[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT OF COLUMBIA UNIVERSITY. No. 380.]

EXPERIMENTAL STUDIES ON CYSTINE.

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Received August 29, 1921.

In preparing cystine for biological experiments it is of importance to know the conditions which favor the precipitation of pure cystine.

It has been shown by Mörner,[1] Embden,[2] Abderhalden[3] and others that cystine may be obtained by the acid hydrolysis of horn, wool and other protein substances containing cystine in combination. When the acid solution is neutralized with alkali or brought to weak acidity with acetic acid after neutralization, the cystine precipitates on standing.

Folin[4] adds sodium acetate to the acid solution of cystine using congo red as indicator. The sodium acetate acts as a buffer salt. He states that this method gives satisfactory results but does not give quantitative directions for the method.

The purpose of this investigation was (1) to determine the concentration of hydrogen ion of solutions of cystine which insures maximum precipitation of cystine; (2) to determine the amounts of sodium acetate to add to the hydrolyzed solution of wool in order to obtain the best yields of cystine and (3) to secure data in regard to purification of the crude cystine from the hydrolyzed solutions. The yield of cystine obtained by Folin's method is compared to the yield obtained by earlier methods.

Experimental Procedure.

The wool used in this investigation was analyzed and found to contain 3.1% sulfur and 11.6% moisture. Two portions of 750 g. each were boiled with 1500 cc. of conc. hydrochloric acid in separate 3-liter flasks with large necks fitted with reflux water condensers. The solutions in the flasks were heated in oil-baths at a temperature of about 115°. In spite of the condensers, hydrogen chloride fumes poured out through the water-cooled tubes during the first part of the hydrolysis and continued to do so until the acid mixture approached the concentration of the acid-water mixture of constant boiling point. The time of hydrolysis was counted from the time at which all the wool was pushed into the hot acid. The hydrolysis was run for 3 hours, when the biuret test became negative.

The solutions on cooling and thorough mixing had a volume of 4065 cc. and an acidity of 6.9 molar strength. Aliquots of 500 cc. representing 188.0 g. of wool were taken and sodium acetate trihydrate crystals added to each aliquot as shown in Table I. The solutions were heated to boiling

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

[2] Embden, *ibid.*, **32**, 94 (1900).

[3] Abderhalden, *ibid.*, **52**, 348 (1907).

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

and boiled for 5 minutes with constant stirring. After standing for 3 days the precipitate of crude cystine was filtered by suction, removed, rubbed up with cold water, filtered and washed once on a Büchner funnel. This process was repeated. The undiluted mother liquors were kept in glass-stoppered bottles and the washings kept separately.

The mother liquors on standing for 2 weeks were filtered by suction and the second precipitates washed and filtered as the first were.

The precipitates were air dried, ground to a powder and kept in desiccators until they came to constant weights.

Sulfur was determined in these weighed precipitates by Osborne's peroxide method. Sulfur was also determined in pure cystine by the Carius method and the results compared with those obtained by the fusion method. It was found that the Osborne method gave results from about 0.2 to 0.3% lower than those obtained by the Carius method on cystine containing 26.69% sulfur. Half-gram samples were fused and an aliquot representing 0.1 g. taken for the precipitation of barium sulfate. Great care was taken to determine and subtract the sulfur in reagents used, to insure barium sulfate precipitates from reduction during ignition and to make determinations in duplicate.

In Table I are shown the grams of sulfur in each precipitate of crude cystine as calculated from the analysis; also the percentages of cystine obtained from the wool. It is considered here that the sulfur determined represents cystine sulfur. This assumption seems correct since these precipitates were almost completely soluble in 1.8 *M* (6.5%) hydrochloric acid and fairly insoluble in cold water as is cystine also, and the yield of pure cystine obtained by experiment corresponds closely to the yield indicated by calculations from sulfur as determined in the crude precipitates.

TABLE I.

SULFUR IN CRUDE CYSTINE PRECIPITATES FROM ALIQUOTS REPRESENTING 188 G. OF WOOL.

$NaC_2H_3O_2(3H_2O)$. G.	(1) S in 1st ppt. G.	(2) S in 2nd ppt. G.	Total S in 1 and 2. G.	Total sulfur in wool. %	Total cystine in wool calc. from sulfur found. %
350	0.077	0.615	0.692	0.368	1.379
450	0.648	0.975	1.623	0.865	3.241
500	1.273	0.746	2.019	1.075	4.028
550	1.260	0.705	1.965	1.046	3.919
650	2.048	0.112	2.160	1.150	4.309
750	2.268	0.055	2.323	1.236	4.631
850	1.706	0.093	1.799	0.957	3.586
950	1.976	0.151	2.127	1.132	4.242

It is evident from Table I that 350 g. of sodium acetate trihydrate precipitated only traces of cystine after standing for 3 days, while 550 g. of sodium acetate gave a yield of only a little over $^1/_2$ of the cystine given by 650 g. of acetate; 750 g. of salt gave the maximum yield of cystine.

