The sedimentation and viscosity data of these polymers (Table III) were fitted to the empirical equations

$$\frac{S^0_{20,w}}{(1 - \bar{v}\rho)} = kM^a \quad (1)$$

$$[\eta] = KM^b \quad (2)$$

where k,a and K,b are constants and M is the molecular weight. In Figs. 3 and 4 the data are plotted on a double logarithmic scale and a straight line drawn by the method of least squares. The points represent the homologous series of polypeptides 7, 8, 9, 10 and 12. Using the constants calculated from the slope and intercept of the lines, equations 1 and 2 become

$$\frac{S^0_{20,w}}{(1 - \bar{v}\rho)} = 0.046\ M^{0.47} \quad (3)$$

$$[\eta] = 0.0008\ M^{0.68} \quad (4)$$

The close similarity of hydrodynamic properties of the polymers, implied by their fitting these equations, gives a further indication that the helical content and molecular weight distribution of each of them are quite similar.

Thus, the polypeptides all have molecular weights in the range of the serum proteins and their residues are linked only by peptide bonds between the α-amino and α-carboxyl groups as in native proteins. Their properties thus recommend them as good protein models for the study of immunological phenomena.

[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE, PITTSBURGH, PENNSYLVANIA]

Insulin Peptides. I. Synthesis of Cysteine-Containing Peptides Related to the A-Chain of Sheep Insulin

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Several protected cysteine-containing peptides with amino acid sequences found in the intra-chain ring region of the A-chain of sheep insulin were synthesized. For the protection of the sulfhydryl functions of these peptides, the *p*-nitrobenzyl, carbobenzoxy and benzylthiomethyl groups which can be removed selectively were employed. Evidence is presented that the S-benzylthiomethyl-L-cysteine does not remain intact on treatment with HBr in acetic acid, contrary to a previous report.

In the structure of sheep insulin determined by Sanger and co-workers[2] the sequence cysteinyl-cysteinyl - alanyl - glycyl - valyl - cysteinyl - seryl-leucyl- is present. This peptide segment is located in the A-chain and is involved both in the inter- and intra-chain disulfide bridges of the insulin molecule (Fig. 1).

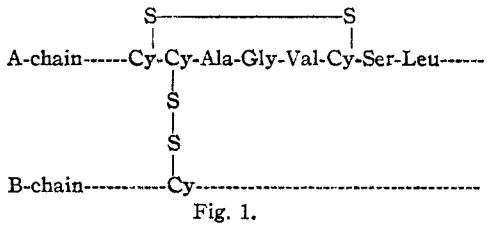

Fig. 1.

A program has been undertaken in this Laboratory directed toward the synthesis and biological evaluation of certain structural features of insulin. To this end certain peptides containing sequences found in the above mentioned peptide segment, with the sulfhydryl function of the cysteine residues protected with groups that can be removed selectively have been synthesized. This selectivity is of key importance if the synthesis of the portion of the insulin molecule containing the inter- and intra-chain disulfide bridges is desired.

For the protection of the sulfhydryl functions we have used the carbobenzoxy, the *p*-nitrobenzyl and the benzylthiomethyl groups. Whereas the S-carbobenzoxy[3] linkage is cleaved by the action of HBr in acetic acid, the S-*p*-nitrobenzyl bond is stable under these conditions but is cleaved by catalytic hydrogenation as was shown by Berse, *et al.*[4] The benzylthiomethyl group has been suggested as a protecting agent for the sulfhydryl group of cysteine by Pimlott and Young[5] who reported that the S-benzylthiomethyl bond is readily cleaved by treatment with mercuric chloride but resisted treatment with HBr in acetic acid. We were unable to substantiate the latter finding. Thus, when S-benzylthiomethyl-L-cysteine was exposed to HBr in acetic acid in the usual manner, paper chromatographic analysis of the product revealed the presence of four ninhydrin-positive components, indicating that the substance had undergone changes. However, when the S-benzylthiomethyl-L-cysteine was exposed to HBr in acetic acid in a mixture with diethylphosphite and methyl ethyl sulfide, paper chromatographic analysis of the product revealed the presence of one main ninhydrin-positive component with traces of two other ninhydrin-positive components. Comparable results were obtained with N-carbobenzoxy-L-valyl-S-benzylthiomethyl-L-cysteine methyl ester. Paper chromatographic analysis of the product which resulted when this dipeptide ester was treated with HBr in acetic acid indicated the presence of four ninhydrin-positive components, while

(1) This work was supported by a Senior Research Fellowship (SF-151) from the Public Health Service and by a grant (A-3067) from the National Institute of Arthritis and Metabolic Diseases, Public Health Service, for which I wish to express my appreciation.

(2) H. Brown, F. Sanger and R. Kital, *Biochem. J.*, **60**, 556 (1955).

(3) A. Berger, J. Noguchi and E. Katchalski, *J. Am. Chem. Soc.*, **78**, 4483 (1956).

(4) C. Berse, R. Boucher and L. Piche, *J. Org. Chem.*, **22**, 805 (1957).

