the calorimetric experiments was incubated at 25° with ATP-Cr transphosphorylase, in the absence of buffer but in the presence of Ca^{++} and KCl as in the calorimetric runs. The *p*H was maintained at 8.00 by adding KOH from a microburet. After some time the enzyme was inactivated by adding *p*-chloromercuribenzoate, and the solution was analyzed for Cr. Two experiments of this type gave *for reaction 1*

$$\phi_H = -0.98 \pm 0.02$$

The published value[8] of ϕ_H for reaction 2 under our experimental conditions is 1.00. These two figures give $+ 0.02 \pm 0.02$ for ϕ_H for reaction 3.

Since ϕ_H differs from zero by less than the experimental error, the correction term $\phi_H \Delta H_b$ in equation 4 has been taken to be zero. Thus the final value for the heat of hydrolysis of CrP is $\Delta H_{hyd} = -9000 \pm 500$ cal. per mole.

The heat of hydrolysis of CrP is thus notably higher than the values found, under similar conditions, for ATP[8,9] (− 5000 cal. per mole) and inorganic pyrophosphate[10] (−5800 cal. per mole). In terms of the heat available on hydrolysis, CrP may thus be considerd a "high-energy phosphate" in a rather more real sense than the polyphosphates. On the other hand, this need not be true of the more significant standard free energy of hydrolysis. In fact, equilibrium studies[11] on reaction 1 indicate that the standard free energy of hydrolysis of CrP is quite close to that of ATP.

Since the standard free energies of hydrolysis of ATP and CrP are nearly equal and the enthalpy of hydrolysis[12] of CrP is much more negative than that of ATP, the standard entropy of hydrolysis of CrP must be more negative than that of ATP by approximately 13 entropy units per mole. In view of the multiply charged character and the unknown extent of binding of Ca^{++}, of the various solute species, and the fact that three molecules are formed in the hydrolysis of ATP whereas only two are formed in the hydrolysis of CrP, it is not possible to give an interpretation of this difference in entropies.

Utilization of the data of Bernhard[13] on the ionization constants and heats of ionization of the intrafibrial buffers of mammalian muscle permits an estimate of the "physiological" heat of hydrolysis of CrP. On the assumption that ΔC_p for the hydrolysis of CrP is zero, the value obtained for 37° and *p*H 6.9 is $\Delta H_{hyd} = -6900$ cal. per mole.

Acknowledgments.—We are indebted to Professor M. F. Morales for suggesting this investigation to us and to him and to Professor L. Noda for many valuable discussions. This work was aided by grants from the National Heart Institute (M.G.) and the National Science Foundation (J.M.S.).

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HANOVER, N. H.
NEW HAVEN, CONN.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE]

Synthetic Work Related to Arginine-Vasopressin[1]

BY ROLF O. STUDER[2] AND VINCENT DU VIGNEAUD

RECEIVED AUGUST 3, 1959

A new synthetic route to arginine-vasopressin was investigated. The approach involved the coupling of either S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine or S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine by the azide method with L-prolyl-L-arginylglycinamide hydrobromide, which was synthesized by using nitroarginine as an intermediate. The resulting crude protected nonapeptide hydrobromide was treated with sodium in liquid ammonia to remove the protecting groups and then oxidized by aeration. The biologically active material thus obtained was purified by countercurrent distribution followed by electrophoresis and ion-exchange chromatography. The active product proved to be identical with natural arginine-vasopressin in potency, electrophoretic mobility, paper chromatography and ion-exchange chromatography on IRC-50 resin.

In earlier synthetic studies[3,4] on arginine-vasopressin, the protected pentapeptide S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine[5] was coupled with the monohydrobromide of the tetrapeptide S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide[6] according to the tetraethyl pyrophosphite method to give the protected nonapeptide amide. Also, the more highly protected pentapeptide S-benzyl-N-carbobenzoxy-L-cysteinyl-*o*-tosyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine was used. In both cases, treatment of the protected nonapeptide amides with sodium in liquid ammonia and oxidation of the resulting sulfhydryl compounds gave active products, which after purification by countercurrent distribution and electrophoresis possessed a lower specific activity than the natural arginine-vasopressin. The lower activity of the products was probably due to the formation of an anhydro compound as a contaminating product in the coupling reaction of the carboxyl group of asparagine.[7,8]

(1) The authors wish to express their appreciation to the National Heart Institute, Public Health Service, for a research grant (H-1675), which has aided greatly in this work.

(2) Appreciation is expressed to the Stiftung für Stipendien auf dem Gebiete der organischen Chemie, Zürich, Switzerland, for a Stipendium.

