ml. of water. Care was taken to keep the *p*H of the mixture above 8 by the addition of more magnesium oxide when necessary. The reaction mixture was shaken for another 20 minutes, diluted with 100 ml. of water and acidified with dilute HCl to *p*H 2. The product was separated by filtration, washed with water and dried. After trituration with ethyl acetate, 9.8 g. was obtained. Purification by precipitation from dilute $KHCO_3$ with HCl gave 8.6 g. (70%); m.p. 193–195°, literature[7] 193–195°.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine.—A solution of 1 g. (0.002 mole) of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosine[9] in 6 ml. of purified tetrahydrofuran and 0.2 g. (0.002 mole) of triethylamine was cooled to −10°, and 0.28 g. (0.002 mole) of isobutyl chlorocarbonate was added with stirring. After 8 minutes at this temperature a cooled solution of 0.85 g. (5% excess) of L-phenylalanyl-L-glutaminyl-L-asparagine[7] and 0.22 g. of triethylamine in 5 ml. of water was added. The mixture was then allowed to come to room temperature over a period of 20 minutes and the triethylamine salt of the product was precipitated with ether. The precipitate was filtered off, washed with ether, suspended in 100 ml. of water and acidified with HCl. The product was separated by filtration, washed with water, dried and triturated with ethyl acetate; wt. 1.1 g. (64%); m.p. 207–209°. The pentapeptide crystallized from 80% aqueous tetrahydrofuran in the form of needles, m.p. 214°, $[\alpha]^{24}D$ −29° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{45}H_{51}O_{11}N_7S$: C, 60.2; H, 5.72; N, 10.9. Found: C, 60.2; H, 5.99; N, 10.6.

O,N-Dicarbobenzoxy-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine. A. Mixed Anhydride Method.—A solution of 1.35 g. (0.003 mole) of O,N-dicarbobenzoxy-L-tyrosine[11] and 0.30 g. (0.003 mole) of triethylamine in 10 ml. of purified tetrahydrofuran was cooled to −10° and 0.41 g. (0.003 mole) of isobutyl chlorocarbonate was added with stirring. After 10 minutes at this temperature a cooled solution of 1.28 g. (5% excess) of L-phenylalanyl-L-glutaminyl-L-asparagine and 0.32 g. of triethylamine in 7 ml. of water was added. The reaction mixture was allowed to come to room temperature over a 20-minute period, and the triethylamine salt of the tetrapeptide was precipitated with ether as a heavy oil, which solidified after standing in the refrigerator for a short time. The product was then filtered off, washed with ether, suspended in 150 ml. of water and acidified with HCl. The tetrapeptide thus obtained was separated by filtration, washed with water, dried, triturated with ethyl acetate and purified by precipitation from 50% aqueous formic acid; wt. 1.78 g. (70.8%); m.p. 219–220°; $[\alpha]^{21}D$ −21° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{43}H_{46}N_6O_{12}$: C, 61.6; H, 5.52; N, 10.0. Found: C, 61.5; H, 5.50; N, 9.50.

B. Acid Chloride Method.—A solution of 0.56 g. (0.0012 mole) of O,N-dicarbobenzoxy-L-tyrosyl chloride[11] in 4 ml. of purified dioxane was added in portions with shaking over a period of 20 minutes to an ice-cold suspension of 0.5 g. (0.0012 mole) of L-phenylalanyl-L-glutaminyl-L-asparagine and 0.1 g. of magnesium oxide in 6 ml. of water. Care was taken to keep the *p*H of the mixture above 8 by the addition of more magnesium oxide when necessary. After all of the acid chloride had been added, 10 ml. of water was added and the mixture was acidified with HCl. The product was filtered off, washed with water, dried and triturated with ethyl acetate; wt. 0.31 g. (30%); m.p. 219–220°.

L-Tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine Hydrobromide.—O,N-Dicarbobenzoxy-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine (1 g.) was suspended in 20 ml. of 2 *N* hydrogen bromide in glacial acetic acid, and the mixture was warmed for 15 minutes at 65° to give a homogeneous solution. After 10 minutes at room temperature the product was precipitated with ether as a white solid which was filtered off, washed several times with ether and purified by reprecipitation from ethanol–ether; wt. 0.695 g. (90%); m.p. indefinite, $[\alpha]^{21}D$ +6.3° (*c* 1, H_2O).

Anal. Calcd. for $C_{27}H_{35}O_8N_6Br$: N, 12.9; Br, 12.3. Found: N, 12.6; Br, 11.9.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine.—A solution of 360 mg. (1.04 mmoles) of S-benzyl-N-carbobenzoxy-L-cysteine[13] and 105 mg. (1.04 mmoles) of triethylamine in 4 ml. of purified tetrahydrofuran was cooled to −10° and 143 mg. (1.04 mmoles) of isobutyl chlorocarbonate added with stirring. After 10 minutes at this temperature a cooled solution of 677 mg. (1.04 mmoles) of L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine hydrobromide and 210 mg. (2.08 mmoles) of triethylamine in 3 ml. of water was added. The reaction mixture was allowed to come to room temperature over a period of 20 minutes and then the triethylamine salt of the product was precipitated with ether. The precipitate was filtered off, washed with ether, suspended in 75 ml. of water and acidified with HCl. The product was separated by filtration, washed with water, dried and triturated with ethyl acetate; wt. 0.8 g. (86.9%); m.p. 205–207°. The pentapeptide crystallized from 80% aqueous tetrahydrofuran, as needles, m.p. 214°, $[\alpha]^{24}D$ −29° (*c* 1, dimethylformamide).

