Synthesis of Polycysteine

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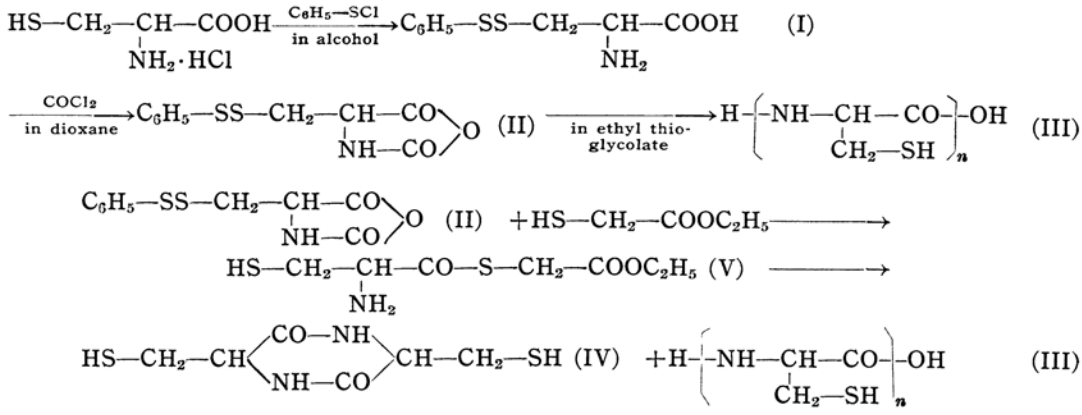
Recently, poly-S-benzylcysteine has been synthesized[1,2,3] for the purpose of preparing polycysteine, which is a basic material to investigate the unique role of cysteine residues in natural proteins, but the reduction of it into polycysteine was unsuccessful because of its insolubility in liquid ammonia. So other protecting groups for sulfhydryl group were desired. Katchalski and Berger[3] published a short communication on the synthesis of polycysteine, which was prepared from poly-S-carbobenzoxycysteine by reducing it with sodium in liquid ammonia, but the details have not been reported. Jones and Lundgren[4] have prepared polycystine and their result that it was soluble in 2-mercaptoethanol in spite of its three dimensional structure suggested that polycysteine might have been formed in that solution. However, the polymerization degree of its polypeptide chain was so low that this route may not be suitable to synthesize the higher molecular weight polycysteine.

In the present paper S-thiophenyl-L-cysteine (I) was synthesized for a starting material, and polycysteine (III) was obtained by heating the solution of N-carboxyanhydride (II) of this amino acid (I) in ethyl thioglycolate, where the polymerization and the reduction occurred simultaneously; on the other hand, the reduction of poly-S-thiophenylcysteine, which was prepared quantitatively by heating the solution of the anhydride (II) in pyridine or nitrobenzene, was unsuccessful for the same reason as in the case of poly-S-benzylcysteine. The synthetic route was as follows:

The synthesis of the compound (I) from benzenesulfenyl chloride and L-cysteine was carried out at first in the cold aqueous solution under the usual condition of the Schotten-Bauman reaction, but the yield was only less than 30% because of the hydrolysis of the reagent. After several trials, it was found that absolute ethanol was an excellent solvent in this case. In the presence of sodium bicarbonate, benzenesulfenyl chloride reacted smoothly with L-cysteine hydrochloride in an alcoholic solution and the compound (I) was obtained in a very good yield. This substance (I) involved a disulfide linkage and could be reduced easily into L-cysteine by simple reactants, for example, by tin and hydrochloric acid or by excess mercaptane through the disulfide-sulfhydryl equilibrium. As the disulfide linkage in this substance was unsymmetrical unlike in cystine, it was



comparatively unstable towards mineral acids or caustic alkalis; when it was dissolved in a cold aqueous solution of more than 1 N sodium hydroxide or boiling hydrochloric acid, decomposition occurred gradually and in the case of the latter L-cystine and diphenyldisulfide were formed. This phenomenon was