(5) P. J. Pimlott and G. T. Young, *Proc. Chem. Soc.* (*London*), 257 (1958); G. T. Young, *Angew. Chem.*, **71**, 741 (1959).

in the presence of methyl ethyl sulfide and diethylphosphite one main ninhydrin-positive component with traces of two other ninhydrin-positive components were obtained. It seems, therefore, that the S-benzylthiomethyl-L-cysteine undergoes changes[6] on exposure to HBr in acetic acid and that these changes are limited in the presence of methyl ethyl sulfide and diethylphosphite.[7] Consequently, exposure of S-benzylthiomethyl-L-cysteine-containing peptides to HBr in acetic acid for removal of other protective groups should be avoided. This could be accomplished by the use of blocking groups such as trityl, *t*-butyloxycarbonyl or formyl, which can be removed by agents other than HBr in acetic acid. When N-formyl-L-valyl-S-benzylthiomethyl-L-cysteine methyl ester was deformylated with methanolic HCl analytically and chromatographically pure L-valyl-S-benzylthiomethyl-L-cysteine methyl ester hydrochloride was obtained.

L-Alanine methyl ester was coupled by the carbodiimide procedure[8] with N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteine to give N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanine methyl ester which upon saponification was converted into the crystalline N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanine. The mixed anhydride method[9] was employed for the preparation of the crystalline peptides, N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanylglycine ethyl ester and N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanylglycyl-L-valine methyl ester by coupling N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanine, with glycine ethyl ester and glycyl-L-valine methyl ester, respectively.

Decarbobenzoxylation of N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanine methyl ester by treatment with HBr in acetic acid and coupling of the resulting product with N,S-dicarbobenzoxy-L-cysteine[3] afforded the tripeptide N,S-dicarbobenzoxy-L-cysteinyl-S-*p*-nitrobenzyl-L-cysteinyl-L-alanine methyl ester in crystalline form.

Exposure to HBr in acetic acid converted N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanylglycine ethyl ester to its deblocked hydrobromide, which on treatment with triethylamine gave the free tripeptide ester. This ester was then condensed by the carbodiimide procedure with N-formyl-S-benzylthiomethyl-L-cysteine and N,S-dicarbobenzoxy-L-cysteine to yield the protected tetrapeptides N-formyl-S-benzylthiomethyl-L-cysteinyl-S-*p*-nitrobenzyl-L-cysteinyl-L-alanylglycine ethyl ester and the crystalline N,S-dicarbobenzoxy-L-cysteinyl-S-*p*-nitrobenzyl-L-cysteinyl-L-alanylglycine ethyl ester, respectively. When the latter tetrapeptide ester was heated in dioxane containing hydrochloric acid the ester group was hydrolyzed to give N,S-dicarbobenzoxy-L-cysteinyl-S-*p*-nitrobenzyl-L-cysteinyl-L-alanylglycine in crystalline form.

Condensation of N-carbobenzoxy-L-serine azide[10] with L-leucinamide[11] afforded N-carbobenzoxy-L-seryl-L-leucinamide. Catalytic hydrogenation of this peptide amide and coupling of the resulting product by the carbodiimide method with N-formyl-S-carbobenzoxy-L-cysteine yielded the crystalline N-formyl-S-carbobenzoxy-L-cysteinyl-L-seryl-L-leucinamide.

The crystalline dipeptide N-formyl-L-valyl-S-benzylthiomethyl-L-cysteine methyl ester was prepared by condensing N-formyl-L-valine[12] by the carbodiimide procedure with S-benzylthiomethyl-L-cysteine methyl ester.[5] Deformylation of this peptide ester with methanolic HCl yielded L-valyl-S-benzylthiomethyl-L-cysteine methyl ester hydrochloride in crystalline form. Coupling of N-carbobenzoxy-L-valine[13] by the mixed anhydride method with S-benzylthiomethyl-L-cysteine methyl ester afforded the crystalline N-carbobenzoxy-L-valyl-S-benzylthiomethyl-L-cysteine methyl ester.

No attempts were made to ascertain by enzymatic methods the optical homogeneity of the peptides reported in the present work. The fact that all but one of these peptides were obtained in crystalline form and exhibit sharp melting points is taken as strong evidence that the steric configuration of the constituent amino acids was preserved during the various synthetic processes.

Experimental

Capillary melting points were determined for all compounds and are corrected.

N-Carbobenzoxy-S-*p*-nitrobenzyl-L-cysteine.—To an ice-cold solution of S-*p*-nitrobenzyl-L-cysteine[4] (15.2 g.) in water (20 ml.) and *N* NaOH (60 ml.), carbobenzoxy chloride (12.8 ml.) and *N* NaOH (80 ml.) were added in portions with vigorous stirring over a period of 30 minutes in the usual manner. The reaction mixture was further stirred for 30 minutes at room temperature and extracted twice with ether. The aqueous layer was acidified with HCl and the precipitated oily product extracted with ethyl acetate. The organic layer was washed successively with dilute HCl and water, dried with $MgSO_4$ and evaporated *in vacuo* to dryness. The product was obtained as a heavy oil; wt. 23 g.

