[CONTRIBUTION FROM THE LABORATORY FOR THE STUDY OF HEREDITARY AND METABOLIC DISORDERS AND THE DEPARTMENTS OF BIOCHEMISTRY AND MEDICINE, UNIVERSITY OF UTAH COLLEGE OF MEDICINE]

A PREPARATION OF L-CYSTATHIONINE AND L-ALLOCYSTATHIONINE[1]

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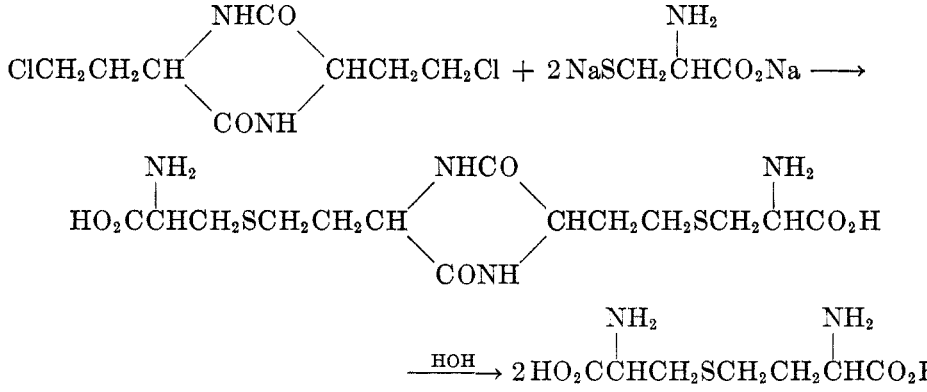
Received October 16, 1950

The original synthesis reported for cystathionine [S-(2-amino-2-carboxy)ethylcysteine] (1) suffers from some disadvantage in that optically active homocysteine and serine must be used as starting materials and because low yields are obtained in the final condensation leading to cystathionine. Inasmuch as satisfactory methods are available for the resolution of homocysteine and of serine, this synthesis was suitable for the successful preparation of the four optically active forms of cystathionine (2). For the synthesis of L-cystathionine and L-allocystathionine, the isomers most likely to be of use in biological experiments, however, it would be desirable to use an alternative synthesis so that the readily available L-cysteine could be used to provide one of the asymmetric centers. The resolution of DL-homoserine was undertaken (3) so that the synthesis of methionine described by Snyder, *et al.* (4) could be adapted to the preparation of L-cystathionine and L-allocystathionine by condensing the appropriate derivative of L-homoserine and D-homoserine, respectively, with L-cysteine.

The successful completion of this synthesis was reported recently by Stekol (5) who condensed the sodium derivative of L-cysteine with inactive 3,6-*bis*-(β-chloroethyl)-2,5-diketopiperazine. The resulting product was hydrolyzed to yield a mixture of L-cystathionine and the diastereoisomeric L-allocystathionine.



This paper reports the application of the above method to the preparation of L-cystathionine and L-allocystathionine. Although the synthesis proceeded smoothly, good yields of optically pure products were not obtained. Some racemization of the labile diketopiperazine ring occurred during the conversion of the hydroxy compound to the chloro derivative, and in addition it seems likely that additional racemization took place under the alkaline conditions used in

[1] This research was supported by a grant from the United States Public Health Service.

the condensation of *bis*-chloroethyldiketopiperazine with cysteine in liquid ammonia, since it is well known that optically active diketopiperazines are sensitive to alkali but are reasonably stable in acidic solutions. L-Cystathionine and L-allocystathionine so obtained could be recrystallized to optical purity, but the losses incurred in the process make this synthesis less advantageous than the improved synthesis from homocysteine and serine recently reported by Rachele, *et al.* (6).