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A more highly active product with a potency similar to that of the natural arginine-vasopressin was obtained by coupling the azide of S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine with L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide hydrobromide.[9] Unfortunately, some of the intermediate peptides required extensive purification by countercurrent distribution.[6,8]

The present paper describes another approach to the synthesis of arginine-vasopressin that has yielded a product with a potency similar to that of the natural arginine-vasopressin. The protected hexapeptide hydrazides S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinehydrazide and S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine hydrazide were synthesized by coupling either S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine[8] or S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanine,[10] by the mixed anhydride or azide method, with L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine methyl ester, and converting the resulting protected hexapeptide esters into their corresponding hydrazides. The latter compounds were coupled by the azide method with L-prolyl-L-arginylglycinamide hydrobromide, which was synthesized by using nitroarginine as an intermediate. Carbobenzoxy-L-prolylnitro-L-arginine was prepared according to Hofmann, Peckham and Rheiner,[11] and the protected dipeptide was condensed with glycinamide in a manner similar to that used by those authors for preparing other peptides through the carboxyl group of arginine. In another experiment, the acetate of the tripeptide was used for coupling with the hexapeptide azide. Treatment of the resulting crude protected nonapeptide with sodium in liquid ammonia, followed by aeration yielded the active material, which was desalted and concentrated on a column of IRC-50 resin by the procedure described for natural arginine-vasopressin.[12] The synthetic product was purified by countercurrent distribution in the system 2-butanol-0.06 M p-toluenesulfonic acid, by electrophoresis at pH 4 in pyridine–acetate buffer[9] and by ion-exchange chromatography on IRC-50 resin (H^+ form) with 0.65 M ammonium acetate.[12]

The behavior of the synthetic product was the same as that of natural arginine-vasopressin on paper electrophoresis at two different pH's (pyridine-acetate buffer pH 5.6 and glycine buffer pH 9), ion-exchange chromatography on IRC-50 and paper chromatography. The pressor activity of the synthetic product, as assayed in the rat,[13] was the same as that of natural vasopressin (350–400 units/mg.) obtained by the method of Acher, Light and du Vigneaud.[12] This level of activity also was found for the synthetic arginine-vasopressin obtained previously by a different method.[9]

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Experimental[14]

L-Glutaminyl-L-asparaginyl-S-benzyl-L-cysteine Methyl Ester Hydrobromide.—Carbobenzoxy-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine methyl ester[8] (8 g.) was suspended in 80 ml. of acetic acid which had been saturated with HBr and the mixture was stirred at room temperature for 1 hr. For removal of the acetic acid and excess HBr, the solution was concentrated *in vacuo*, the residual oil was dissolved in 50 ml. of methanol and the solvent was distilled *in vacuo*. The hydrobromide was crystallized and recrystallized from methanol–ether; wt. 6.9 g. (95%), m.p. 184–185°, $[\alpha]^{21.5}D$ −37.5° (c 1, water).

Anal. Calcd. for $C_{20}H_{29}O_6N_5S \cdot HBr$: C, 43.8; H, 5.51; N, 12.8. Found: C, 43.7; H, 5.61; N, 12.7.

S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine Methyl Ester.—A solution of S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine[8] (3 g., 4.4 mM) in 30 ml. of dimethylformamide and triethylamine (0.61 ml., 4.4 mM) was cooled to −15° and isobutyl chlorocarbonate (0.61 ml., 4.4 mM) was added. The mixture was stirred for 5 minutes at −15° and L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine methyl ester hydrobromide (2.5 g., 4.5 mM) and triethylamine (0.62 ml., 4.5 mM) in 20 ml. of dimethylformamide were added. The reaction was allowed to proceed for 16 hr. at 5°. After addition of water to the reaction mixture, the precipitate was filtered off and washed successively with N HCl, water, 5% $NaHCO_3$ and water, and dried. It was recrystallized from acetic acid–water; wt. 3.3 g. (67%), m.p. 247–248°, $[\alpha]^{23}D$ −38.8° (c 1, acetic acid).

Anal. Calcd. for $C_{55}H_{64}O_{12}N_8S_3$: C, 58.7; H, 5.73; N, 9.95. Found: C, 58.6; H, 5.92; N, 9.65.

This protected hexapeptide ester was also prepared by the azide method. The yield was 60%, m.p. 247–248°.

S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine Hydrazide.—Hydrazine hydrate (1.5 ml.) was added to a solution of S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine methyl ester (2.65 g., 2.3 mM) in 20 ml. of dimethylformamide. The solution was allowed to stand at room temperature overnight and then cooled to 0°. The crystalline hydrazide was filtered off, washed with water and dried; wt. 1.9 g. (75%), m.p. 261–262°, $[\alpha]^{21}D$ −15.2° (c 1, dimethylformamide).