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, WEIZMANN INSTITUTE OF SCIENCE]

Poly-L-cysteine

BY ARIEH BERGER, JUNZO NOGUCHI[1] AND EPHRAIM KATCHALSKI

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S-Carbobenzoxy-N-carboxy-L-cysteine anhydride (III) was prepared from N,S-dicarbobenzoxy-L-cysteine (I) or from S-carbobenzoxy-L-cysteine (II). Poly-S-carbobenzoxy-L-cysteine (IV), obtained by the polymerization of III, yielded on reduction with sodium in liquid ammonia poly-L-cysteine (V). The reactivity of the thiol groups of V toward various SH-reagents was investigated. V yielded poly-S-carboxymethylcysteine (VI) on treatment with iodoacetic acid. VI was also obtained by the polymerization of S-carbomethoxymethyl-N-carboxycysteine anhydride (XI), followed by de-esterification. Poly-L-cysteic acid (VIII) was derived from IV by oxidation with performic acid.

The sulfhydryl groups of proteins have been extensively investigated, because of their importance in biological processes, their high chemical reactivity and the ease with which they may be detected and estimated. Native proteins have been found to contain thiol groups of three grades of reactivity[2]: (a) freely reacting –SH groups, reacting readily with nitroprusside and with mild oxidizing agents, (b) sluggish –SH groups, which do not give the nitroprusside reaction but may react with iodine and mercaptide forming compounds, and (c) masked –SH groups which can be detected only after the denaturation of the native protein.

Because of the complexity of the protein molecule, however, this different reactivity of the various types of sulfhydryl groups has as yet not been satisfactorily explained. The study of the chemical behavior of the –SH groups of polypeptides containing cysteine residues may shed new light on this prob-

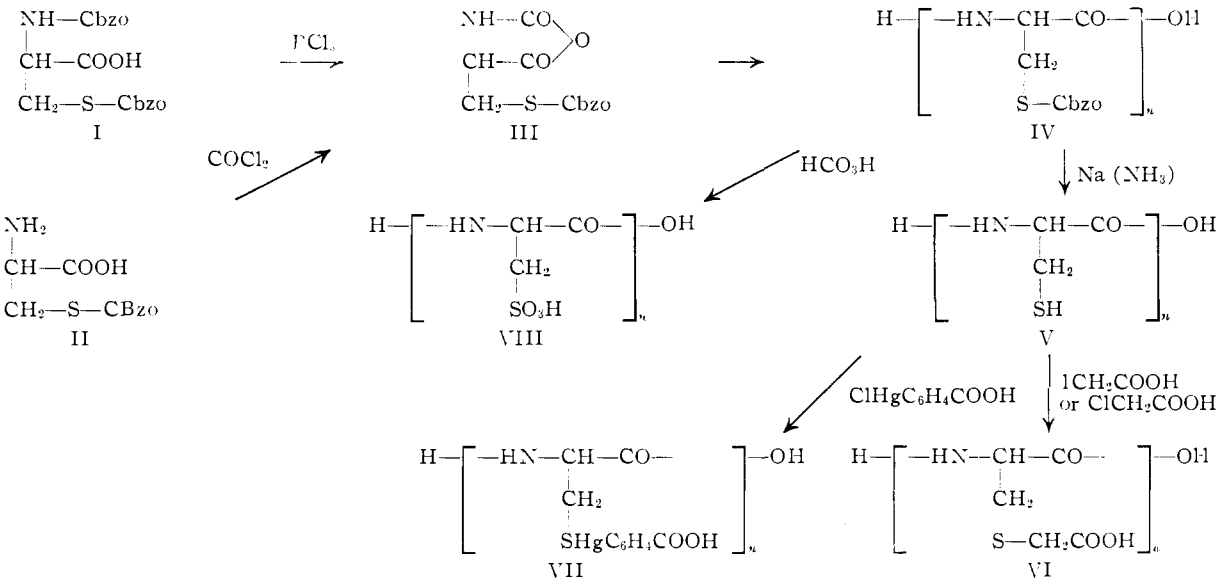
(1) Weizmann Fellow, 1955–1956; on leave of absence from Kanazawa University, Kanazawa, Japan.

(2) E. S. G. Barron in "Advances in Enzymology," Vol. 11, Interscience Publishers, New York, N. Y., 1951, p. 219.

lem. As a first step in this direction polycysteine was synthesized and its properties investigated. A preliminary report on this work has been published.[3]

In view of the successful use of N-carboxy-α-amino acid anhydrides as monomers in the synthesis of polyamino acids the synthesis of a suitable N-carboxy-L-cysteine anhydride derivative was undertaken. Because of the high reactivity of the thiol group its reversible masking was required.

In preliminary experiments S-benzyl-N-carboxy-L-cysteine anhydride[3,4] was chosen as monomer. However, the benzyl groups of the poly-S-benzyl-L-cysteine obtained could not be removed by means of sodium in liquid ammonia[5] or phosphonium iodide in glacial acetic acid.[6] The successful synthesis of poly-L-cysteine (V) was finally accomplished using S-carbobenzoxy compounds as intermediates.[7] The route of synthesis of V and some of its derivatives is given in the following scheme



Cbzo = $-OCOCH_2C_6H_5$

L-Cysteine was coupled with benzyl chloroformate in aqueous alkali to yield N,S-dicarbobenzoxy-L-cystine (I) which gave, on treatment with phosphorus pentachloride, S-carbobenzoxy-N-carboxy-L-cysteine anhydride (III). The anhydride III can also be prepared by the action of phosgene on S-carbobenzoxy-L-cysteine (II). The latter was obtained on coupling benzyl chloroformate with a slight excess of cysteine in aqueous solution in the presence of sodium bicarbonate. III was polymerized in solution and the carbobenzoxy groups of the poly-S-carbobenzoxy-L-cysteine (IV) obtained were removed by treatment with sodium in liquid ammonia. Poly-L-cysteine (V) was precipitated on acidification of the aqueous solution of its sodium salt left after the evaporation of the ammonia from the reduction mixture.