For the synthesis of L-cystathionine and L-allocystathionine on a considerable scale, however, it was desirable to prepare the mixture reported by Stekol and to ascertain whether the diastereoisomers could be separated by recrystallization of the compounds from suitable solvents. This was attempted and it was found that L-allocystathionine is considerably less soluble than L-cystathionine in ammoniacal solution; L-cystathionine, on the other hand, is more insoluble than L-allocystathionine in slightly acid solution. Since the specific rotation in 1 *N* acid is +23.9° for L-cystathionine and −25.8° for L-allocystathionine, a measurement of the rotation of a mixture of the two provides a satisfactory assay of the amount of each present. By three recrystallizations of each of the initially obtained fractions, L-cystathionine and L-allocystathionine were obtained optically pure in 15% and 17% yield respectively. The separation could be repeated on the material recovered from the mother liquors, so that essentially complete resolution eventually could be attained.

EXPERIMENTAL

Inactive homoserine diketopiperazine [*3,6-bis(β-hydroxyethyl)-2,5-diketopiperazine*]. Prepared according to the directions of Livak, *et al.* (7), from 3.34 g. of DL-α-aminobutyrolactone hydrobromide; yield, 1.1 g. (62%), m.p. 187–190°.[2]

Anal. Calc'd for $C_8H_{14}N_2O_4$: N, 13.86. Found: N, 13.61.

L-*Homoserine diketopiperazine.* This was prepared as above from 3.34 g. of L-α-aminobutyrolactone hydrobromide $[\alpha]_D^{23}$ −21.5° (*c*, 1, H_2O); yield, 1.25 g. (67%). After one recrystallization from hot ethanol containing a trace of water, m.p. 188.5–190°, $[\alpha]_D^{22}$ −32.4° (*c*, 1, H_2O).

Anal. Calc'd for $C_8H_{14}N_2O_4$: N, 13.86. Found: N, 13.61.

D-*Homoserine diketopiperazine.* This was prepared in the manner described above from 9.5 g. of D-α-aminobutyrolactone hydrobromide $[\alpha]_D^{24}$ +21.2° (*c*, 1, H_2O); yield, 3.6 g. (68%). After one recrystallization, m.p. 190–191°, $[\alpha]_D^{20}$ +32.1° (*c*, 1, H_2O).

Anal. Calc'd for $C_8H_{14}N_2O_4$: N, 13.86. Found: N, 13.82.

Inactive 3,6-bis(β-chloroethyl)-2,5-diketopiperazine. Prepared by the method of Snyder, *et al.* (4) from 26.0 g. of crude inactive homoserine diketopiperazine; yield, 24.6 g. (80%), m.p. 224–228° d.

L-*3,6-Bis(β-chloroethyl)-2,5-diketopiperazine.* Prepared according to the preceding directions from 3.2 g. of L-homoserine diketopiperazine; yield, 3.55 g. (94%). A small sample was recrystallized from 95% ethanol for analysis; m.p. 220–223°, partially solidified, m.p. 230–232° d.; $[\alpha]_D^{24}$ −88.1° (*c*, 1, pyridine). Crude samples of this compound gave much lower values, indicating that a considerable amount of racemization had occurred under the conditions of its preparation.

Anal. Calc'd for $C_8H_{12}Cl_2N_2O_2$: N, 11.71; Cl, 29.24.
Found: N, 11.60; Cl, 28.74.

D-*3,6-Bis(β-chloroethyl)-2,5-diketopiperazine.* Prepared as above from 2.7 g. of D-homo-

[2] All melting points were taken on the micro hot stage and are corrected.

serine diketopiperazine; yield, 3.0 g. (94%). A small sample was recrystallized from 95% ethanol for analysis; m.p. 220–228° d.; $[\alpha]_D^{24}$ +87.0° (*c*, 1, pyridine).

Anal. Calc'd for $C_8H_{12}Cl_2N_2O_2$: N, 11.71; Cl, 29.24.

Found: N, 11.64; Cl, 28.68.

