulfide 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

[33] Doniges, *J. Soc. Pharm. Bordeaux*, **58**, 8 (1920); *C. A.*, **14**, 1689 (1920).

the most, only about 10% 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 principal constituent of the hydrolysate. It was found to be optically inactive, and its phosphotungstate was much more soluble than that of the plate cystine. Certain derivatives of this cystine have been 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% hydrochloric acid but is changed into another compound that is optically inactive and has different physical and chemical properties.* This "isomeric" cystine showed the required amount of amino nitrogen, total nitrogen and total sulfur 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 proved, but in view of the fact that the cystine synthesized by Fischer and Raske[26] and by Erlenmeyer and Stoop[25] crystallized in the form of needles *and the synthetic l-cystine prepared by Fischer and Raske 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 we 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 which we intend to study in the near future.

Summary and General Conclusions.

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

The data seem to warrant the following conclusions.

1. Cystine is only slowly decomposed or destroyed during long boiling with 20% hydrochloric acid. There would be no appreciable decomposition during the time of an ordinary protein hydrolysis (12–24 hours).

2. There is but little decarboxylation when cystine is boiled for 192 hours with 20% hydrochloric acid. Small amounts of carbon dioxide are evolved during the boiling.

3. The sulfur of cystine is not broken off to any appreciable extent by

boiling with 20% hydrochloric acid for 192 hours. A small amount of hydrogen sulfide is evolved, some elementary sulfur separates, no sulfates are formed, but about 90% of the original sulfur is still in an unchanged and unoxidized condition at the end of the boiling period.

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

5. The nitrogen of cystine was not appreciably changed during this experiment. The amount of total nitrogen remains constant, the amount of amino nitrogen slowly decreases and there is a corresponding slowly 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 from the original cystine. It was approximately 2.5 times as soluble in water and the phosphotungstate was about 4 times as 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 analyzed for its nitrogen and sulfur content and was found to be isomeric with the original cystine.

9. A number of derivatives of the two forms of cystine have been prepared and in every instance the isomeric derivatives possessed different properties from those possessed by corresponding derivatives of natural "plate" cystine. A description of the derivatives is reserved for a later communication.

10. It is suggested that the "isomeric" cystine as described in this paper is actually the cystine synthesized by Fischer and Raske, and by Erlenmeyer, and Erlenmeyer and Stoop, and that the plate cystine of protein hydrolysis has never been synthesized.

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