1) H. Tani, H. Yūki, and S. Sakakibara, *Seni Kagaku Kenkyusho Nenpo* (*Mem. Inst. Fiber Research*), **7**, 100 (1953).
2) E.R. Blakley, A.K. Sumner, and E.Y. Spencer, *Can. J. Technol.*, **30**, 258 (1952); cf. *C.A.*, **47**, 9264 (1953).
3) E. Katchalski and A. Berger, *Bull. Research Council of Israel*, **2**, 314 (1952).
4) H.W. Jones and H.P. Lundgren, *J. Am. Chem. Soc.*, **73**, 5465 (1951).

observed also in glacial acetic acid containing dry hydrogen chloride. The specific rotation of L-cystine formed in this way was compared with that of L-cystine used as a starting material, and then it was confirmed that no racemization occurred in this synthetic route. N-Benzoyl derivative and ethyl ester hydrochloride of the compound (I) were prepared, respectively. From these results it may be expected that it will serve as a useful material for the synthesis of simple peptides containing cysteine.

N-Carboxy-S-thiophenyl-L-cysteine anhydride (II) was prepared by the usual method from the compound (I) and phosgen. By heating the anhydride (II) in ethyl thioglycolate, a white amorphous substance (IIIa) was precipitated.

The infrared spectrum of this substance had three absorption bands, 3050, 3190, and 3285 cm^{-1}, within the amide NH stretching frequency region; one of which, 3190 cm^{-1}, corresponded to the band of *cis*-hydrogen bonds formed between two amide linkages, suggesting the presence of diketopiperazine-type compounds[5]. After extraction of this substance with thioglycolic acid and methanol in order to remove any soluble matter, this band, 3190 cm^{-1}, completely vanished in the spectrum of the insoluble substance (III b), and a band at 2545 cm^{-1}, indicating the presence of the sulfhydryl-group, remained (Fig. 1). From the fact that the substance (III b) displayed two major absorption bands in the spectrum at 1520 and 1635 cm^{-1} and very small bands at 1540 and 1670 cm^{-1} within the C=O stretching and amide NH deformation frequency regions, it was suggested, according to Ambrose and Elliott[6], that the conformation of polypeptide chain in the

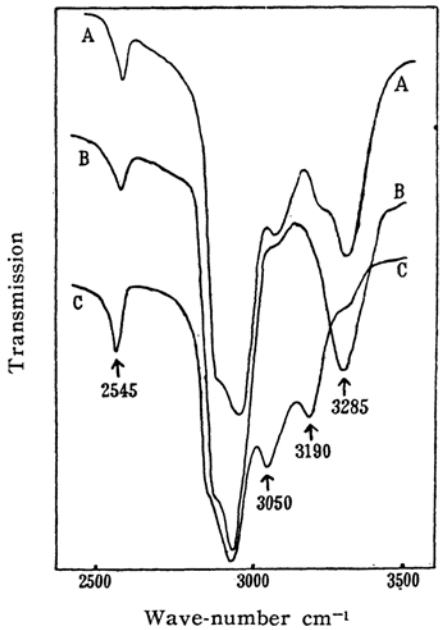


Fig. 1. Infrared spectra of IIIa(A), IIIb(B), and IV(C). Nujol suspension. Adam-Hilger H-800 double beam system.

substance (III b) was mainly in the β-keratin-type (Fig. 2). Absorption bands due to the thiophenyl group were not detected at all in this spectrum by comparison with that of poly-S-thiophenylcysteine and also no absorption bands due to ethyl thioglycolate were observed.

This substance was insoluble in usual organic solvents except ethanolamine and soluble in aqueous alkali but its alkaline solution decomposed gradually under evolution of hydrogen sulfide at room temperature. The nitroprusside test was extremely positive; the brown copper salt was formed under the condition of the biuret reaction and its mother liquor was brownish violet; the picric acid test for diketopiperazine was negative.