The retention of the steric configuration in N,S-dicarbobenzoxycysteine derived from L-cysteine was ascertained by the almost quantitative recovery of optically pure L-cystine after decarbobenzoxylation followed by oxidation of the resulting cysteine by means of hydrogen peroxide. The S-carbobenzoxy group of N,S-dicarbobenzoxycysteine was found to be relatively stable toward dilute aqueous alkali but it is removed easily in aqueous ammonia to give benzyl carbamate with the liberation of the theoretical amount of thiol groups.

The S-carbobenzoxy groups of poly-S-carbobenzoxy-L-cysteine are much less reactive toward ammonia than those of I. No free –SH groups were formed on treatment of IV with concentrated aqueous ammonia for several days, neither were –SH groups liberated by the treatment of a pyridine solution of IV with diethylamine at 80°. IV can be decarbobenzoxylated by phosphonium iodide in dichloroacetic acid solution; in this case, however, considerable loss of sulfur occurs due to hydrogen sulfide evolution. None of the poly-S-carbobenzoxy-L-cysteine preparations obtained contained free amino groups. This might be due to an intramolecular termination reaction, similar to the one observed in the case of polyglutamic acid,[8] leading to the formation of a terminal thiazolidone ring. On treatment of IV with sodium in liquid ammonia, terminal amino groups appear. Degrees of polymerization in the range of 18 to 38 were found for V by amino-end group analysis.

By the oxidation of IV with performic acid in formic acid practically all the S-carbobenzoxy groups of the polymer were oxidized to yield poly-L-cysteic acid (VIII). VIII which is a strong polyacid, dissolves readily in water and alcohol. It is not precipitated from its aqueous solution by bar-

(3) E. Katchalski and A. Berger, *Bull. Research Council of Israel*, **2**, 314 (1952).

(4) E. R. Blakley, A. K. Sumner and E. Y. Spencer, *Canad. J. Technol.*, **30**, 258 (1952).

(5) J. L. Wood and V. du Vigneaud, *J. Biol. Chem.*, **130**, 110 (1939).

(6) C. R. Harington and T. H. Mead, *Biochem. J.*, **30**, 1594 (1936).

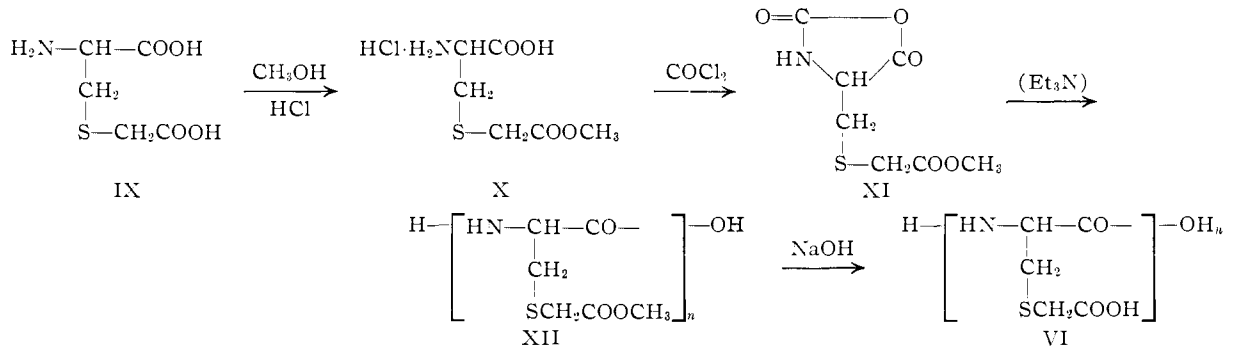
(7) S. Sakakibara and H. Tani (Osaka University, Japan) communicated recently to us their successful synthesis is of polycysteine by an independent route. N-Carboxy-S-thiophenyl-L-cysteine anhydride was used as starting monomer and the simultaneous polymerization and reduction was carried out in ethyl thioglycolate.

(8) W. E. Hanby, S. G. Waley and J. Watson, *J. Chem. Soc.*, 3239 (1950).

ium, silver, lead or cupric ions. A polymeric water-insoluble salt was obtained by the addition of polylysine to an aqueous solution of VIII at neutral *p*H. Polycysteic acid yields on acid hydrolysis cysteic acid identical with an authentic sample. A chromatogram of the hydrolysate gave, with ninhydrin, one spot corresponding to that of cysteic acid.

Polycysteine is soluble in ethanolamine and in water at *p*H values higher than *p*H 8 and its alkaline solutions give a deep violet color with nitroprusside. On acid hydrolysis the total nitrogen of V is transformed into amino nitrogen (Van Slyke). The amount of sulfhydryl groups of polycysteine, as determined from the amount of iodide liberated on reaction with iodoacetic acid, corresponded to the theoretical.

From the reaction mixture of polycysteine and iodoacetic acid poly-S-carboxymethylcysteine was isolated and identified by its neutralization equivalent weight. The same product was obtained directly by the reaction of chloroacetic acid with the mixture derived from poly-S-carbobenzoxycysteine by reduction with sodium in liquid ammonia. For comparison, poly-S-carboxymethylcysteine was also prepared by an independent route according to the scheme



S-Carboxymethylcysteine (IX)[9] was esterified to yield S-carbomethoxymethylcysteine (X). The latter, on treatment with phosgene in dioxane solution, gave S-carbomethoxymethyl-N-carboxycysteine anhydride (XI). On polymerization in dioxane using triethylamine as initiator, XI gave the polyester XII, which yielded the required poly-S-carboxymethylcysteine (VI) on saponification.