The sulfur in the precipitates obtained from the mother liquors which stood for 2 weeks after the first filtration was greatest in those in which insufficient sodium acetate had been added to secure good yields of cystine in the first precipitation.

The total yield of cystine in the first and second precipitates from the aliquot in which 500 g. of acetate had been added was almost as large as with greater amounts of sodium acetate, but required 2 weeks' standing instead of 3 days.

Determination of Hydrogen-ion Concentrations.

In order to determine the concentration of the hydrogen ion more accurately in the cystine solutions, a set of buffer solutions was made up. The acetic acid-sodium acetate mixtures of Walpole[5] were used in place of the potassium hydrogen phthalate solutions recommended by Clark and Lubs,[6] as the latter gave a decided drift when determining the hydrogen-ion concentration by the electrometric method, due possibly to reduction occurring. The set of standard solutions of Clark and Lubs[6] was also made up and used with the one exception stated above. The hydrogen-ion concentrations of these standard buffer solutions were determined directly in the solutions using the hydrogen electrode. By use of the indicators listed by Clark and Lubs the standard solutions were compared colorimetrically with the cystine solutions. It was considered inadvisable to determine the hydrogen-ion concentration in the cystine solutions by the electrometric method because of probable reduction by the hydrogen electrodes.

Since the hydrolyzed solutions were colored dark brown they were diluted to 5 times the original volume as recommended by Walpole.[5] Measured amounts of indicator were added to given volumes of cystine solutions and compared with standard solutions containing the same amount of indicator. That the tints might be comparable the standard solutions plus the indicator were superimposed against the dark solution according to the method of Walpole[7] but modified as follows.

Modified Comparator Used.—Difficulty was experienced in the use of the comparator of Humitz, Mayer and Ostenberg[8] due to the reflection of the light from the round sides of the glass tubes. It was believed that glass cells with flat sides and fitted together in pairs would give better results. Four flat-sided glass cells generally used with colorimeters were therefore picked out. These cells were $10 \times 35 \times 53$ mm. inner dimensions and were placed together in pairs, the broad sides fitted and held together

[5] Walpole, *J. Chem. Soc.*, **105**, 2501 (1914).
[6] Clark and Lubs, *J. Biol. Chem.*, **25**, 479 (1916).
[7] Walpole, *Biochem. J.*, **5**, 207 (1910).
[8] Humitz, Meyer and Ostenberg, *Proc. Soc. Exp. Biol. Med.*, **13**, 24 (1915).

by elastic bands. The cells were filled with solutions and superimposed according to the Walpole combination.

The cells were placed on dull black card board, placed before the screen described by Clark and Lubs[9] and lighted by 4 electric bulbs. A dull black card board with vertical rectangular windows cut close together was drawn over the cells and the color of each as seen through the apertures compared. The cells were also compared in daylight when suitable indicators were used. This substitute for the black box comparator was very satisfactory as well as simple to use.

Concentration of Hydrogen Ion of Hydrolyzed Solutions.—Using the modified comparator and brom phenol blue indicator, it was found that the hydrolyzed solution giving maximum yields of cystine after addition of sodium acetate showed a concentration of hydrogen ion equal to 10^{-4}.

It was noticed that congo red gives a red color when sodium acetate is added to a hydrolyzed solution in amounts still insufficient to give maximum yields of cystine, and therefore this red color does not indicate that sufficient sodium acetate has been added.

Hydrolysis of Wool.

A series of hydrolyses of wool was next made to determine the effect of increasing the length of time of hydrolysis on the yield of cystine. Portions of 500 g. of wool were boiled with 1000 cc. of conc. hydrochloric acid for different periods of time and 750 g. of sodium acetate added to aliquots representing 185 g. of wool. Sulfur was determined in the precipitates formed on standing 3 days and also on precipitates in the mother liquor. Alcohol was also added to the mother liquors until they became turbid, and the precipitates thus formed were analyzed.

TABLE II.

HYDROLYSIS WITH CONCENTRATED HYDROCHLORIC ACID. SULFUR AND CYSTINE IN PRECIPITATES FROM ALIQUOTS REPRESENTING 185 G. of WOOL.

