[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF ILLINOIS]

THE REDUCTION OF CYSTINE IN LIQUID AMMONIA BY METALLIC SODIUM[1]

BY VINCENT DU VIGNEAUD, L. F. AUDRIETH AND H. S. LORING[2]

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From a consideration of amino acids as ammono compounds it would seem likely indeed that liquid ammonia would be a most suitable solvent for the study of not only these substances, but also of the simple derivatives of the amino acids and possibly for the investigation of the more complex derivatives, the proteins. A study of the solubilities of a number of amino acids in liquid ammonia showed they were quite soluble in most instances. Of particular interest to us was the ready solubility of cystine in this medium, in contrast to its behavior in water. In view of this fact it was decided to investigate the possibility of the reduction of cystine to cysteine, the sulfhydryl form, by means of metallic sodium. It was found that this reaction readily takes place and, in fact, affords an excellent method for the preparation of cysteine.

The general applicability of a solution of sodium in liquid ammonia as a reducing agent has been demonstrated by numerous investigators.[3,4] The experimental procedures outlined below, however, involve the first recorded case which we know of in which reduction of the disulfide linkage to the sulfhydryl group has been effected in this manner.

The slight possibility of a change in cystine itself on treatment with liquid ammonia and subsequent evaporation was eliminated by the quantitative recovery of the cystine having an unchanged rotation. Preliminary reactions were then run to prove that the reduction of the cystine actually leads to the formation of cysteine. Benzylcysteine was obtained in very good yield by adding benzyl chloride directly to the reduced cystine solution in the liquid ammonia. This procedure is the simplest method for the preparation of this derivative of cysteine and should offer a general method for the preparation of similar derivatives. The reduced cystine solution obtained after evaporation of the ammonia and careful neutralization in the cold with acid gave a very strong positive nitroprusside test and a very strong Sullivan reaction.[5] The nitroprusside test was carried out in the usual fashion in aqueous solution but later it was found that the reaction

[1] A preliminary report of this paper was given before the meeting of the American Society of Biological Chemists in Chicago, March 26–29, 1930.

[2] This communication is an abstract of a thesis submitted by H. S. Loring in partial fulfilment of the requirements for the Degree of Master of Science in Chemistry at the University of Illinois.

[3] Kraus and White, THIS JOURNAL, **45**, 768 (1923).

[4] White and Knight, *ibid.*, **45**, 1780 (1923).

[5] Sullivan, *Public Health Repts.*, U. S. P. H. S., **44**, 1599 (1929).

could be carried out directly in the liquid ammonia. Sodium nitroprusside dissolves in liquid ammonia and gives a deep purple-red color with cysteine.

The amount of sodium required for the reduction of the cystine was next investigated. It is well known that sodium dissolved in liquid ammonia gives a deep blue solution. The disappearance of this blue color was used as the end-point in the reaction with cystine. To a weighed amount of sodium dissolved in liquid ammonia in a liquid ammonia titration apparatus, described by Johnson and Fernelius,[6] cystine was added very slowly until the blue color disappeared. It was found that four atoms of sodium were necessary to reduce one molecule of cystine.

For completeness of reduction probably the best criterion is the optical rotation. Vickery and Leavenworth[7] had come to this same conclusion in their study of the formation of cysteine when cystine is precipitated with silver sulfate. They based their conclusions on the great difference between the rotation of levo cystine and the positive rotation of cysteine obtained from the reduction of levo cystine. Andrews[8] in his study of the reduction of cystine obtained cysteine hydrochloride with a rotation of $+9°$ calculated as cysteine from cystine having a rotation of $-215°$. This positive rotation of cysteine hydrochloride was confirmed by Vickery[7] in the above-mentioned work. Bergmann and Michalis[9] have recently published a method for the preparation of cysteine depending on catalytic reduction with palladium black. These investigators obtained free cysteine, which had a negative rotation of $-10.14°$ in water solution, but a positive rotation of $+6.9°$ in 1 N acid solution. In the present work the rotations of the reduced solutions were followed and found in a great number of runs to have values close to $+7°$. In a number of runs higher values were obtained, in one instance $+12°$.

The method for the preparation of cysteine suggested by the present investigation is particularly simple where the presence of sodium chloride and a trace of ammonium chloride is not undesirable. For the preparation of the free cysteine hydrochloride it is necessary to extract the mixture with alcoholic hydrochloric acid and recrystallize the extracted cysteine hydrochloride from 20% hydrochloric acid. Free cysteine can then be prepared in the usual manner by dissolving the hydrochloride in alcohol and precipitating the cysteine by exactly neutralizing with ammonium hydroxide. The method is particularly advantageous where it is desirable to avoid the introduction of traces of heavier metals in the preparation of cysteine.