Both the poly-S-carboxymethylcysteine derived from S-carboxymethylcysteine and that derived from polycysteine yielded S-carboxymethylcysteine quantitatively on acid hydrolysis. Both polymers showed the same infrared absorption bands and similar potentiometric titration curves.

On titration with iodine, polycysteine consumed only 85% of the theoretical amount. This is probably due to the fact that the three-dimensional network formed through the extensive cross-linking by S–S bonds still contains masked –SH groups.

When polycysteine was titrated with standard methyl mercuric nitrate or with *p*-chloromercuric benzoate in the presence of nitroprusside as internal indicator, the color of the indicator faded after the addition of only 46 and 68% of the theoretical, respectively. Addition of guanidine hydrobromide (5 mole/l.) had no effect on the titration values. The low value obtained in the case of mercuric methyl nitrate might be due, at least partially, to the formation of an insoluble reaction product. In the case of *p*-chloromercuric benzoate, however, a quantitative reaction between this reagent and V could be demonstrated by the isolation of VII from a reaction mixture containing polycysteine and a slight excess of *p*-chloromercuric benzoate. It seems therefore that the free –SH groups left after the interaction of approximately two thirds of the thiol groups, are incapable of reacting with nitroprusside, although they can still react with the organic mercury compound which is known to combine even with the "sluggish –SH groups" of proteins.

Polycysteine is readily oxidized by hydrogen peroxide, or by air, in the presence of cupric ions. The oxidation product precipitates from the alkaline aqueous solution and is insoluble in the usual organic solvents. It consists probably of a three-dimensional network similar to the one obtained on oxidation of polycysteine with iodine. Its elementary composition corresponds to that of polycystine. The oxidized product is resistant to cyanide and no soluble products are formed after treatment with this reagent even after several days.

A preliminary investigation was carried out in order to examine the possibility of oxidative cross-linking of amino acid copolymers containing cysteine residues. A lysine–cysteine copolymer was prepared by bulk polymerization of a mixture of ε,N-carbobenzoxy-α,N-carboxylysine anhydride[10] and S-carbobenzoxy-N-carboxycysteine anhydride (9:1 mole/mole) and decarbobenzoxylation of the product obtained by means of phosphonium iodide in glacial acetic acid. When the water-soluble copolymer was oxidized with dilute hydrogen peroxide, a highly viscous solution was obtained, indicating the formation of high molecular weight molecules due to S–S cross-linking. The high viscosity disappeared on the addition of an excess of cysteine.

Experimental

All melting points are uncorrected.

Poly-S-benzyl-L-cysteine was prepared by bulk polymerization of S-benzyl-N-carboxy-L-cysteine anhydride (m.p. 138°)[4] at 140° *in vacuo* (10^{-3} mm.); soluble in dichloroacetic acid, insoluble in dimethylformamide and glacial acetic acid.

(9) S. A. Harris, N. R. Easton, D. Heyl, A. N. Wilson and K Folkers, THIS JOURNAL, **66**, 1757 (1944).

(10) M. Bergmann, L. Zervas and W. F. Ross, *J. Biol. Chem.*, **111**, 245 (1935).

Anal. Calcd. for $(C_{10}H_{11}NOS)_n$: C, 62.1; H, 5.7; N, 7.3; S, 16.6. Found: C, 61.7; H, 5.8; N, 7.3; S, 15.7.

Attempted Debenzylation.—Phosphonium iodide (1.0 g.) was added in four portions during five hours to a solution of poly-S-benzyl-L-cysteine (0.5 g.) in dichloroacetic acid (5.0 ml.). A stream of dry hydrogen was passed through the reaction mixture kept at 50°. Finally ether (20 ml.) was added to the clear solution and the precipitate obtained was filtered and washed with water, alcohol and ether. The product left was insoluble in aqueous alkali and gave no color with nitroprusside. Calcd. for polycysteine: N, 13.6. Found: N, 7.2.

A suspension of poly-S-benzylcysteine (0.5 g.) in liquid ammonia (20 ml.) was treated with metallic sodium (0.1 g.). The excess of sodium was destroyed by the addition of ammonium chloride. The residue left after the evaporation of the ammonia was water-insoluble and gave no color with nitroprusside.

N,S-Dicarbobenzoxy-L-cysteine (I).—To a solution of L-cysteine hydrochloride (12.0 g.) in 2 *N* sodium hydroxide (115 ml.) were added simultaneously benzyl chloroformate (27 ml.) and 2 *N* sodium hydroxide (43 ml.) during 30 minutes. The reaction mixture was ice-cooled, stirred mechanically and its *p*H kept between 9 and 10. The sodium salt of the dicarbobenzoxycystine which settled out as a heavy oil was separated, washed with ether and acidified with hydrochloric acid. The dicarbobenzoxycysteine was extracted with ethyl acetate (200 ml.) and the organic layer washed with water, dried over sodium sulfate and concentrated *in vacuo*. The residue solidified on trituration with petroleum ether. The crude product (30 g., m.p. 92–95°) was recrystallized from carbon tetrachloride (100 ml.); yield 21 g. (71%); m.p. 97–98°, $[\alpha]^{20}D$ −32.4° (*c* 8.5, in glacial acetic acid).

Anal. Calcd. for $C_{19}H_{19}NO_6S$: C, 58.6; H, 4.9; N, 3.6; S, 8.2; neut. equiv., 389. Found: C, 58.7; H, 5.2; N, 3.6; S, 8.0; neut. equiv., 396.

Dicarbobenzoxy-L-cysteine is soluble in ethyl acetate, glacial acetic acid, alcohol and acetone. It is sparingly soluble in cold carbon tetrachloride and benzene and is insoluble in water and petroleum ether.