Anal. Calcd. for $C_{54}H_{64}O_{11}N_{10}S_3$: N, 12.4. Found: N, 12.1.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine Methyl Ester.—S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanine azide (prepared from 1.6 g. (2.4 mM) of the corresponding hydrazide[10]) was added to a solution of L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine methyl ester hydrobromide (1.15 g., 2.1 mM) and triethylamine (0.3 ml., 2.1 mM) in 15 ml. of dimethylformamide and stirred for 24 hr. at 4° and for 12 hr. at room temperature. After addition of 50 ml. of water to the reaction mixture, the precipitate was filtered off and washed with 100 ml. of hot ethyl acetate, N HCl and water, and dried. It was precipitated from acetic acid–water; wt. 1.6 g. (70%), m.p. 256–257°, $[\alpha]^{20}D$ −31.5° (c 1, acetic acid).

Anal. Calcd. for $C_{56}H_{64}O_{12}N_8S_2$: C, 60.8; H, 5.83; N, 10.1. Found: C, 60.7; H, 5.91; N, 10.1.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine Hydrazide.—Hydrazine hydrate (0.3 ml.) was added to a solution of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine methyl ester (0.5 g., 0.45 mM) in 5 ml. of dimethylformamide. The solution was allowed to stand at room temperature overnight and then was cooled to 0°. Cold water (20 ml.) was added and the precipitate was filtered

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

off, washed with water and dried; wt. 0.4 g. (80%), m.p. 250–251°, $[\alpha]^{20}D$ −40.4° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{55}H_{64}O_{11}N_{10}S_2$: C, 59.8; H, 5.83; N, 12.7. Found: C, 59.6; H, 5.87; N, 12.5.

Carbobenzoxy-L-prolylnitro-L-arginine Methyl Ester.—Nitro-L-arginine methyl ester hydrochloride[11] (2.7 g., 0.01 *M*) was dissolved in 25 ml. of methanol, and 0.5 *N* methanolic sodium methoxide (20 ml.) was added. The mixture was evaporated to dryness *in vacuo* and the residue was triturated with dioxane (25 ml.). This solution was added to a mixed anhydride prepared from carbobenzoxy-L-proline (2.5 g., 0.01 *M*), tri-*n*-butylamine (2.4 ml., 0.01 *M*) and ethyl chlorocarbonate (0.96 ml., 0.01 *M*) in 25 ml. of dioxane for 15 minutes at 10°. The mixture was allowed to stir for 45 minutes at room temperature and then the dioxane was replaced by ethyl acetate; the solution was washed successively with *N* HCl, water, 5% $NaHCO_3$ and water, dried over anhydrous magnesium sulfate and evaporated to dryness. The compound was recrystallized from methanol-ether; wt. 3.3 g. (70%), m.p. 130–132°, $[\alpha]^{20}D$ −44.9° (*c* 2, methanol).

Anal. Calcd. for $C_{20}H_{28}O_7N_6$: C, 51.7; H, 6.07; N, 18.1. Found: C, 51.7; H, 6.15; N, 18.0.

Carbobenzoxy-L-prolylnitro-L-arginine.—Carbobenzoxy-L-prolylnitro-L-arginine methyl ester (1.4 g., 3 m*M*) was saponified by stirring the material for 45 minutes at room temperature with 0.5 *N* NaOH (9 ml.). The reaction mixture was cooled in ice and acidified with concd. HCl. The resulting crystalline product was filtered off, washed with water and dried. It was recrystallized from acetone–water; wt. 1.25 g. (92%), m.p. 197–198°, $[\alpha]^{20}D$ −32.5° (*c* 3.92, methanol). Hofmann, *et al.*,[11] reported m.p. 197–198°, $[\alpha]D$ −32.7° (*c* 3.92, methanol).

Carbobenzoxy-L-prolylnitro-L-arginylglycinamide.—A solution of carbobenzoxy-L-prolylnitro-L-arginine (1.8 g., 4 m*M*) in dimethylformamide (3 ml.), tetrahydrofuran (30 ml.) and triethylamine (0.56 ml., 4 m*M*) was cooled to −5° and isobutyl chlorocarbonate (0.52 ml., 4 m*M*) was added. The mixture was stirred for 20 minutes at −5° and glycinamide (330 mg., 4.4 m*M*) in dimethylformamide (5 ml.) was added. The reaction was allowed to proceed at room temperature for 2 hr., when the mixture was filtered and the filtrate evaporated to dryness *in vacuo*. The residue was first extracted with chloroform (100 ml.), and then the gummy material was triturated with ethyl acetate, when it solidified. It was filtered off and precipitated twice from pyridine–ethyl acetate as an amorphous powder; wt. 1.5 g. On exposure to air the substance picked up one molecule of water.