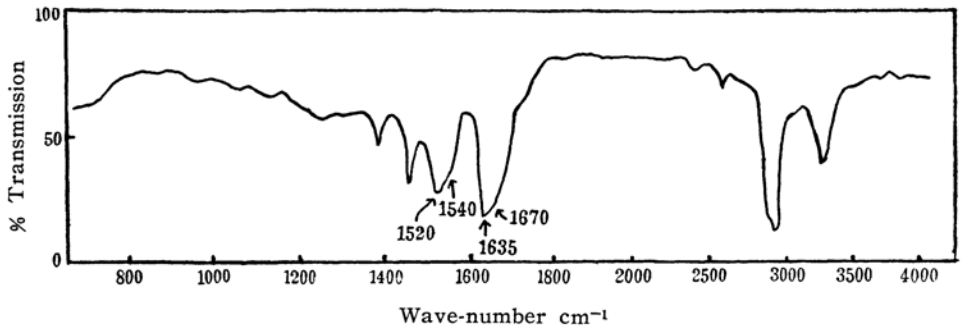


Fig. 2. Infrared spectrum of polycysteine (IIIb): Nujol suspension. Adam-Hilger H-800 double beam system.

5) M. Tsuboi, This Bulletin, 22, 255 (1949).

6) E.J. Ambrose and A. Elliott, *Proceed. Roy. Soc.*, A205, 47 (1951); cf. S. Mizushima and S. Akabori, "Chemistry of Proteins", Kyoritsu Shuppan Co. Ltd., Tokyo, Japan (1954), p 455-479.

The ammoniacal solution of this material was coagulated oxidatively to form gel on treatment with iodine. It displayed an amorphous X-ray diffraction pattern and the average polymerization degree calculated from the ratio of total nitrogen and amino nitrogen was 36.

The substance (IV) extracted from the polymer (IIIa) by thioglycolic acid and methanol was obtained as colourless fine prisms; its chemical properties have all been identified with those of cysteinyl-cysteine anhydride described by Greenstein[7] and its infrared spectrum has agreed with that of glycine anhydride, except the bands due to the presence of the mercaptomethyl group.

From these results, it may be concluded that the thioester of cysteine (V), formed from the anhydride (II) and ethyl thioglycolate, should take a part in the condensation reaction in parallel with the ordinary polymerization reaction of the anhydride (II)[8].

Experimental

S-Thiophenyl-L-cysteine (I).—Benzenesulfenyl chloride was prepared from 5 g. of thiophenol according to the method of Lecher[9], and, after removal of the reaction solvent, it was used as the solution of its residual syrup in 30 ml. of ether. A solution of 6.2 g. of L-cysteine hydrochloride in 120 ml. of absolute ethanol was cooled at 0°C, 3.8 g. of powdered sodium bicarbonate was added in one portion and into the whole mixture was dropped the chloride solution under stirring. After the complete addition of the reagent, the reaction mixture was allowed to stand at room temperature and sodium chloride was removed by filtration. After the addition of 5 ml. of pyridine into the filtrate, the fine precipitate was centrifuged, washed well with alcohol and dried; wt. 8.6 g.. After recrystallization from 0.5N hydrochloric acid, 8.1 g. (88%) of colourless plates was obtained; m.p. 192° (decomp.), $[\alpha]_D^{18}$-78.3° (c 0.38, 1N HCl). Anal. Found: C, 47.43; H, 4.64: N, 6.19; S, 27.4. Calcd. for $C_9H_{11}O_2NS_2$: C, 47.14; H, 4.84; N, 6.12; S, 27.9%. The solubility of this substance in water was about 50 mg./100 ml. at 17°C and increased about three times at 100°C. Paper chromatography showed this amino acid to have an Rf value of 0.69 in *n*-butanol: water: acetic acid (4 : 1 : 1).