L-Cystine from I.—N,S-Dicarbobenzoxy-L-cysteine (4.0 g.) was treated with a solution of hydrogen bromide (30%) in glacial acetic acid (10 ml.). When gas evolution had ceased (10 min.) anhydrous ether (50 ml.) was added and the precipitate formed was washed thoroughly with ether. The dried substance was dissolved in water (5 ml.) and ammonia was added to *p*H 8.5. On the addition of 3% hydrogen peroxide (5 ml.), cystine (1.30 g.) separated. It was identified by paper chromatography and nitrogen analysis; $[\alpha]^{20}D$ +208° (*c* 1.0, in 1 *N* hydrochloric acid). An authentic sample of cystine prepared by the oxidation of L-cysteine hydrochloride as described above, had $[\alpha]^{20}D$ +206° (*c* 1.0, in 1 *N* hydrochloric acid).

Ammonolysis of I.—N,S-Dicarbobenzoxy-L-cysteine (1.0 g.) was dissolved in concentrated aqueous ammonia and the solution left at room temperature for one hour. The crystals of benzyl carbamate formed (m.p. 86°, 0.30 g., 85% of the theoretical) were collected and the filtrate was found to contain 2.3-milliequivalents sulfhydryl (90% of the theoretical) as determined by titration with methyl mercuric nitrate.

S-Carbobenzoxy-L-cysteine (II).—To an ice-cooled solution of L-cysteine hydrochloride (8.8 g., 0.056 mole) in 1 *N* aqueous sodium bicarbonate (100 ml.), covered with ether (50 ml.) was added benzyl chloroformate (8.6 g., 0.05 mole) in one portion with vigorous stirring. After one hour at 0° the temperature was allowed to rise to 10° and maintained for one more hour. The crystalline mass was transferred to a buchner funnel, washed with water, sucked as dry as possible, washed with acetone and ether, and dried *in vacuo;* yield 9.5 g. (67%), m.p. 177°, unchanged on recrystallization from aqueous acetic acid (1:1 v./v.), $[\alpha]^{20}D$ −50.0° (*c* 1.0, in glacial acetic acid).

Anal. Calcd. for $C_{11}H_{13}NO_4S$: C, 51.7; H, 5.1; N, 5.5; S, 12.6; neut. equiv., 255. Found: C, 51.8; H, 5.1; N, 5.4; S, 12.5; neut. equiv., 253 (determined by anhydrous titration with 0.1 *N* perchloric acid in glacial acetic acid).

S-Carbobenzoxy-N-carboxy-L-cysteine Anhydride (III).

(a) From I.—To an ice-cooled solution of I (10.0 g.) in benzene (70 ml.), phosphorus pentachloride (6.0 g.) was added and the mixture was shaken for ten minutes. Unreacted phosphorus pentachloride was removed and the clear solution was heated to 50° for five minutes. The anhydride formed was precipitated with petroleum ether, dissolved in ethyl acetate (20 ml.), reprecipitated, washed as above and dried; yield 6.5 g. (90%), m.p. 75° dec.

Anal. Calcd. for $C_{12}H_{11}NO_5S$: C, 51.2; H, 3.9; N, 5.0; S, 11.4; CO_2, 15.6. Found: C, 51.5; H, 3.9; N, 4.9; S, 10.9; CO_2, 15.4 (loss of weight on heating to 110° for 10 hours).

(b) From II.—Through a suspension of S-carbobenzoxy-L-cysteine (II) (10 g.) in dry dioxane (200 ml.), phosgene was passed at 40–50° for 30 minutes. Carbon dioxide was then passed through the resulting solution, which was filtered, if necessary, and concentrated *in vacuo* at 50°. The sirupy residue crystallized on trituration with petroleum ether; m.p. 76–77° dec., unchanged on recrystallization from ethyl acetate–petroleum ether; yield after recrystallization, 9.3 g. (84%).

Anal. Calcd. for $C_{12}H_{11}NO_5S$: C, 51.2; H, 3.9; N, 5.0; S, 11.4. Found: C, 50.7; H, 3.8; N, 5.0; S, 11.5.

Poly-S-carbobenzoxy-L-cysteine (IV).—To a solution of III (4.0 g.) in dry benzene (100 ml.) was added diethylamine (0.5 ml., of a 1% w./v. solution in benzene) and the mixture kept at 50° for three days. Moisture was excluded by means of a calcium chloride tube. After three days, petroleum ether (400 ml.) was added and the polymer was collected, washed with petroleum ether and dried; yield quantitative (3.3 g.).

Anal. Calcd. for $C_{11}H_{11}NO_3S$: C, 55.6; H, 4.7; N, 5.9; S, 13.5. Found: C, 55.3; H, 4.5; N, 6.0; S, 13.1; amino N, 0.0 (Van Slyke, or titration with perchloric acid in anhydrous dioxane).

IV is soluble in dichloroacetic acid, trifluoroacetic acid, dioxane and pyridine. It is insoluble in the usual organic solvents.

The anhydride III (5 g.) was also polymerized in dioxane (100 ml.) for three days at room temperature using triethylamine (0.07 ml.) as catalyst. The polymerization was brought to completion by heating to 100° for two hours, when a clear solution was obtained. The polymer was precipitated with water (500 ml.) and washed thoroughly; yield 2.5 g. It contained no free amino groups. The titration of the terminal carboxyl groups with sodium methoxide gave erratic results, probably as a result of the reaction of the titrant with the S-carbobenzoxy groups of IV.

Attempted Ammonolysis of IV.—(a) Poly-S-carbobenzoxy-L-cysteine (IV) was suspended in concentrated aqueous ammonia and left at room temperature for two days. (b) IV was kept in a sealed tube, covered with liquid ammonia, for two days at room temperature. (c) To a solution of IV (5 mg.) in pyridine (3 ml.) diethylamine (1 ml.) was added and the mixture heated to 80° for four hours. On addition of water (10 ml.) the polymer separated out.