Experimental

The liquid ammonia used in the actual preparation of cysteine was obtained from

[6] Johnson and Fernelius, *J. Chem. Ed.*, **6**, 441 (1929).

[7] Vickery and Leavenworth, *J. Biol. Chem.*, **86**, 129 (1930).

[8] Andrews, *ibid.*, **68**, 209 (1926).

[9] Bergmann and Michalis, *Ber.*, **63**, 987 (1930).

the usual standard laboratory tank. In those experiments in which the molecular ratio of sodium to cystine was determined, the ammonia was dried over metallic sodium in an auxiliary tank, and then passed into the titration apparatus, where it was condensed by means of a bath of solid carbon dioxide in ether.

The cystine was prepared by the method given in "Organic Syntheses,"[10] the samples having specific rotations of $-205°$ to $-208°$.

Determination of the Molecular Ratio of Sodium to Cystine.—The liquid ammonia titration apparatus[6] used consists of a cylindrical flask 26 cm. long and 3.5 cm. in diameter. A weighing tube is fitted by means of ground-glass connection into a side arm 2 cm. in diameter, located approximately 8 cm. from the top of the apparatus. A second side arm, 7 mm. in diameter, is located opposite the first and contains a stopcock. The flask is closed by means of a ground-glass stopper, through which a 7-mm. tube also containing a stopcock passes to the bottom of the flask.

Approximately 40 cc. of liquid ammonia was condensed into the titration apparatus after all the air had been displaced by first passing ammonia through the flask. A carefully weighed piece of sodium was then quickly added through the side arm of the apparatus; the mixture was stirred by bubbling gaseous ammonia through the solution until the sodium had dissolved. Cystine was then added slowly from the dropping tube, which had previously been weighed. The addition was continued until the blue color due to free sodium had just disappeared. The amount of cystine was then determined by the difference in the weight of the tube before and after the addition of the cystine. The values of the ratios found are given in Table I.

TABLE I

MOLES OF SODIUM NECESSARY TO REDUCE CYSTINE

	Grams	Moles used	Molecular ratio
	Run No. 1		
Wt. of sodium	0.3648	0.01586	4.092
Wt. of cystine	.9302	.003876	1.0
	Run No. 2		
Wt. of sodium	0.185	0.008043	4.179
Wt. of cystine	.462	.001925	1.0

The fact that the amount of sodium used was very slightly higher than the theoretical may be explained by the presence of a small amount of sodium hydroxide, which formed during the weighing, and the transference of the sodium to the reaction flask.

Preparation of Benzylcysteine.—The presence of a sulfhydryl group in the liquid ammonia solution was shown by a positive sodium nitroprusside reaction. To prove the presence of cysteine, benzylcysteine was prepared. An ammonia solution of the reduced cystine as obtained above was allowed to evaporate spontaneously at room temperature. The flask containing the residue was evacuated to remove the last traces of ammonia. A part of the solid remaining was tested for cysteine by the Sullivan reaction,[5] and was found to give a strong positive test. The remainder was treated with benzyl chloride by the method of Gortner and Hoffman[11] for the preparation of benzylcysteine. On acidifying the solution with acetic acid, a large white precipitate formed. This was filtered, washed and recrystallized from boiling water; m. p. 214° (uncorr.); m. p. of benzylcysteine, 213°. This product was soluble in dilute hydrochloric acid and sodium hydroxide and gave a negative nitroprusside reaction.

[10] "Organic Syntheses," John Wiley and Sons, Inc., New York, 1925, Vol. V, pp. 39–41.

[11] Gortner and Hoffman, *J. Biol. Chem.*, **72**, 444 (1927).

Anal. Calcd. for $C_9H_{13}O_2NS$: N, 6.63. Found: N, 6.54, 6.39.

Benzylcysteine in Liquid Ammonia Solution.—Weighed amounts of cystine were reduced with sodium as described above. To the resulting colorless solution the theoretical amount of benzyl chloride (two moles of benzyl chloride for every mole of cystine) was added slowly with stirring. The ammonia solution was evaporated and the sodium salt remaining was dissolved in water and acidified with acetic acid. Benzylcysteine separated out and was purified by recrystallization from hot water; m. p. 212° (uncorr.). Yields of about 70% of the theoretical were obtained.

Anal. Calcd. for $C_9H_{13}O_2NS$: N, 6.63. Found: N, 6.74, 6.88.