Cyclohexylamine Salt.—By the addition of an ethereal solution of cyclohexylamine to a solution of N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteine in ether, the cyclohexylamine salt of the product was precipitated as a heavy oil that crystallized upon the addition of a few drops of acetone. On recrystallization from ethanol–ether, the cyclohexylamine salt of N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteine was obtained as long needles; m.p. 129–130°, $[\alpha]^{28}D$ −2.2° (c = 1, ethanol).

Anal. Calcd. for $C_{24}H_{31}O_6N_3S$: C, 58.9; H, 6.38; N, 8.6. Found: C, 59.1; H, 6.41; N, 8.6.

N-Carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanine Methyl Ester.—L-Alanine methyl ester hydrochloride (4.2 g.) was suspended in freshly distilled tetrahydrofuran (50 ml.) and triethylamine (4.1 ml.) was added. The mixture was stirred for 20 minutes, cooled and the triethylamine hydrochloride was removed by filtration. To the filtrate

(6) It is interesting to note that side reactions also have been observed during the acid-treatment of methionine derivatives which have structural similarities to S-benzylthiomethyl-cysteine. See C. A. Dekker and J. S. Fruton [*J. Biol. Chem.*, **178**, 471 (1948)], HCl-treatment of carbobenzoxymethionine; N. F. Albertson and F. C. McKay, [*J. Am. Chem. Soc.*, **75**, 5323 (1953)], HBr in nitromethane treatment of carbobenzoxy-methionylglycine, and St. Guttmann and R. A. Boissonnas [*Helv. Chim. Acta*, **41**, 1852 (1958)], HBr in acetic acid treatment of methionine-containing peptides.

(7) This solvent mixture was used successfully by St. Guttmann and R. A. Boissonnas (see ref. 6) for preventing side reactions during HBr in acetic acid treatment of methionine-containing peptides.

(8) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

(9) T. Wieland and R. Sehring, *Ann.*, **569**, 122 (1950); R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 847 (1951); J. R. Vaughan, Jr., *J. Am. Chem. Soc.*, **73**, 3547 (1951).

(10) J. S. Fruton, *J. Biol. Chem.*, **146**, 463 (1942).

(11) P. S. Yang and M. M. Rising, *J. Am. Chem. Soc.*, **53**, 3183 (1931).

(12) J. C. Sheehan and D. D. H. Yang, *ibid.*, **80**, 1154 (1958).

(13) J. R. Vaughan, Jr., and J. A. Eichler, *ibid.*, **75**, 5556 (1953).

was added a solution of N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteine (11 g.) and N,N'-dicyclohexylcarbodiimide (6.2 g.) dissolved in tetrahydrofuran (50 ml.), and the reaction was allowed to proceed overnight at 0°. The precipitated N,N'-dicyclohexylurea was removed by filtration and the tetrahydrofuran was replaced by ethyl acetate (600 ml.). The ethyl acetate solution was washed successively with water, *N* HCl, water, *N* $KHCO_3$, water and dried over $MgSO_4$. Concentration of the ethyl acetate solution to a small volume yielded 8.95 g. (70%) of the crystalline product, m.p. 173–174°, $[\alpha]^{28}D$ −39.6° (*c* = 1.5, dimethylformamide).

Anal. Calcd. for $C_{22}H_{25}O_7N_3S$: C, 55.6; H, 5.29; N, 8.9. Found: C, 56.2; H, 5.51; N, 9.0.

N-Carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanine.—To a solution of N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanine methyl ester (4.75 g.) in dioxane (50 ml.) and acetone (50 ml.) was added, with stirring, *N* NaOH (11 ml.) over a period of 30 minutes at ice-bath temperature. The reaction mixture was stirred at room temperature for 45 minutes, diluted with 150 ml. of cold water and acidified with 6 *N* HCl. The crystalline precipitate was collected, washed with water and recrystallized from 50% aqueous acetic acid or 50% aqueous ethanol; wt. 3.65 g. (79%), m.p. 157–159°, $[\alpha]^{28}D$ −45.5° (*c* = 1, dimethylformamide). Neutral equivalent calcd., 461; found, 458.

Anal. Calcd. for $C_{21}H_{23}N_3O_7S$: C, 54.7, H, 5.10; N, 9.0. Found: C, 54.9; H, 4.94; N, 8.9.

N-Carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanylglycine Ethyl Ester.—A solution of N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanine (6.93 g.) and triethylamine (2.1 ml.) in tetrahydrofuran (70 ml.) was cooled to −5° and isobutyl chlorocarbonate (2 ml.) added. After 10 minutes a solution of glycine ethyl ester in tetrahydrofuran (25 ml.) (prepared from 3.5 g. of glycine ethyl ester hydrochloride by the dry ammonia procedure of Bailey[14]) was added. The mixture was kept at −5° for 10 minutes and at room temperature for 45 minutes. The reaction mixture (solid) was then triturated successively with water, 5% aqueous $KHCO_3$ twice, water, *N* HCl twice, water, and dried. On recrystallization from 60% aqueous acetic acid, 7 g. (85%) of product was obtained as long needles; m.p. 212°, $[\alpha]^{28}D$ −25.4° (*c* = 1, dimethylformamide).