Anal. Calcd. for $C_{21}H_{30}O_7N_8 \cdot H_2O$: C, 48.1; H, 6.14; N, 21.4; H_2O, 3.43. Found: C, 48.3; H, 6.02; N, 21.2; H_2O, 3.48.

For further characterization a sample (385 mg., 1.0 m*M*) was hydrogenated[11] and the L-prolyl-L-arginylglycinamide was crystallized as the diflavianate; wt. 600 mg. (60%), m.p. 181–183°, $[\alpha]^{21}D$ −18° (*c* 1, acetone-water 1:1). Lit.[6] m.p. 180°, $[\alpha]^{24}D$ −18.2° (*c* 1, acetone-water 1:1).

L-Prolyl-L-arginylglycinamide Dihydrobromide.—Carbobenzoxy-L-prolylnitro-L-arginylglycinamide (1.5 g.) was dissolved in methanol containing 10% glacial acetic acid (100 ml.) and was hydrogenated over a palladium catalyst for 10 hr.[11] The catalyst was then removed by filtration and the filtrate was evaporated to dryness *in vacuo*. The acetate was dissolved in methanol (20 ml.) and acetic acid saturated with HBr (2 ml.) was added. The solution was evaporated to dryness *in vacuo*, the residual oil dissolved in methanol and the dihydrobromide[6] precipitated by ether; wt. 1.05 g. (75%).

Coupling of the Azide of S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine with L-Prolyl-L-arginylglycinamide Hydrobromide and Conversion of the Product to Active Material.—S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine hydrazide (1.9 g., 1.7 m*M*) was dissolved in glacial acetic acid (160 ml.), concd. HCl (16.4 ml.) and water and cooled to −2°, when sodium nitrite (0.1 *N*, 16.4 ml.) was added. The mixture was allowed to stir for 10 minutes at −2°. The azide was precipitated by addition of cold water (300 ml.), filtered and washed successively with cold water, cold 5% $NaHCO_3$, cold water and dried *in vacuo* over P_2O_5 at 4°. It was then added to a solution of L-prolyl-L-arginylglycinamide monohydrobromide (0.85 g., 1.7 m*M*) in dimethylformamide (15 ml.). The monohydrobromide was prepared from 1.92 g. of the dihydrobromide by dissolving the latter in 4 ml. of dimethylformamide and adding 0.67 ml. of triethylamine. After the mixture was cooled in ice, it was filtered, and the precipitate was washed with a little dimethylformamide. The monohydrobromide (1.3 g.) was precipitated from the filtrate with chloroform. The solution of azide and tripeptide amide was allowed to stir overnight at 4°. The product was precipitated by ether, washed with ethanol and ether and dried; wt. 2.2 g. This product was combined with a similar product (0.4 g.) from another run on a smaller scale. The material was divided into four portions and reduced with sodium in liquid ammonia.[15,16] A few drops of glacial acetic acid were added to discharge the blue color. The residue after the evaporation of the ammonia from each portion was dissolved in 650 ml. of 0.2% acetic acid and, after adjustment of the *p*H to 6.7, the solution was aerated for 1 hr. with a slow stream of CO_2-free air.[9] The solutions, representing 120,000 units of pressor activity, were then combined, the *p*H adjusted to 4.9 and the activity absorbed on a column (2 × 25 cm.) of IRC-50 (H^+ form). The column was washed with 0.25% acetic acid until the *p*H reached about 3.9, then with 50 ml. of water, and the active material was eluted with pyridine-acetic buffer.[12] Lyophilization of this material yielded a white, fluffy powder representing 120,000 units of pressor activity. The material was purified by countercurrent distribution in 2-butanol-0.06 *M* *p*-toluenesulfonic acid and by electrophoresis on a cellulose block at *p*H 4 in pyridine–acetate buffer.[9] It was then subjected to chromatography on a column of IRC-50 (H^+ form) with 0.65 *M* ammonium acetate, *p*H 6.5.[12]

The synthetic material (28 mg.) was compared with natural arginine-vasopressin by electrophoresis on paper, ion exchange chromatography, paper chromatography and assay for pressor activity in the rat. They proved to be indistinguishable in these respects. In addition, a starch column analysis of the synthetic product showed the eight amino acids and ammonia to be present in the expected ratios.

Acknowledgments.—The authors are indebted to Miss Dade W. Tull and Miss Maureen O'Connell for carrying out the assays for pressor activity, Mrs. Lorraine S. Abrash for the amino acid analyses and Mr. Joseph Albert for the microanalyses.

NEW YORK, N. Y.

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