Free thiol groups could not be detected in any of the above experiments. The starting material was recovered in all three cases without significant change in elementary composition.

Poly-L-cysteine (V).—To a suspension of IV (0.56 g.) in liquid ammonia (50 ml.), finely cut sodium (or preferably sodium sand) (0.3 g.) was added in small portions with magnetic stirring. Each portion was added when the blue color caused by the previous one had faded. After the addition of the last portion, the excess sodium was destroyed by addition of a few crystals of ammonium chloride. The ammonia was allowed to evaporate and the residue was dissolved in air-free water. The aqueous solution was washed with ether and acidified with a few drops of concentrated hydrochloric acid. The poly-L-cysteine which separated was collected on a glass filter under hydrogen pressure, washed with air-free water, alcohol and peroxide-free ether and dried in a stream of hydrogen; yield 0.15 g. (62%).

Anal. Calcd. for $(C_3H_5NOS)_n$: C, 34.9; H, 4.9; N, 13.6; S, 31.1; thiol-S, 31.1. Found: C, 34.6; H, 5.2; N, 13.2 (micro Dumas with the addition of cupric acetate to the sample); S, 31.3 (determined gravimetrically as described below); thiol-S, 31.2 (determined with iodoacetic acid as described below). Amino-N for different samples, 0.36 to 0.76%, corresponding to degrees of polymerization $n = 38$ to 18.

Poly-L-cysteine is soluble in aqueous alkali and is pre-

cipitated on the addition of acid to *p*H 8, or by saturation with carbon dioxide. It is soluble in ethanolamine and dichloroacetic acid. In the latter, however, some decomposition accompanied by hydrogen sulfide evolution occurs. It is insoluble in alcohol, ether, dioxane, glacial acetic acid and dimethylformamide.

Sulfur Determination in Poly-L-cysteine.—The Carius method applied to polycysteine gave low and irreproducible results. Reproducible values were obtained by the following procedure. A sodium plumbite solution was prepared by the addition of 4 *N* sodium hydroxide to lead acetate (10% in water) until a clear solution was obtained. Samples of 15 to 25 mg. of polycysteine were digested with 5 ml. of the plumbite solution on a steam-bath for two hours. Water (50 ml.) was added and the lead sulfide was collected on an ash free-filter paper and washed with hot water until the washings were neutral. The precipitate together with the filter paper was transferred to a porcelain crucible, incinerated and the residue treated first with a few drops of concentrated nitric acid and then heated repeatedly with a few drops of concentrated sulfuric acid until white. The last traces of sulfuric acid were removed by ignition for five minutes and the lead sulfate was weighed after cooling in the desiccator. The method was found suitable for the determination of sulfur in cystine, polycarbobenzoxycysteine and several other cysteine derivatives.

Estimation of -SH Groups in V. (a) With Iodoacetic Acid.—To a solution of polycysteine (2.34 mg.) in 0.1 *N* sodium hydroxide (0.3 ml.) was added a solution of iodoacetic acid (8.6 mg.) in 0.1 *N* sodium hydroxide (1.0 ml.) and the reaction mixture was left at room temperature for 15 minutes. An excess of 0.01 *N* silver nitrate (3.0 ml.) was added and the silver iodide formed was removed by centrifugation and washed twice with 1-ml. portions of water. In the combined solution and washings, the excess silver was back-titrated with 0.01 *N* thiocyanate after acidification with nitric acid. The 2.34 mg. of polycysteine was found equivalent to 2.28 ml. of 0.01 *N* silver nitrate. Hence thiol-S = 31.2%. The same value was obtained when polycysteine and iodoacetic acid were left to react for 30 minutes. No silver nitrate was consumed in blank experiments where the polycysteine was omitted.

From a reaction mixture containing larger quantities of the reactants, poly-S-carboxymethylcysteine (VI) was isolated as follows: poly-L-cysteine (100 mg.) and iodoacetic acid (560 mg.) were dissolved in 0.5 *N* sodium hydroxide (50 ml.) and the solution adjusted to *p*H 9.0. After 20 minutes at room temperature, it was acidified with hydrochloric acid and the precipitate was centrifuged, washed with water and acetone and dried.

Anal. Calcd. for $(C_6H_7NO_3S)_n$: neut. equiv., 169. Found: neut. equiv., 167.

(b) With Iodine.—Polycysteine (2.66 mg.) was suspended in 80% acetic acid (2 ml.) and an excess of 0.01 *N* iodine in 80% acetic acid was added. The mixture was kept in a stoppered vessel for two hours in the dark, diluted with 100 ml. of water containing a few milligrams of potassium iodide and the excess of iodide titrated with standard 0.01 *N* thiosulfate. 2.66 mg. of polycysteine reacted with 2.20 ml. of 0.01 *N* iodine. Hence thiol-S = 26.5%.

(c) With *p*-Chloromercuric Benzoate.—Polycysteine (3.17 mg.) was dissolved in 0.1 *N* bicarbonate buffer, *p*H 8.5 (5 ml.). A few drops of a 1% sodium ethylenediamine tetraacetate solution were added and a 5% sodium nitroprusside was used as indicator. The solution was titrated at 0° with 0.01 *N* sodium *p*-chloromercuric benzoate solution (2.08 ml.) to the disappearance of the violet color. Hence thiol-S = 21.0%. The addition of guanidine hydrobromide (5 mole/l.) did not affect the amount of titrant consumed.

From a reaction mixture containing equimolar amounts of the reactants, the reaction product VII was isolated as follows.

To a solution of poly-L-cysteine (50 ml.) in 0.5 *N* aqueous ammonia (5 ml.) was added 0.1 *N* sodium *p*-chloromercuric benzoate (5 ml.). The solution was acidified with hydrochloric acid and the precipitate was collected, washed with water, alcohol and ether and redissolved in 0.1 *N* potassium bicarbonate (10 ml.). The filtered solution was acidified with hydrochloric acid and the precipitate was collected, washed as above and dried; yield 180 mg. (88%).