Mixed isomers of 3,6-bis[2-(2-amino-2-carboxyethylmercapto)ethyl]-2,5-diketopiperazine. (Mixed diketopiperazines). Prepared essentially according to the procedure described by Stekol (5) from 24.6 g. of *bis*(β-chloroethyl)diketopiperazine,[3] 24.6 g. of L-cystine, and 9 g. of sodium in 500 ml. of liquid ammonia. The yield of crude product was 30.5 g. (73%). In practice it was found that a second treatment of the material with sodium cyanide was necessary in order to insure the complete removal of traces of cystine from the product. (If the material is not purified to a degree such that it gives a completely negative sodium-cyanide-nitroprusside test for cystine at this point, a greater loss is encountered in rendering free from cystine the cystathione-allocystathione mixture obtained upon hydrolysis of the mixed diketopiperazines). The yield of once recrystallized material was 27.1 g. (65%).

3,6-Bis[2-(2-amino-2-carboxyethylmercapto)ethyl]-2,5-diketopiperazine (L-*Cystathionine diketopiperazine*). Prepared as above from 3.2 g. of L-*bis*(β-chloroethyl)diketopiperazine, 3.3 g. of L-cystine, and 1.3 g. of sodium in 150 ml. of liquid ammonia. Yield, 2.95 g. (55%) m.p. 270–275° d.

Anal. Calc'd for $C_{14}H_{24}N_4O_6S_2$: N, 13.71; S, 15.70.

Found: N, 13.47; S, 15.50.

3,6-Bis[2-(2-amino-2-carboxyethylmercapto)ethyl]-2,5-diketopiperazine(L-*Allocystathionine diketopiperazine*). Prepared as above from 2.8 g. of D-*bis*(β-chloroethyl)diketopiperazine, 2.9 g. of L-cystine, and 1.1 g. of sodium in 150 ml. of liquid ammonia. Yield, 3.3 g. (69%); m.p. 275–280° d.

Anal. Calc'd for $C_{14}H_{24}N_4O_6S_2$: N, 13.71; S, 15.70.

Found: N, 13.29; S, 15.61.

L-*Cystathionine*-L-*allocystathionine mixture.* A solution of 27.9 g. of the cystathionine diketopiperazine mixture in 400 ml. of 6 *N* hydrochloric acid was refluxed 4 hours. The resulting solution was concentrated *in vacuo* to a sirup, 100 ml. of water was added, and the concentration was repeated. The sirup was dissolved in 120 ml. of water, treated with charcoal, and filtered. The clear colorless solution obtained by this treatment was heated to 90°, neutralized to *p*H 5 with ammonium hydroxide, diluted with sightly more than an equal volume of absolute ethanol, and cooled. The white solid that separated was washed with cold water, with absolute ethanol, and was air-dried. Yield, 28.0 g. (92%) of material giving a negative sodium cyanide-nitroprusside test for cystine, $[\alpha]_D^{25}$ −1.0° (*c*, 1, *N* HCl (indicating approx. 50% of each isomer).

L-*Cystathionine.* L-Cystathionine diketopiperazine (2.5 g.) was hydrolyzed with 20 ml. of 6 *N* hydrochloric acid and the mixture was worked up as described in the preceding experiment. The product was treated with sodium cyanide to remove the traces of cystine present; one recrystallization then produced 1.15 g. of L-cystathionine, $[\alpha]_D^{25}$ +21.5° (*c*, 1, *N* HCl) (corresponding to 96.2% L-cystathionine). A second crop of 1.15 g. was obtained from the mother liquors; $[\alpha]_D^{26}$ +12.4° (*c*, 1, *N* HCl) (76.8% L-cystathionine). Total yield, 2.30 g. (88%).

L-*Allocystathionine.* The preceding hydrolysis was repeated with 1.0 g. of L-allocystathionine diketopiperazine. The yield of L-allocystathionine was 0.90 g. (82%). One recrystallization from water yielded 0.55 g., $[\alpha]_D^{24}$ −21.0° (*c*, 1, *N* HCl) (90% L-allocystathionine).