Anal. Calcd. for $C_{25}H_{30}O_8N_4S$: C, 54.9; H, 5.52; N, 10.3. Found: C, 54.9; H, 5.63; N, 10.3.

N-Carbobenzoxyglycyl-L-valine Methyl Ester.—To a cooled (−5°) solution of carbobenzoxyglycine (8.20 g.) in freshly distilled tetrahydrofuran (50 ml.) containing triethylamine (5.6 ml.) was added isobutyl chlorocarbonate (5.28 ml.) followed after 10 minutes at −5° by a cooled solution of valine methyl ester in tetrahydrofuran prepared in this way: valine methyl ester hydrochloride (6.8 g.) was suspended in tetrahydrofuran (80 ml.) containing triethylamine (5.6 ml.); after 20 minutes, the precipitated triethylamine hydrochloride was filtered off, washed with 20 ml. of tetrahydrofuran and the combined filtrates added to the mixed anhydride prepared as described above. The reaction mixture was stirred at −5° for 15 minutes and at room temperature for 1 hr. and evaporated to dryness *in vacuo*. The residue was dissolved in 150 ml. of ethyl acetate and 50 ml. of water. The organic layer was separated and washed successively with 5% aqueous $KHCO_3$ thrice, water, *N* HCl and water, dried with $MgSO_4$ and evaporated to a small volume *in vacuo*. Upon addition of petroleum ether and storage in the refrigerator for several hours, the product crystallized out; wt. 8.5 g. (66%), m.p. 78°, $[\alpha]^{28}D$ −15.5° (*c* = 1.5, ethanol). A sample for analysis was recrystallized from ethyl acetate–petroleum ether.

Anal. Calcd. for $C_{16}H_{22}O_6N_2$: C, 59.6; H, 6.87; N, 8.7. Found: C, 59.6; H, 6.70; N, 8.8.

N-Carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanylglycyl-L-valine Methyl Ester.—A suspension of carbobenzoxyglycyl-L-valine methyl ester (2.21 g.) in acetic acid (4 ml.) was mixed with 4 *N* HBr in acetic acid (10 ml.). After 1 hr. at room temperature the resulting solution was concentrated to one third of its original volume *in vacuo* and mixed with anhydrous ether (100 ml.). The precipitated hydrobromide was washed several times with ether, dried over NaOH *in vacuo* and then used directly for condensation with N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanine.

A solution of N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanine (2.9 g.) and triethylamine (0.86 ml.) in tetrahydrofuran (20 ml.) was cooled to −5° and isobutyl chlorocarbonate (0.82 ml.) was added with subsequent addition after 10 minutes of a cooled solution of glycyl-L-valine methyl ester in tetrahydrofuran prepared as noted: the hydrobromide, which had been made as described above from carbobenzoxyglycyl-L-valine methyl ester (2.21 g.), was suspended in tetrahydrofuran (10 ml.) and triethylamine (0.87 ml.), stirred 10 minutes, and the precipitated triethylamine hydrobromide was filtered off and washed with 10 ml. of tetrahydrofuran; the combined filtrates then were added to the mixed anhydride prepared as described above. The reaction mixture was stirred at −5° for 15 minutes, at room temperature for 1 hr. and then evaporated to dryness *in vacuo*. The solid residue was triturated successively with *N* HCl, water, 5% aqueous $KHCO_3$, and water. On recrystallization from 50% aqueous acetic acid 2.60 g. (66.5%) of product was obtained as needles, m.p. 200°, $[\alpha]^{28}D$ −18° (*c* = 1, dimethylformamide).

Anal. Calcd. for $C_{29}H_{35}O_9N_5S$: C, 55.1; H, 6.04; N, 11.1. Found: C, 54.9; H, 5.93; N, 11.0.

N,S-Dicarbobenzoxy-L-cysteinyl-S-*p*-nitrobenzyl-L-cysteinyl-L-alanine Methyl Ester.—N-Carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanine methyl ester (6 g.) was suspended in 2 *N* HBr in acetic acid (40 ml.), and the mixture was stirred at room temperature for 1 hr. For removal of the acetic acid and HBr, the mixture was concentrated *in vacuo*, the residual oil was dissolved in 15 ml. of methanol and the solvent was removed *in vacuo*. The hydrobromide was crystallized from methanol–ether; wt. 5 g., m.p. 126–128°. For conversion into the free base, the hydrobromide was dissolved in tetrahydrofuran (50 ml.) containing triethylamine (1.65 ml.) and the mixture was stirred for 15 minutes and cooled. The triethylamine hydrobromide was removed by filtration and to the filtrate was added a solution of N,S-dicarbobenzoxy-L-cysteine[3] (4.66 g.) in tetrahydrofuran (20 ml.) and N,N'-dicyclohexylcarbodiimide (2.7 g.). The reaction was allowed to proceed at 0° for 1 hr. and at room temperature for 16 hr. To the reaction mixture a few drops of acetic acid was added, the precipitated N,N'-dicyclohexylurea was removed by filtration and the filtrate was evaporated *in vacuo* to dryness. The residue was triturated with 100 ml. of warm ethyl acetate and the product isolated by filtration; wt. 6.4 g. (69%), m.p. 169–171°. The protected tripeptide ester was crystallized from 60% warm aqueous acetic acid; m.p. 173–174°, $[\alpha]^{28}D$ −54° (*c* = 1, dimethylformamide).