Anal. Calcd. for $(C_{10}H_9NO_3SHg)_n$: C, 28.3; H, 2.1; N, 3.3; neut. equiv., 424. Found: C, 28.2; H, 2.2; N, 3.1; neut. equiv., 427.

(d) With Methylmercuric Nitrate.—The titration was performed as in (c), 0.01 *N* methylmercuric nitrate being used as titrant. The methylmercury derivative of polycysteine precipitated from the reaction mixture during the titration. 4.14 mg. of polycysteine consumed 1.86 ml. of titrant. Hence thiol-S = 14.4%.

The titrants used in (c) and (d) were standardized against cysteine hydrochloride.

Polycystine.—Through a solution of poly-L-cysteine in 0.5 *N* aqueous ammonia, containing traces of copper sulfate, oxygen was bubbled for four hours. The precipitate formed was centrifuged, washed with water, alcohol and ether and dried. Polycystine is insoluble in the usual organic solvents and aqueous sodium hydroxide.

Anal. Calcd. for $(C_3H_4NOS)_n$: C, 35.3; H, 3.9. Found: C, 35.6; H, 4.1.

S-Carboxymethyl-L-cysteine (IX) was prepared from L-cysteine and chloroacetic acid.[9] From the potentiometric titration at an ionic strength of 0.1, the three acidic dissociation constants, $pK_1 = 2.54$, $pK_2 = 3.03$ and $pK_3 = 9.32$ were calculated by the method given by Simms.[11] These three dissociation constants may be assigned to the α-carboxyl, the S-carboxymethyl and the ammonium group, respectively.

S-Carbomethoxymethylcysteine Hydrochloride (X).—A stream of dry hydrogen chloride was passed through a suspension of S-carboxymethylcysteine[9] (3 g.) in anhydrous methanol (30 ml.). When nearly all the starting material had disappeared, the solution was filtered and evaporated to dryness *in vacuo* below 35°. The crystalline residue was washed with acetone and ether; yield 3.0 g. (78%). The m.p. 142°, did not change on recrystallization from methanol–ether. An additional quantity of material (0.8, 21%, m.p. 142°) was recovered from the washings by evaporation to dryness and treatment of the residue with a small volume of acetone and ether as above.

Anal. Calcd. for $C_6H_{12}NO_4SCl$: C, 31.4; H, 5.3; N, 6.1; CH_3O, 13.5; neut. equiv., 114.8. Found: C, 31.3; H, 5.4; N, 6.1; CH_3O, 13.5; neut. equiv., 116 (determined by titration in dimethylformamide with 0.1 *N* sodium methoxide in benzene–methanol, thymol blue being used as indicator).

S-Carbomethoxymethyl-N-carboxycysteine Anhydride (XI).—A stream of dry phosgene was passed through a suspension of S-carbomethoxymethylcysteine hydrochloride (3 g.) in dioxane (60 ml.) at 40° for one hour with magnetic stirring. Through the resulting clear solution, dry carbon dioxide was passed for ten minutes. On concentration *in vacuo* at 40° a sirupy residue was obtained which solidified on repeated trituration with petroleum ether. The solid was collected, dried and recrystallized from ethyl acetate–petroleum ether; yield 2.5 g. (87%) m.p. 74°.

Anal. Calcd. for $C_7H_9NO_5S$: C, 38.3; H, 4.1; N, 6.4; CH_3O, 14.2; neut. equiv., 219. Found: C, 38.3; H, 4.4; N, 6.4; CH_3O, 14.2; neut. equiv., 216.[12]

Poly-S-carbomethoxymethylcysteine (XII).—To a solution of XI (2.0 g.) in dioxane (40 ml.) was added triethylamine (0.04 ml.). The mixture was stirred magnetically for two days at room temperature and finally refluxed for two hours. Moisture was rigorously excluded. The resulting polymer was precipitated with petroleum ether, centrifuged, washed with alcohol and ether and dried; yield 1.3 g. (81%). The average degree of polymerization ($n = 35$) was determined by end-group analysis.[13]

Anal. Calcd. for $(C_6H_9NO_3S)_n$: C, 41.1; H, 5.2; N, 8.0; S, 18.3. Found: C, 40.9; H, 5.3; N, 7.9; S, 18.5.

Poly-S-carboxymethylcysteine (VI). (a) From XII.—Finely powdered poly-S-carbomethoxymethylcysteine (X11) (0.3 g.) was suspended in 0.1 *N* aqueous sodium hydroxide (20 ml.) and the suspension was kept for two days at room temperature. The resulting clear solution was acidified with concentrated hydrochloric acid and the precipitate was centrifuged, washed with dilute hydrochloric acid, alcohol and ether and dried *in vacuo;* yield 0.14 g. (52%).

(11) H. S. Simms, THIS JOURNAL, **48**, 1239 (1926).

(12) A. Berger, J. Sela and E. Katchalski, *Anal. Chem.*, **25**, 1554 (1953).

(13) M. Sela and A. Berger, THIS JOURNAL, **77**, 1893 (1955).

Anal. Calcd. for $(C_5H_7NO_3S)_n$: C, 37.0; H, 4.4; N, 8.6; neut. equiv., 162. Found: C, 37.0; H, 4.5; N, 8.8; neut. equiv., 165.