Separation of L-*cystathionine—*L-*allocystathionine mixture.* A suspension of 30.0 g. of a 50% mixture of L-cystathione and L-allocystathione, $[\alpha]_D^{20}$ −1.0° (*c*, 1, *N* HCl), in 400 ml. of water was heated nearly to boiling and 50 ml. of conc'd ammonium hydroxide was added. The clear solution that resulted was diluted with an equal volume of hot absolute ethanol,

[3] The author wishes to thank Dr. H. R. Snyder of the University of Illinois for a supply of this intermediate.

the solution was allowed to cool slowly to room temperature, and was then cooled in a refrigerator for three days. The solid was washed with cold water and absolute ethanol and was air-dried. *Fraction I*, 7.5 g., $[\alpha]_D^{25}$ −11.0° (*c*, 1, *N* HCl) (corresponding to 70% L-allocystathione—30% L-cystathionine). The filtrate from fraction I was concentrated to dryness *in vacuo* and the residue was dissolved in approximately 150 ml. of hot water plus enough conc'd hydrochloric acid to effect complete solution. This hot solution was neutalized to *p*H 3–4 by the careful addition of conc'd ammonium hydroxide and was allowed to cool slowly to room temperature and stand overnight. The solid was washed and dried. *Fraction II*, 10.0 g., $[\alpha]_D^{25}$ +8.7° (*c*, 1, *N* HCl) (corresponding to 69% L-cystathionine—31 L-allocystathionine). The above order of separation is preferable, since excess ammonia can be removed completely by concentrating the solution to dryness, whereas the reverse procedure results in the retention of a considerable amount of hydrochloric acid. The inorganic salts formed when the acid residue is neutralized retard crystallization greatly and result in a loss of material.

The filtrate from fraction II was heated almost to boiling, adjusted to *p*H 5 by the addition of conc'd ammonium hydroxide, was diluted with an equal volume of hot absolute ethanol, and was cooled in a refrigerator overnight. The resulting solid was washed and dried as above. *Fraction III*, 11.6 g., $[\alpha]_D^{23}$ −3.7° (*c*, 1, *N* HCl) (corresponding to 56% L-allocystathionine mixture). The total recovery of material in these three fractions was 97%.

Since fraction III contained an excess of L-allocystathionine, a repetition of the above separation yielded a larger amount of 70% allocystathionine in the first crop obtained. The efficient recovery of material makes the complete resolution of a large batch of mixture quite practical.

L-*Allocystathionine*. Fraction I (7.5 g., 70% L-allocystathionine) was dissolved by suspending it in 75 ml. of hot water and adding 15 ml. of conc'd ammonium hydroxide. The hot solution was diluted *slowly* by the addition of an equal volume of hot absolute ethanol and the resulting solution was allowed to cool to room temperature and was left in a refrigerator overnight. After washing and drying the solid, 3.8 g. was obtained; $[\alpha]_D^{25}$ −21.0° (*c*, 1, *N* HCl) (90% L-allocystathionine). The filtrate was heated to remove some of the ammonia, was neutralized to *p*H 5 with conc'd hydrochloric acid, and was cooled. An additional 2.0 g. was recovered, $[\alpha]_D^{25}$ −15.0° (*c*, 1, *N* HCl) (79% L-allocystathionine).

Next, 3.8 g. of material (90% L-allocystathionine) was dissolved in 40 ml. of hot water containing 12 ml. of conc'd ammonium hydroxide. The hot solution was *slowly* diluted by the addition of an equal volume of hot absolute ethanol and was allowed to cool very gradually to room temperature. A definite crystalline sheen was noticed in the precipitate at this time and examination under the microscope showed the presence of elongated flat hexagonal crystals of L-allocystathionine. After the mixture had stood overnight at room temperature the solid was washed and dried. Yield, 2.6 g., $[\alpha]_D^{25}$ −25.0° (*c*, 1, *N* HCl) (99% L-allocystathionine). This material was dissolved in hot water containing a small amount of hydrochloric acid, the solution was treated with charcoal, and filtered. The hot filtrate was brought to *p*H 5 by the addition of ammonium hydroxide, was allowed to cool slowly to room temperature, and was then cooled in a refrigerator overnight. The crystals were washed thoroughly with cold water and air-dried. Yield, 2.5 g. of pure L-allocystathionine, $[\alpha]_D^{24}$ −25.8° (*c*, 1, *N* HCl).[4] The rotation was not changed when the material was subjected to further recrystallizations.