Decomposition of (I) by 6N hydrochloric acid.—The solution of 0.5 g. of (I) in 20 ml. of 6N hydrochloric acid was boiled for about thirty minutes, cooled to room temperature and the crystalline diphenyldisulfide was filtered off. This was separated out as light oil and crystallized after cooling; the yield of recrystallized needles was 230mg., m.p. 60–61°, undepressed on admixture with an authentic sample. The filtrate was treated with charcoal and neutralized at pH 3 with aqueous sodium acetate; 220 mg. (84%) of L-cystine was obtained; $[\alpha]_D^{17}$-233° (c 0.85 1N HCl); cf. original L-cystine $[\alpha]_D^{17}$-233° (c 2.0 1N HCl).

Decomposition of (I) by dry hydrogen chloride in glacial acetic acid.—A suspension of 0.5g. of (I) in 50 ml. of glacial acetic acid was heated at 60 °C and dry hydrogen chloride was introduced for about thirty minutes; (I) was dissolved gradually within about ten minutes and then another crystalline precipitate was separated; this was filtered off after cooling. The filtrate was concentrated to dryness under reduced pressure, washed with petroleum ether, then some second crop was obtained. The total crop was 350 mg. in the form of L-cystine hydrochloride; $[\alpha]_D^{16.5}$-232° (c 0.85 1N HCl) (after recrystallization from 0.3 N HCl).

N-Benzopl-S-thiophenyl-L-cysteine.—A solution of 1g. of (I) in 90 ml. of 0.05N sodium hydroxide was cooled at 0°--5°C, into the solution was added 0.73 g. of benzoyl chloride, and 43.5 ml. of 0.1N sodium hydroxide was added in portions under stirring. After thirty minutes the reaction mixture was slightly acidified with hydrochloric acid, and the crystallized crude product was filtered off. This substance was dissolved in sodium bicarbonate solution, an insoluble matter, which was unchanged (I), wt. 0.5 g., was filtered off and the filtrate was extracted with ether and then slightly acidified by the addition of 6N hydrochloric acid; the N-benzoyl derivative of (I) was crystallized in fine needles, wt. 0.7 g.; m. p. 154–5° (after recrystallization from either 50% ethanol or benzene), $[\alpha]_D^{16.5}$-232° (c 1.0 ethanol). The yield based on reacted (I) was quantitative. Anal. Found: N, 4.16; S, 19.3. Calcd. for $C_{16}H_{15}O_3NS_2$: N, 4.21; S, 19.2%.

N-Carboxy-S-thiophyenyl-L-cysteine anhydride (II).—Dry phosgen was introduced into a suspension of 2 g. of finely powdered (I) in 80 ml. of dioxane at 40°C until the solid matter disappeared. After concentration to dryness under reduced pressure, the residual syrup was crystallized at 50°C in vacuo. N-carboxy anhydride was obtained as colourless plates after recrystallization from ethyl acetate and petroleum ether; wt. 1.2 g., m.p. 117–9°; a second crop was 0.7 g., total yield 85%. Anal. Found: N, 5.32. Calcd. for $C_{10}H_9O_3NS_2$: N, 5.50%.

Ethyl S-thiophenyl-L-cysteinate hydrochloride.—A solution of 0.5 g. of (II) in 50 ml. of ethanol containing 0.55 g. of dry hydrogen chloride was allowed to stand over night at room temperature. Removal of ethanol left a viscous syrup, which was crystallized by the addition of some ether; slightly coloured needles 0.57 g., m.p. 130–131.5° (after recrystallization from either ethylacetate or benzene); $[\alpha]_D^{18.5}$—43.6°C (c 0.87 ethanol). Anal. Found: N, 4.58. Calcd. for $C_{11}H_{13}O_2NS_2Cl$: N, 4.77%.