Anal. Calcd. for $C_{33}H_{36}O_{10}N_4S_2$: C, 55.6; H, 5.07; N, 7.9. Found: C, 55.8; H, 5.15; N, 8.0.

S-Benzylthiomethyl-L-cysteine.—Since no experimental details are given by Pimlott and Young, who first synthesized this compound, we describe here its synthesis following essentially the route suggested by these authors.[5]

A suspension of finely powdered L-cysteine hydrochloride (50 g.) in methanol (70 ml.) and benzylthiomethyl chloride[15] (58 g.) was refluxed for 30 minutes. The resulting solution was concentrated to dryness *in vacuo*, and the residue was dissolved in a mixture of dioxane (150 ml.) and water (60 ml.). This solution was stirred for 2 hr. at room temperature the *p*H being maintained at *ca.* 9 by the stepwise addition of 4 *N* NaOH. The reaction mixture was diluted with 500 ml. of water and extracted twice with ether. Acidification of the aqueous phase to *p*H 5.5 with dilute HCl precipitated the product in crystalline form. After cooling for several hours the S-benzylthiomethyl-L-cysteine was isolated by filtration, washed with cold water and recrystallized as follows: The still wet product was suspended in 1.5 l. of boiling water and brought into solution by the addition of 6 *N* HCl. The hot solution was treated with Norit and filtered through a pre-heated funnel. Adjusting the *p*H of the filtrate to 5.5 with dilute NH_4OH caused the crystallization of the product. After cooling, the crystals were collected, washed with cold water and dried; wt. 45 g., m.p. 200° (lit.[5] 193°).

Stability of S-Benzylthiomethyl-L-cysteine. A. Treatment with HBr in Acetic Acid.—A 200 mg. sample of S-

(14) J. L. Bailey, *J. Chem. Soc.*, 3461 (1950).

(15) J. L. Wood and V. du Vigneaud, *J. Biol. Chem.*, **131**, 267 (1939).

benzylthiomethyl-L-cysteine was added to 10 ml. of 2 *N* HBr in acetic acid. After 45 minutes the reaction mixture was concentrated to a small volume *in vacuo*, and the product was precipitated by addition of dry ether. Paper chromatography of this product on Whatman #1 paper using the Partridge[16] system revealed the presence of four ninhydrin-positive components with R_f's 0.19, 0.32, 0.77 and 0.87.

B. Treatment with HBr in Acetic Acid in the Presence of Diethylphosphite and Methyl Ethyl Sulfide.—A 200 mg. sample of S-benzylthiomethyl-L-cysteine was added in a mixture consisting of 6 ml. of 4 *N* HBr in acetic acid, 3 ml. of diethylphosphite and 3 ml. of methyl ethyl sulfide and treated as described previously. Paper chromatography of the product (Whatman #1 paper, Partridge system) revealed the presence of one main ninhydrin-positive component with R_f = 0.77 and only traces of two other ninhydrin-positive components with R_fs 0.24 and 0.87.

N-Formyl-S-benzylthiomethyl-L-cysteine.—To a precooled solution of S-benzylthiomethyl-L-cysteine (5.2 g.) in 98% formic acid (42 ml.), acetic anhydride (14 ml.) was added dropwise over a period of 15 minutes keeping the temperature of the reaction mixture between 8 to 12°. After stirring for an additional hour, cold water (200 ml.) was added and the precipitated product was filtered off, washed with cold water and recrystallized from water; wt., 5.2 g. (81%), m.p. 138°, $[\alpha]^{27}D$ −38.4° (*c* = 1.24, 90% aqueous dimethylformamide).

Anal. Calcd. for $C_{12}H_{15}O_3NS_2$: C, 50.5; H, 5.29; N, 4.9. Found: C, 50.5; H, 5.32; N, 4.7.