Recovery of Cystine from the Reduced Solution.—To determine whether racemization had taken place during reduction, the material obtained after evaporation of the ammonia solution was dissolved in water, neutralized with dilute hydrochloric acid and allowed to stand in contact with air for several days. Cysteine is readily oxidized under these conditions. White particles separated from the solution. Upon recrystallization of this precipitate from dilute acid by the addition of sodium hydroxide, characteristic hexagonal plates of cystine were obtained with an optical rotation of −210°. The optical activity of the original cystine was −208°.

Preparation of Cysteine Solutions and their Optical Activity.—In preparing cysteine solutions for measurements of the optical rotation, it was found that the reaction could be carried out best in a 100-cc. graduated cylinder, cooled in the carbon dioxide–ether cooling bath. A typical procedure consisted in dissolving a weighed amount of sodium, approximately 0.5 g., in about 40 cc. of liquid ammonia, and adding approximately 1 g. of cystine from a weighing bottle, leaving an excess of sodium in the solution. The amount of cystine used was accurately determined. The resulting blue solution, which contained an excess of sodium, was then neutralized by the addition of an amount of ammonium chloride equivalent to the sodium used (1.16 g. for 0.5 g. of sodium). The mixture, which contained sodium chloride and the ammonium salt of cysteine, was evaporated to dryness at room temperature in the absence of air. To remove traces of free ammonia the cylinder was evacuated and finally flushed with oxygen-free nitrogen. The mixture of salts remaining was dissolved in a small volume of cold air-free water (25 cc.) and enough standard acid was added to make an approximately 1% solution in 1 *N* acid (75 cc. of 1.3 *N* hydrochloric acid). This solution was then forced over by means of a pressure of nitrogen into a polariscope tube for determination of the optical rotation. The values found are given in Table II.

TABLE II

ROTATION OF CYSTEINE PREPARATIONS

Cystine $\alpha^{D}_{28°}$	Wt. of cystine reduced, g.	α^{D}, calcd. as cysteine	Evidence of decomposition[a]
205°	0.2699	12^{23}	Negative
205°	4.2352	5^{23}	Slightly positive
208°	2.0580	4^{28}	Slightly positive
208°	0.8065	5^{28}	Negative
208°	1.2341	3^{30}	Negative
208°	1.3456	7^{30}	Slightly positive
208°	1.1203	5^{31}	Slightly positive

[a] Tested by bubbling pure nitrogen through the solution and passing the gases obtained through a solution of neutral cadmium sulfate.

When the salt remaining after evaporation of the ammonia was acidified directly, it was found that considerable decomposition with the liberation of hydrogen sulfide

and the formation of a cloudy solution took place. This decomposition could be reduced and in many cases entirely prevented by the use of an ice–salt cooling bath during the addition of the acid to the water solution. The liberation of hydrogen sulfide during the reduction of cystine with zinc and hydrochloric acid has been mentioned by Okuda.[12] In a few experiments in which cystine was reduced with tin and hydrochloric acid, we have also found small amounts of hydrogen sulfide to be liberated.

Preparation of Cysteine Hydrochloride and Free Cysteine.—The preparation of pure cysteine hydrochloride involves its separation from the sodium chloride and small amounts of ammonium chloride present in the reaction mixture. The reduction as described above is carried out in a Dewar test-tube and the ammonia solution evaporated to dryness. The sodium salt remaining is dissolved in a small amount of cold 95% alcohol and then acidified with cold 2 *N* alcoholic hydrochloric acid. The precipitate of sodium chloride is filtered and washed several times with alcoholic hydrochloric acid. The filtrate and washings are evaporated to dryness at 45° under diminished pressure in an atmosphere of nitrogen, and the residue recrystallized from a small volume of hot 20% hydrochloric acid; 3.44 g. of pure cysteine hydrochloride was obtained from 10 g. of cystine. An approximately 1% solution of this product in 1 *N* hydrochloric acid had an optical rotation of 4.4° at 28°.

Preparation of Cysteine.—Free cysteine may be prepared by dissolving the hydrochloride in a small volume of absolute alcohol at room temperature and neutralizing with a solution of ammonia in absolute alcohol. The precipitate of free cysteine is filtered, washed with alcohol and ether and remains as a white powder. On standing an aqueous solution of this material deposits a white precipitate, which when recrystallized from dilute acid with sodium hydroxide gives characteristic hexagonal plates of cystine.

Summary

Cystine is soluble in liquid ammonia and can be reduced readily by metallic sodium in this medium. The reduction product has been shown to be cysteine.

A new method for the preparation of cysteine has been worked out on the basis of this reaction.

Benzylcysteine was prepared by adding benzyl chloride directly to the liquid ammonia solution of cysteine which had been prepared by the sodium reduction method. This procedure offers an exceptionally convenient method for preparing this and similar derivatives.

URBANA, ILLINOIS

[12] Okuda, *J. Biochem.*, 5, 220 (1925).