Time of hydrolysis. Hours.	Sulfur. G.	Calc. cystine in wool. %.
3	1.618	3.27
4	2.037	4.12
5	2.120	4.29
6	2.135	4.32
12	2.578	5.22
74	0.120	0.24

A study of Table II suggests that 3 hours' hydrolysis is not a sufficient length of time but that there is an increased yield of cystine up to the 12-hour period. The 74-hour period gives practically no yield of cystine. The addition of alcohol gave a slight increase in yield.

[9] Clark and Lubs, *J. Bact.*, **2**, 134 (1917).

Hydrolysis was carried out with 6.8 *M* (22%) hydrochloric acid. Two hundred and fifty g. of wool was boiled with 900 cc. of dil. acid for 12 hours. From an aliquot representing 185 g. of wool only 1.532 g. of sulfur was precipitated, but on adding alcohol until the solution became turbid 1.354 g. of sulfur was further precipitated, giving a total calculated yield of cystine equal to 5.84% of the wool. This indicates that hydrolysis with dilute hydrochloric acid gives good yields of cystine, but as the mother liquors obtained from dilute acid contain less hydrolyzed wool per volume they tend to retain more cystine in solution after neutralization. The advantage of using dil. hydrochloric acid is that while pushing the wool into the hot acid no strong hydrogen chloride fumes are evolved to burn the hands.

Preparation of Pure Cystine.

The quantitative yields of pure cystine obtained from the crude cystine precipitated by sodium acetate as above were determined.

Two portions of 500 g. of wool were boiled with 1000 cc. of conc. hydrochloric acid each, for 6 hours as usual. Sodium acetate sufficient to give a maximum yield of cystine was added. Each washed and dried precipitate was treated as follows. The precipitate was dissolved in 250 cc. of 1.8 *M* (6.5%) hydrochloric acid, filtered by suction, the insoluble portion digested with 2 portions of 50 cc. of hydrochloric acid and filtered. Norite freed from iron and calcium was added equal to 50 g. on the water-free basis, to the combined filtrates, heated and filtered. The Norite was boiled with 2 portions of 100 cc. of 5% hydrochloric acid[10] and filtered. The filtrates were practically colorless. Five hundred cc. of hot sodium acetate trihydrate solution (1:1) was added to the hot combined acid solution. Almost pure cystine was precipitated. Analysis of the white crystals of cystine showed them to be 98.6% pure. By analysis for sufur before and after use it was found that the Norite had adsorbed 0.313 g. of sulfur. The yield of cystine thus obtained was 4.3% of the wool and practically equal to the yield as calculated from sulfur determined in the crude precipitate.

Analysis of Pure Cystine.

Pure cystine was obtained by repeatedly dissolving in 1.8 *M* (6.5%) hydrochloric acid and precipitating with sodium acetate solution. Sulfur in one sample was found by 3 determinations by the Carius method to be 26.66%, 26.63% and 26.86% respectively; the calculated sulfur is 26.69%. By the peroxide method with the same sample 26.37% was obtained.

Cystine Obtained by the Method used by Abderhalden.

Two hundred and fifty g. of wool was boiled under a reflux condenser with 900 cc. of 6.8 *M* (22%) hydrochloric acid for 12 hours, and evaporated to a gummy mass *in vacuo*. This was dissolved in water, made up to 1000 cc., decolorized with Norite, and 100 g. of sodium hydroxide in solution added to the cold filtrate. After standing for 3 days the fine white crystals were filtered with suction. The concentration of hydrogen ion in the filtrate was found to be $10^{-4.4}$. The filtrate was evaporated *in vacuo* and a further yield of cystine obtained.

[10] Miss Dennis recommends that bone-black used in decolorizing these precipitates be boiled with dilute acid. *J. Biol. Chem.*, **9**, 369 (1911).

TABLE III.

CYSTINE BY ABDERHALDEN'S METHOD.

	Sulfur. G.	Cystine. G.	Cystine in Wool. %
1st Ppt.	2.910	10.904	4.36
2nd Ppt.	1.429	5.354	2.14
Total	4.339	16.258	6.50

The total yield of cystine obtained by this method was 6.50% of wool. Here again it is noticed that concentration of the mother liquor results in further precipitation of cystine from solution.

The advantage of using sodium hydroxide solution over that of using sodium acetate is that less salt is formed in the mother liquor and concentration of the latter is easier. In Folin's method the mother liquors are saturated with salt.

The Determination of the Concentrations of Hydrogen Ion in Solutions in which Maximum Yields of Cystine are Obtained.

The hydrogen-ion concentration of the solutions of hydrolyzed wool which on addition of sodium acetate had given the maximum yield was found to be 10^{-4}.