N-Formyl-S-benzylthiomethyl-L-cysteinyl-S-*p*-nitrobenzyl-L-cysteinyl-L-alanylglycine Ethyl Ester.—A suspension of N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanylglycine ethyl ester (3.3 g.) in acetic acid (8 ml.) was mixed with 18 ml. of 4 *N* HBr in acetic acid. After 45 minutes at room temperature the resultant solution was concentrated to one half of its original volume *in vacuo* and mixed with anhydrous ether (300 ml.). The precipitate which formed was filtered, washed with ether and re-precipitated from ethanol–ether; wt., 2.8 g. A solution of this product in dimethylformamide (10 ml.) was treated with triethylamine (0.8 ml.) and the precipitated triethylamine hydrobromide filtered off and washed with dimethylformamide (5 ml.). To the combined filtrates a solution of N-formyl-S-benzylthiomethyl-L-cysteine (1.8 g.) in dioxane (20 ml.) was added, followed, after cooling at 0°, by N,N′-dicyclohexylcarbodiimide (1.35 g.). After 18 hr. at 5°, the reaction mixture was diluted with dimethylformamide (20 ml.), warmed to 45°, cooled to room temperature and acidified with acetic acid. The precipitated N,N′-dicyclohexylurea (1.38 g.) was filtered off and the filtrate mixed with 400 ml. of water containing 1 ml. of acetic acid. The precipitated product was filtered off, washed with water, triturated with 5% aqueous $KHCO_3$, washed thoroughly with water and reprecipitated from 50% aqueous acetic acid; wt., 2.5 g. (58%), m.p. 212–215°, $[\alpha]^{27}D$ − 32.1° (*c* = 0.99, dimethylformamide).

Anal. Calcd. for $C_{29}H_{37}O_8N_5S_3$: C, 51.2; H, 5.48; N, 10.3. Found: C, 51.3; H, 5.70; N, 10.3.

N,S-Dicarbobenzoxy-L-cysteinyl-S-*p*-nitrobenzyl-L-cysteinyl-L-alanylglycine Ethyl Ester.—N-Carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-alanylglycine ethyl ester (7 g.) was suspended in acetic acid (10 ml.) and 4 *N* HBr in acetic acid (25 ml.). After 45 minutes at room temperature, the resultant solution was concentrated to one half of its original volume and then mixed with dry ether (400 ml.). The precipitated hydrobromide was filtered, washed thoroughly with ether and reprecipitated from ethanol–ether. A solution of this product in dimethylformamide (20 ml.) and tetrahydrofuran (60 ml.) was treated with triethylamine (1.8 ml.) and the precipitated triethylamine hydrobromide was filtered off. The filtrate was concentrated to one fourth of its original volume *in vacuo*, mixed with a solution of N,S-dicarbobenzoxy-L-cysteine (4.7 g.) in dioxane (45 ml.) and, after cooling at 5°, N,N′-dicyclohexylcarbodiimide (2.9 g.) in dioxane (20 ml.) was added. After 18 hr. at 5° the reaction mixture was diluted with dioxane (20 ml.) brought to room temperature and acidified with 1 ml. of acetic acid. The precipitated N,N′-dicyclohexylurea (2.8 g., m.p. 234°) was filtered off, the filtrate concentrated to *ca.* 50 ml. and mixed with 400 ml. of 5% aqueous $KHCO_3$. The solid formed was isolated by filtration, washed with water, triturated with *N* HCl, filtered off, washed with water and crystallized from 60% aqueous acetic acid; wt., 7 g. (76%), m.p. 197–198°, $[\alpha]^{28}D$ −36.8° (*c* = 1, dimethylformamide).

Anal. Calcd. for $C_{35}H_{41}O_{11}N_5S_2$: C, 55.1; H, 5.26; N, 8.9. Found: C, 55.2; H, 5.21; N, 9.1.

N,S-Dicarbobenzoxy-L-cysteinyl-S-*p*-nitrobenzyl-L-cysteinyl-L-alanylglycine.—N,S-Dicarbobenzoxy-L-cysteinyl-S-*p*-nitrobenzyl-L-cysteinyl-L-alanylglycine ethyl ester (1.54 g.) was dissolved with warming in dioxane (80 ml.) and *N* HCl (10 ml.) added. The solution was heated for 1.5 hr. at 90°, concentrated to one fourth of its original volume *in vacuo* and mixed with water. The precipitated product was filtered, washed with water and crystallized from 60% aqueous acetic acid as needles; wt., 1.4 g. (94%), m.p. 170–172°, $[\alpha]^{28}D$ −36.2° (*c* = 1, dimethylformamide). The material has the analysis of a monohydrate.

Anal. Calcd. for $C_{34}H_{37}O_{11}N_5S_2 \cdot H_2O$: C, 52.8; H, 5.07; N, 9.1. Found: C, 53.0; H, 5.23; N, 9.1.

N-Carbobenzoxy-L-seryl-L-leucinamide.—To a cold solution of L-leucinamide[11] (4.6 g.) in dimethylformamide (40 ml.) was added an ethyl acetate solution (150 ml.) of N-carbobenzoxy-L-serine azide[10] (prepared from 8.5 g. of the hydrazide and 2.35 g. of sodium nitrite in 75 ml. of 1 *N* HCl at 0°). The reaction mixture was stirred at 5° for 20 hr. and at room temperature for 1 hr. The mixture was diluted with ethyl acetate (100 ml.) and ethanol (20 ml.), washed successively with dilute HCl, water, 5% aqueous $KHCO_3$ and water and dried over $MgSO_4$. Concentration of the solvent to a small volume and then addition of petroleum ether afforded a solid which was recrystallized from 50% aqueous ethanol; wt., 6.6 g. (60% based on hydrazide), m.p. 181–183°; $[\alpha]^{27}D$ +9.1° (*c* = 1.07, dimethylformamide).