Anal. Calc'd for $C_7H_{14}N_2O_4S$: C, 37.82; H, 6.35; N, 12.61; S, 14.43.
Found: C, 37.84; H, 6.37; N, 12.58; S, 14.25.

L-*Cystathionine*. Fraction II (10.0 g., 69% L-cystathionine) was recrystallized by the addition of enough hydrochloric acid to cause solution of a suspension in 90 ml. of hot water and adjustment of the hot solution to *p*H 4 by the careful addition of conc'd ammonium hydroxide. The hot solution was allowed to cool slowly and after it had stood overnight at room temperature the separated solid was washed and dried. Yield, 5.1 g.; $[\alpha]_D^{25}$ +15.8°

[4] A value of −25.0° (*c*, 1, *N* HCl) is reported in the literature (2).

(*c*, 1, *N* HCl) (84% L-cystathionine). This material was recrystallized by dissolving it in 50 ml. of hot water plus enough hydrochloric acid to cause complete solution and neutralizing the acid solution to *p*H 4 with ammonium hydroxide. It was then allowed to stand overnight at room temperature and was washed and dried; yield, 2.8 g. of boat-shaped plates, $[\alpha]_D^{25}$ +23.1° (*c*, 1, *N* HCl) (98.5% L-cystathionine). One more recrystallization of this material from 25 ml. of water in the same manner as in the preceding step yielded 2.3 g. of analytically pure L-cystathionine, $[\alpha]_D^{20}$ +23.9° (*c*, 1, *N* HCl),[5] as elongated rectangular plates; the rotation was not changed by further recrystallization.

Anal. Calc'd for $C_7H_{14}N_2O_4S$: C, 37.82; H, 6.35; N, 12.61; S, 14.43.
Found: C, 37.69; H, 6.42; N, 12.55; S, 14.27.

Acknowledgments. The author wishes to thank Mr. Joy D. Lewis for his valuable assistance in preparing some of the intermediates used in this work. The microanalyses were performed by Dr. A. Elek of the Elek Microanalytical Laboratory.

SUMMARY

A convenient preparation of L-cystathionine and L-allocystathionine is accomplished by the separation of the mixture of diastereoisomers resulting from the condensation of L-cysteine with a derivative of DL-homoserine.

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REFERENCES

(1) BROWN AND DU VIGNEAUD, *J. Biol. Chem.*, **137**, 611 (1941); DU VIGNEAUD, BROWN, AND CHANDLER, *J. Biol. Chem.*, **143**, 59 (1942).
(2) ANSLOW, SIMMONDS, AND DU VIGNEAUD, *J. Biol. Chem.*, **166**, 35 (1946).
(3) ARMSTRONG, *J. Am. Chem. Soc.*, **70**, 1756 (1948).
(4) SNYDER, ANDREEN, CANNON, AND PETERS, *J. Am. Chem. Soc.*, **64**, 2083 (1942).
(5) STEKOL, *J. Biol. Chem.*, **173**, 153 (1948).
(6) RACHELE, REED, KIDWAI, FERGER, AND DU VIGNEAUD, *J. Biol. Chem.*, **185**, 817 (1950).
(7) LIVAK, BRITTON, VAN DER WEELE, AND MURRAY, *J. Am. Chem. Soc.*, **67**, 2219 (1945).

[5] A value of +23.7° (*c*, 1, *N* HCl) is reported in the literature (1).