Of special interest are the results obtained in determining the concentration of hydrogen ion in solutions which give maximum and minimum precipitates of cystine from a solution of pure cystine. Three series were run to obtain these results. In each, 10 g. of pure cystine was dissolved in 1.8 *M* hydrochloric acid and made up to 500 cc. with 1.8 *M* hydrochloric acid.

Fifty cc. aliquot portions containing 1 g. of cystine each were run into separate beakers. Sodium acetate trihydrate in solution (1:1) was added from a buret in various amounts to each beaker and distilled water added to make the volume in each case 150 cc. In another series 4.34 *M* sodium hydroxide solution was added first to 50 cc. of the acetate solution and rinsed with water into the aliquots of cystine solution; the volume was then made up as before to 150 cc. After standing for 48 hours at room temperatures the precipitates formed were filtered into weighed Gooch crucibles with asbestos pads, washed thrice with cold water and once each with alcohol and ether. The precipitates were dried at 100°, cooled in the desiccator and weighed.

The concentration of hydrogen ion was determined in the filtrates kept separate from the washings. Standard buffer solutions, the hydrogen-ion concentrations of which had been determined by the electrometric method, were used for comparison in determining the concentration of hydrogen ion in the filtrates by the colorimetric method.

In Table IV are shown the amounts of reagents used, the percentages of cystine obtained and the corresponding concentrations of hydrogen ion.

The maximum yield of cystine was 96.4%. It is clear that addition of the 1 : 1 sodium acetate solution to 1.8 *M* hydrochloric acid gives a maxi-

TABLE IV.

PERCENTAGES OF TOTAL CYSTINE IN SOLUTION PRECIPITATED AND CONCENTRATIONS OF HYDROGEN ION.

$NaC_2H_3O_2.3H_2O$ sol. 1 : 1. Cc.	NaOH 4.34 *M.* Cc.	Cystine precipitated. %.	Log C_{H+}.
10.0	....	00.00	.....
12.0	....	00.00	.....
14.0	....	00.00	−1.0
16.0	....	11.93	−1.1
16.5	....	16.06	−1.2
17.0	....	53.11	−1.3
17.5	....	62.18	−1.4
18.0	....	75.85	−1.6
19.0	....	93.97	−2.3
20.0	....	96.22	−3.4
30.0	....	96.37	−4.3
40.0	....	96.10	−4.5
50	....	96.29	−4.6
50	18.0	94.54	−6.9
50	18.5	84.91	−8.3
50	19.0	54.64	−8.9
50	19.5	30.77	−8.9
50	20.0	2.67	−9
50	20.5	0.	−9

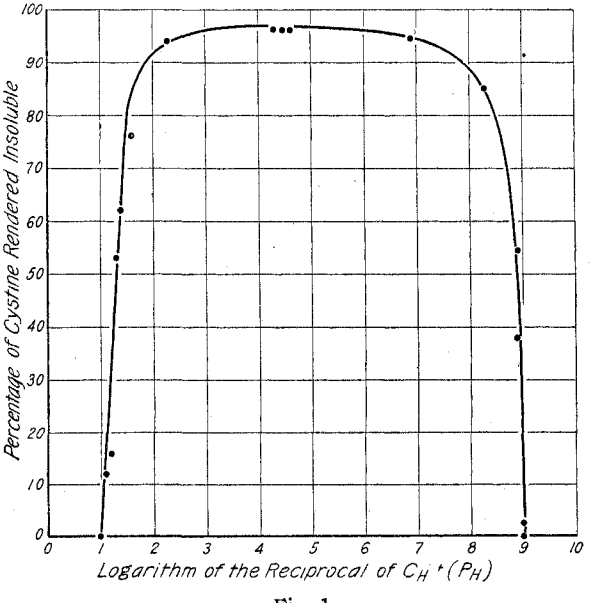

Fig. 1.

mum yield when 20 to 30 cc. of the acetate solution is added to 50 cc. of the dil. hydrochloric acid solution of cystine. As the iso-electric point of tyrosine, calculated from the values $ka=4\times10^{-9}$ and $kb=2.6\times10^{-12}$ as determined by Kanitz[11] and using the formula[12] $L=\sqrt{\frac{ka}{kb}\times kw}$ is $10^{-5.4}$, it is well to precipitate the cystine at a hydrogen-ion concentration greater than that of $10^{-5.4}$ to obtain it free from tyrosine.

The curve in Fig. 1 follows the values of Table IV. The iso-electric zone for cystine as indicated is a broad one and shows that cystine can be precipitated in solutions of quite varying strengths.