Anal. Calcd. for $C_{17}H_{25}O_5N_3$: C, 58.1; H, 7.16, N, 12.0. Found: C, 57.9; H, 7.15; N, 12.0.

N-Formyl-S-carbobenzoxy-L-cysteine.—To a solution of S-carbobenzoxy-L-cysteine[3] (12.75 g.) in 98% formic acid (105 ml.) cooled at 5°, acetic anhydride (42 ml.) was added dropwise over a period of 15 minutes. The reaction mixture then was stirred for 1 hr. at 10 to 15° after which period it was diluted with cold water (350 ml.). The precipitated product was filtered off, washed with cold water and recrystallized from hot water (650 ml.); wt., 9.8 g. (70%), m.p. 141–142°, $[\alpha]^{27}D$ −41.6° (*c* = 1.34, dimethylformamide)

Anal. Calcd. for $C_{12}H_{13}O_5NS$: C, 50.9; H, 4.62; N, 4.9. Found: C, 50.6; H, 4.56; N, 4.9.

N-Formyl-S-carbobenzoxy-L-cysteinyl-L-seryl-L-leucinamide.—N-Carbobenzoxy-L-seryl-L-leucinamide (5.25 g.) was hydrogenated over 1 g. of 10% palladium-charcoal catalyst, in ethanol (120 ml.) containing concentrated HCl (1.35 ml.) until the evolution of carbon dioxide had ceased (*ca.* 2 hr). The catalyst was filtered off and the filtrate evaporated to dryness *in vacuo*. The residue of dipeptide amide hydrochloride was dried by the addition of ethanol and then evaporated *in vacuo*. The residue finally was dissolved in dimethylformamide (25 ml.), triethylamine (2.1 ml.) added and the triethylamine hydrochloride was removed by filtration. To the filtrate was added a solution of N-formyl-S-carbobenzoxy-L-cysteine (4.25 g.) in dioxane (20 ml.) followed by N,N′-dicyclohexylcarbodiimide (3.3 g.). The reaction mixture was stirred at 5° for 20 hr., brought to room temperature and acidified with acetic acid. The N,N′-dicyclohexylurea which separated was filtered off and washed with dioxane. Addition of ether to the combined filtrates caused the precipitation of a solid which was triturated consecutively with water, dilute HCl, water, 5% aqueous $KHCO_3$ and water and dried. Crystallization from 80% aqueous ethanol yielded 4.2 g. (57%) of tripeptide amide; m.p. 219°, $[\alpha]^{27}D$ −13.3° (*c* = 1.12, dimethylformamide).

Anal. Calcd. for $C_{21}H_{30}O_7N_4S$: C, 52.3; H, 6.26; N, 11.6. Found: C, 52.3; H, 6.29; N, 11.4.

N-Formyl-L-valyl-S-benzylthiomethyl-L-cysteine Methyl Ester.—A suspension of S-benzylthiomethyl-L-cysteine methyl ester hydrochloride[5] (9.7 g.) in tetrahydrofuran (60 ml.) was treated with triethylamine (4.3 ml.) and the

(16) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

precipitated triethylamine hydrochloride was filtered off and washed with tetrahydrofuran (10 ml.). To the combined filtrates was added a solution of N-formyl-L-valine (4.35 g.) in tetrahydrofuran (25 ml.) followed by N,N'-dicyclohexylcarbodiimide (6.8 g.). The reaction mixture was stirred at 5° for 5 hr., at room temperature for 1 hr. and acidified with drops of acetic acid. The precipitated N,N'-dicyclohexylurea was filtered off (6.9 g., m.p. 234°) and the filtrate concentrated to 15 ml. *in vacuo* and mixed with cold water (150 ml.) containing acetic acid (1 ml.). The precipitated crystalline product was isolated by filtration, washed with water and recrystallized from 60% aqueous methanol; wt., 6.8 g. (56%), m.p. 126–128°. A sample for analysis was recrystallized from methanol; m.p. 128–129°, $[\alpha]^{27}D$ −50.0° (c = 1.46, dimethylformamide).

Anal. Calcd. for $C_{18}H_{26}O_4N_2S_2$: C, 54.2; H, 6.52; N, 7.0. Found: C, 54.1; H, 6.63; N, 6.8.