7) J.S. Greenstein, *J. Biol. Chem.*, **118**, 321 (1937).
8) Th. Wieland und W. Schäfer, *Angew. Chem.*, **63**, 146 (1951); cf. Th. Wieland, *Angew. Chem.*, **66**, 510 (1954).
9) H. Lecher und F. Holschneider, *Ber.*, **57**, 757 (1924).

Ethyl thioglycolate.—The procedure of Fiesselmann and Schipprak[10] for methyl thioglycolate was followed. A mixture of 34 g. of thioglycolic acid, 60 ml. of ethanol, 60 ml. of chloroform, and 4 g. of conc. sulfuric acid was placed in a flask of the Soxhlet extraction apparatus and about three quarters of the thimble was filled with freshly dehydrated magnesium sulfate. After refluxing for fifteen hours, the solution was washed with water, dried over anhydrous sodium sulfate and fractionally distilled; 39 g. of ethyl thioglycolate boiling between 68°/30 mm.–64°/21 mm. was obtained.

Polymerization of N-carboxy-S-thiophenyl-L-cysteine anhydride.—A solution of 1 g. of (II) in 10 ml. of ethyl thioglycolate was sealed in a glass tube and heated for about five hours on a boiling water bath, where a white amorphous substance was precipitated spontaneously. The reaction mixture was allowed to cool; it was filtered after adding enough ethyl acetate and washed well with the same solvent, then the precipitate was suspended in 5 ml. of thioglycolic acid at 30–40°C for about thirty minutes. After adding enough methanol, the precipitate was filtered and washed well with the same solvent, 227 mg. (50% based on II) of white amorphous powder (III) was obtained after drying over phosphorus pentoxide in vacuo at room temperature for thirty minutes. Although this polymer diminished its weight 7% more by complete dryness, it was stored for any uses as it was, because after complete drying it might become insoluble, and then the weight was corrected in every analysis. Anal. Found: C, 35.1; H, 4.64; N, 13.2; S, 31.2; amino-N, 0.367; SHa, 26.9; SHb, 30.1. Calcd. for $(C_3H_5ONS)_n$: C, 34.9; H, 4.88; N, 13.6; S, 31.1; SH, 32.0%. The analytical procedures for the sulfhydryl group used here were the *p*-chloromercuribenzoate method[11] and the iodoacetic acid method[3]. In the former the sample was reacted with *p*-chloromercuri-benzoate in 0.1 N sodium hydroxide for twenty minutes, pH 5.2 acetate buffer solution was added and titrated with standardized cysteine solution (SHa). In the latter the sample was mixed with excess iodoacetic acid, and, after addition of 0.2 N sodium hydroxide, was allowed to stand at room temperature for thirty minutes, then the liberated iodide ion was titrated by the Volhard method (SHb). The filtered solution of thioglycolic acid and methanol was concentrated under reduced pressure and white powder was precipitated with ether; wt. 100 mg., m.p. 195–200° (after recrystallization from water). This substance was easily soluble in pyridine and thioglycolic acid, soluble in ethanol, methanol, and water; the nitroprusside and picric acid tests were positive respectively.

10) Fiesselmann und Schipprak, *Ber.*, **87**, 839 (1954).
11) R.E. Feeney, L.R. Mac Donnell and R.S. Silva, *Arch. Biochem. Biophys.*, **32**, 288 (1951).

Summary

The synthesis of polycysteine via S-thiophenyl-L-cysteine was described. S-thiophenyl-L-cysteine was prepared from benzenesulfenyl chloride and L-cysteine hydrochloride in absolute alcohol and its chemical properties were studied. This amino acid was polymerized by the N-carboxy anhydride method in ethyl thioglycolate containing an active sulfhydryl group, and it was confirmed by the studies of infrared spectra of these substances that the resultant polymer was polycysteine contaminated with diketopiperazine. Pure polycysteine was isolated by treating this polymer with thioglycolic acid and methanol. The benzoyl derivative and ethylester hydrochloride of S-thiophenyl-L-cysteine were prepared.

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