Summary.

It has been shown that the zone of hydrogen-ion concentration most favorable for the precipitation of cystine from solution is between 10^{-3} and 10^{-6}. To obtain cystine free from tyrosine it is advisable to precipitate it from solution at a concentration of hydrogen ion of about 10^{-3}.

The quantity of sodium acetate required as a buffer in the hydrolyzed solution of wool was determined. When 100 g. of wool is hydrolyzed with 200 cc. of conc. hydrochloric acid, sp. gr. 1.19, 750 g. of sodium acetate trihydrate crystals should be added to 500 cc. of the hydrolyzed solution to insure maximum precipitation of cystine. The concentration of hydrogen ion was determined and found to be 10^{-4}. The highest yield of cystine thus obtained was 5.2% of the weight of the wool taken.

When sodium hydroxide solution was added to a hydrolyzed solution which had been evaporated *in vacuo* and taken up in water, the yield of cystine obtained was 6.5% of the wool. The filtrate contained less salt than that obtained by Folin's method and could therefore be concentrated with less difficulty. Cystine was obtained pure from the crude cystine precipitated by sodium acetate from the hydrolyzed solution of wool and the percentage yield of pure cystine actually obtained agreed with the yield calculated by determination of sulfur in the crude precipitate.

Comparisons were made between the yields of cystine obtained after boiling wool with conc. hydrochloric acid, sp. gr. 1.19, for from 3 to 12 hours with the yield obtained using 6.8 *M* (22%) hydrochloric acid for hydrolysis. It was found that boiling wool with conc. hydrochloric acid for 12 hours gave the highest yield of cystine. When about 20% hydrochloric acid was used, the solution was so dilute that alcohol had to be added to the buffered mixture. When alcohol was added, the yield of cystine was as high from the dilute acid hydrolysis as with the concentrated. Dil. hydrochloric acid does not evolve the strong acid fumes that the concen-

[11] Kanitz, *Z. physik. Chem.*, **47**, 476 (1906).

[12] Michaelis and Mostynski, *Biochem. Z.*, **24**, 79–91 (1910).
Michaelis and Davidsohn, *ibid.*, **30**, 143–50 (1911).

trated acid does and is therefore more convenient to handle although it takes more space.

The amount of cystine adsorbed by the Norite used in decolorizing the crude cystine was found to be small when the Norite had been boiled with dil. hydrochloric acid and filtered after decolorizing.

In determining the hydrogen-ion concentration of dark colored solutions a modification was made in the comparator used by Humitz, Meyer and Ostenberg.

The above investigation was suggested by Dr. H. C. Sherman. Appreciation is due also to Dr. A. W. Thomas and Dr. H. T. Beans for helpful advice.

NEW YORK CITY.

[CONTRIBUTION FROM THE OIL, FAT AND WAX LABORATORY, BUREAU OF CHEMISTRY, U. S. DEPARTMENT OF AGRICULTURE.]

THE CHEMICAL COMPOSITION OF CORN OIL.

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Received September 1, 1921.

Corn oil is produced from the germ of the corn kernel which is obtained by degerminating corn in the hominy, starch and glucose industries. The germ represents approximately 10% of the dry kernel and contains about 50% of oil. However, the commercially separated germs contain considerable portions from other parts of the corn which reduce the percentage of oil. About 75% of the corn oil output is used after refining for edible purposes. The poorer grades are used for soap making. It has also been used for the preparation of rubber substitutes.[1]

Very little work on the composition of corn oil is reported in the literature. Lewkowitsch[2] states that the unsaturated acids consist of a mixture of oleic and linolic acids and quotes Vulte and Gibson as authority for the statement that the saturated acids consist of palmitic, stearic and arachidic acids. But Hehner and Mitchell[3] could not detect stearic acid. Leathes[4] states that hypogaeic acid occurs in corn oil.

The oil used in this investigation was pressed by means of an oil expeller from corn germs which had been produced by the so-called dry process.

Physical and Chemical Characteristics.—The more important physical and chemical characteristics are given in Table I. The saturated and unsaturated acids were determined by the lead-salt ether method.

[1] Sievers, "The Production and Utilization of Corn Oil in the United States," *U. S. Dept. Agriculture Bull.*, No. 904, 1920.

[2] Lewkowitsch, "Chemical Technology and Analysis of Oils, Fats and Waxes," Macmillan and Co., 5th ed. Vol. 2, p. 168.

[3] Hehner and Mitchell, *Analyst*, 21, 328 (1896).

[4] Leathes, "The Fats," Longmans, Green and Co., 1910, p. 15.