L-Valyl-S-benzylthiomethyl-L-cysteine Methyl Ester Hydrochloride.—A solution of N-formyl-L-valyl-S-benzylthiomethyl-L-cysteine methyl ester (1.6 g.) in methanol (30 ml.) and 1 *N* HCl (8 ml.) was refluxed for 1 hr. and the solvent removed under reduced pressure. The residue was dissolved in methanol, the solvent evaporated and this procedure was repeated three times. The solid residue then was dissolved in methanol (15 ml.). Addition of ether (100 ml.) caused the crystallization of the dipeptide ester hydrochloride; wt., 1.12 g. (70%), m.p. 161–162°, $[\alpha]^{27}D$ −4.9° (c = 1.12, dimethylformamide).

Anal. Calcd. for $C_{17}H_{27}O_3N_2S_2Cl$: C, 50.2; H, 6.68; N, 6.9. Found: C, 50.0; H, 6.72; N, 7.3.

Paper chromatography on Whatman #1 paper using the Partridge system revealed the presence of only one ninhydrin positive component with R_f = 0.74.

N-Carbobenzoxy-L-valyl-S-benzylthiomethyl-L-cysteine Methyl Ester.—A solution of N-carbobenzoxy-L-valine (3.8 g.) and triethylamine (2.1 ml.) in tetrahydrofuran (30 ml.) was cooled to −5° and isobutyl chlorocarbonate (2 ml.) added with stirring. After 10 minutes at this temperature a solution of S-benzylthiomethyl-L-cysteine methyl ester (prepared as described previously from 4.7 g. S-benzylthiomethyl-L-cysteine methyl ester hydrochloride, 2.1 ml. of triethylamine and 30 ml. of tetrahydrofuran) was added with stirring. After 30 minutes at −5° and 6 hr. at room temperature the reaction mixture was concentrated *in vacuo* to *ca.* 15 ml. and poured into cold water (300 ml.) containing concentrated HCl (2 ml.). The precipitated product was separated by filtration, washed with water, triturated with 5% aqueous $KHCO_3$, washed again with water and crystallized from 70% aqueous methanol; wt., 6.3 g. (83%), m.p. 112°, $[\alpha]^{30}D$ −28.8° (c = 1.05, dimethylformamide).

Anal. Calcd. for $C_{25}H_{32}N_2O_5S_2$: C, 59.50; H, 6.38; N, 5.55. Found: C, 60.0; H, 6.45; N, 5.55.

Decarbobenzoxylation of N-Carbobenzoxy-L-valyl-S-benzylthiomethyl-L-cysteine Methyl Ester. A. Treatment with HBr in Acetic Acid.—A 100 mg. sample of the protected dipeptide ester was added to 4 ml. of 2 *N* HBr in acetic acid. After 1 hr. at room temperature dry ether was added and the precipitate which formed was washed with ether. Paper chromatography of this product on Whatman #1 paper using the Partridge system revealed the presence of four ninhydrin-positive components with R_f's 0.38, 0.48, 0.60 and 0.77.

B. **Treatment with HBr in Acetic Acid in the Presence of Diethylphosphite and Methyl Ethyl Sulfide.**—A 100 mg. sample of the dipeptide ester was added in a mixture containing 2 ml. of 4 *N* HBr in acetic acid, 1 ml. of diethylphosphite and 1 ml. of methyl ethyl sulfide. After 1 hr. a mixture of ethyl acetate–petroleum ether (1:1) was added and the product which separated as a heavy oil was washed with ether. Paper chromatography of this product, carried out as described in A, revealed the presence of one main ninhydrin-positive component with R_f = 0.76 and traces of two other ninhydrin-positive components with R_f's 0.42 and 0.54.

[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE, PITTSBURGH, PENNSYLVANIA]

Insulin Peptides. II. Synthesis of a Protected Pentapeptide Containing the C-Terminal Sequence of the A-Chain of Insulin[1]

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A protected pentapeptide containing the C-terminal sequence of the A-chain of insulin, namely N-carbobenzoxy-γ-benzyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester, has been prepared. Stepwise elongation of the peptide chain from the carboxyl toward the amino end using the *p*-nitrophenyl esters of the appropriate N-carbobenzoxyamino acids was employed mainly in the synthesis of this compound and its intermediates.

In the preceding paper[3] we reported the synthesis of certain peptides containing amino acid sequences found in the intra-chain ring region of the structure of the A-chain of sheep insulin postulated by Sanger, *et al.*[4]

In connection with our synthetic studies on peptides with amino acid sequences found in insulin, we would like to report the synthesis of the protected pentapeptide N-carbobenzoxy-γ-benzyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester. The sequence corresponding to this peptide is found in the C-terminal portion of the A-chain of insulin. For the synthesis of this pentapeptide and its intermediates the conventional methods of peptide chemistry were employed.

Stepwise elongation of the peptide chain from the carboxyl toward the amino end was the principal approach used. The appropriate N-carbobenzoxy amino acids served as the "carboxyl component" and they were activated by conversion to the corresponding *p*-nitrophenyl esters.[5] Since the use of HBr in acetic acid was unavoidable for the decarbobenzoxylation of the intermediate cysteine-containing peptides, the carboxyl group of the C-terminal amino acid, asparagine, was protected by conversion to its *p*-nitrobenzyl ester.

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