For potentiometric titration, the polymer (13.28 mg.) was dissolved in 0.1 *N* sodium hydroxide (2.76 ml.), the solution made up to 25 ml. with 0.1 *N* sodium chloride, and back-titrated with 0.1 *N* hydrochloric acid. The titration curve obtained fitted closely a theoretical curve constructed with the aid of eq. 1, used to describe the titration of proteins[14] and poly-α-amino acids[15]

$$pH = pK_0 - \log \frac{1 - \alpha}{\alpha} + 0.868\, n\alpha w \qquad (1)$$

An intrinsic ionization constant $pK_0 = 3.03$ (the pK_2 of S-carboxymethylcysteine), a number of ionizable carboxyl groups $n = 35$ (corresponding to the average degree of polymerization of VI) and an electrostatic interaction factor $w = 0.064$, were assumed.

(b) From IV.—Poly-S-carbobenzoxycysteine (IV) (2.4 g.) was treated with sodium (3.0 g.) in liquid ammonia (50 ml.), as in the preparation of poly-L-cysteine. After the destruction of excess sodium, chloroacetic acid (3.0 g.) was added in small portions. The ammonia was left to evaporate, the residue was dissolved in water, and the aqueous solution was extracted with ether. The precipitate formed on the acidification of the aqueous layer was centrifuged, washed with alcohol and ether and dried *in vacuo;* yield 1.07 g. (65%).

Anal. Calcd. for $(C_5H_7NO_3S)_n$: C, 37.0; H, 4.4; N, 8.6; neut. equiv., 162. Found: C, 37.0; H, 4.2; N, 8.4; neut. equiv., 167.

The potentiometric titration curve of this polymer, obtained under the conditions described for the titration of poly-S-carboxymethylcysteine derived from XII, fitted closely the theoretical curve deduced from eq. 1, assuming the same intrinsic dissociation constant $pK_0 = 3.03$, $n = 18$ (corresponding to the average degree of polymerization of polycysteine derived from IV) and $w = 0.073$.

Quantitative Chromatographic Determination of S-Carboxymethylcysteine.—In preliminary experiments it was found that S-carboxymethylcysteine gives with ninhydrin a well-defined spot separated from cysteine on paper chromatograms developed with propanol–water–concentrated aqueous ammonia (100:50:1 v./v.) ($R_f = 0.35$).

Samples of 20, 40, 60, 80 and 100 μg. of S-carboxymethylcysteine were applied as a 1% solution in 6 *N* hydrochloric acid to Whatman No. 1 paper and developed with the propanol–ammonia mixture. The spots were detected, eluted and determined colorimetrically according to Conell, *et al.*[16] From the values obtained, a calibration curve was constructed. The relationship between optical density and amount of amino acid was found to be linear.

A solution of S-carboxymethylcysteine (0.5 g.) in 6 *N* hydrochloric acid (10 ml.) was kept in a sealed tube at 110° for 12 hours. When subjected to paper chromatography, the above procedure being used, only one spot with $R_f = 0.35$ appeared. Chromatographic analysis showed that the recovery of the starting material was quantitative.

By evaporation of the acid solution to dryness and washing of the residue with dilute hydrochloric acid, 0.48 g. of material (m.p. 197°) was recovered. Mixed m.p. with an authentic sample of S-carboxymethylcysteine (m.p. 196°) was 196°.

Hydrolysis of Poly-S-carboxymethylcysteine.—Poly-S-carboxymethylcysteine (prepared from IV, 10.0 mg.) was hydrolyzed with 6 *N* hydrochloric acid (1 ml.) in a sealed tube at 110° for 10 hours. The hydrolysate was analyzed chromatographically as above. Only one spot with an R_f corresponding to S-carboxymethylcysteine was observed. The amount of S-carboxymethylcysteine found in the hydrolysate was 99.0% of the theoretical. By the same method a recovery of 94.5% of S-carboxymethylcysteine was obtained from poly-S-carboxymethylcysteine prepared from XII.

Poly-L-cysteic Acid (VIII).—To a mixture of 90% formic acid (20 ml.) and 30% hydrogen peroxide (2 ml.) was added poly-S-carbobenzoxy-L-cysteine (IV) (0.95 g.). The suspension was stirred magnetically until a clear solution was obtained (50 min.). The solution was evaporated to dryness at 40° (1 mm.), the residues was redissolved in water (10 ml.) and the solution brought to dryness as above. A light brown powder was obtained on trituration with acetone. It was washed with ether by decantation and dried *in vacuo* at 100° over phosphorus pentoxide; yield quantitative.

Anal. Calcd. for $(C_3H_5NO_4S$: C, 23.8; H, 3.3; N, 9.3; neut. equiv., 151. Found: C, 23.5; H, 3.2; N, 9.3; neut. equiv., 146.

Poly-L-cysteic acid is very hygroscopic. It is soluble in water and alcohol, and is insoluble in acetone, ether and benzene.

A comparison of the amino N content of polycysteic acid and of polycysteine, both prepared from the same sample of poly-S-carbobenzoxycysteine, showed that practically no degradation occurred during oxidation.

Polycysteic acid (100 mg.) was hydrolyzed with 6 *N* hydrochloric acid in a sealed tube at 110° for 12 hours. The residue (115 mg.), left on evaporation of the solvent, gave on a paper chromatogram (developed with butanol–acetic acid) one spot with ninhydrin, corresponding to that of an authentic sample of L-cysteic acid.[17]

Anal. Calcd. for $C_3H_7NO_5S$: amino N, 8.3. Found: amino N, 8.5.

The specific rotation of the cysteic acid obtained was $[\alpha]^{20}D + 8.4°$ (*c* 5.0, in water). The authentic sample of L-cysteic acid gave under the same conditions $[\alpha]^{20}D + 8.5°$.

The ammonium salt of polycysteic acid was obtained by the evaporation of a solution of VIII in excess aqueous ammonia; dried *in vacuo* over sulfuric acid before analysis.

Anal. Calcd. for $(C_3H_8N_2O_4S)_n$: N, 16.7. Found: N, 16.4.

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