Ethyl α-carbethoxy-α-nitro-β-(4-cyano-3-indole)-propionate was prepared for study as a possible precursor of α-amino substituted β-(4-carboxy-3-indole)-propionic acid derivatives. To a solution of 23 mg. (0.001 mole) of sodium in 3 ml. of absolute ethanol was added 205 mg. (0.001 mole) of ethyl nitromalonate[37] and 341 mg. (0.001 mole) of 4-cyanogramine methiodide (V). After the mixture had been maintained at reflux temperature for 15 hours, the ethanol was distilled under reduced pressure. The residue was dissolved in ether and was washed with dilute hydrochloric acid and with water. The remainder from the dried ($MgSO_4$) ether solution was recrystallized from absolute ethanol; yield 170 mg. (47%), m.p. 131–133°.

Anal. Calcd. for $C_{17}H_{17}N_3O_6$ (359.33): C, 56.82; H, 4.77; N, 11.70. Found: C, 56.75; H, 4.48; N, 11.57.

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BOSTON 15, MASS.

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

Oxypressin, a Synthetic Octapeptide Amide with Hormonal Properties[1]

BY PANAYOTIS G. KATSOYANNIS

RECEIVED AUGUST 13, 1956

A cyclic octapeptide amide containing a cyclic pentapeptide amide moiety identical with the one existing in vasopressin and a tripeptide amide side chain identical with the one existing in oxytocin has been synthesized and tested for biological activity. It was found to possess oxytocic, avian depressor and pressor activities to quantitatively different degrees and in different ratios from those existing in oxytocin and vasopressin.

Oxytocin, the principal uterine-contracting and milk-ejecting hormone of the posterior pituitary gland, has been obtained in highly purified form[2–4] in this Laboratory. Structure I was suggested for

CyS—Tyr—Ileu—Glu(NH_2)—Asp(NH_2)—CyS—Pro—
Leu—Gly(NH_2)

I

the hormone on the basis of degradative studies[5–7] and confirmed by synthesis.[7] Arginine vasopressin, the principal pressor and antidiuretic hormone of the beef posterior pituitary gland, has also been isolated in highly purified form[8] and degradative studies have resulted in the postulation of structure II to represent this hormone.[9–11] It

CyS—Tyr—Phe—Glu(NH_2)—Asp(NH_2)—CyS—Pro—
Arg—Gly(NH_2)

II

will be noted that both oxytocin and vasopressin are octapeptide amides composed of a cyclic pentapeptide amide moiety linked to a tripeptide amide side chain. Six amino acids are common to both hormones and they differ from each other only in the other two amino acids; phenylalanine, present in vasopressin, is replaced by isoleucine in oxytocin and the arginine of vasopressin is replaced by leucine in oxytocin. However, in spite of their closely related composition and structures, oxytocin and vasopressin differ considerably in their biological properties.

It is therefore of interest to find out what effect changes in their structures will have on the biological properties of the hormones, with the aim of possibly correlating these changes in chemical structure with changes in biological properties. This paper describes the synthesis and purification of such a "modified hormone" which we have tentatively named "Oxypressin." This compound is the cyclic disulfide of L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. It contains a cyclic pentapeptide amide portion identical with the one existing in vasopressin, linked to the tripeptide amide side chain that is present in oxytocin. In other words oxypressin, III, can be con-

CyS—Tyr—Phe—Glu(NH_2)—Asp(NH_2)—CyS—Pro—
Leu—Gly(NH_2)

III

sidered either as oxytocin with phenylalanine replacing isoleucine in the ring or as vasopressin with leucine replacing arginine in the side chain.

The synthesis of this octapeptide followed the pattern introduced in this Laboratory for the synthesis of the posterior pituitary hormones, in that it involved the preparation of a protected nonapeptide (in this case, S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide), cleavage of the protecting groups with sodium in liquid ammonia and oxidation of the resulting sulfhydryl nonapeptide to the cyclic octapeptide amide. The synthesis of the

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(11) An octapeptide amide was synthesized [V. du Vigneaud, D. T. Gish and P. G. Katsoyannis, THIS JOURNAL, **76**, 4751 (1954)] according to the structure proposed for arginine–vasopressin and found to possess biological properties associated with the natural hormone.

nonapeptide intermediate for oxypressin was accomplished by coupling the pentapeptide, S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine,[12] with the tetrapeptide amide, S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide,[13] by the tetraethyl pyrophosphite method.[14] The product was thus obtained as an amorphous solid, m.p. 197–201°, in a yield of 57%. After purification for analysis, the compound melted at 210–212°. The protected nonapeptide was then treated with sodium in liquid ammonia and the reduced material thus obtained was oxidized, after evaporation of the ammonia, by aeration in aqueous solution at *p*H 6.7 and tested for biological activity. The product was tested for oxytocic, avian depressor and pressor activity and found to possess these activities to quantitatively different degrees and in different ratios from those existing between these activities in oxytocin and vasopressin. The avian depressor activity[15] of the synthetic preparation was used as a criterion of purity during the purification of the crude material. The material was distributed by countercurrent distribution in the system *sec*-butyl alcohol–0.1% acetic acid for 900 transfers. The active material was concentrated in a single peak having a partition coefficient (*K*) of 0.48. The contents of the tubes containing the active material were combined, concentrated and lyophilized. During electrophoresis on paper[16] in a pyridine–acetic acid buffer at *p*H 5.6[17] this preparation behaved as an homogeneous compound and exhibited the same electrophoretic mobility as oxytocin. Starch column chromatography[18] of the product after acid hydrolysis showed the constituent amino acids to be present in molar ratios of approximately 1:1, namely, cystine 0.8, tyrosine 0.8, phenylalanine 1.0, glutamic acid 1.1, aspartic acid 1.0, proline 0.9, leucine 1.0, glycine 1.1, and ammonia 3.5. The preparation possessed the specific rotation $[\alpha]^{22}D$ −33° (*c* 0.57, 0.1 *N* acetic acid).

This purified material was then assayed for oxytocic, avian depressor and pressor activity.[19] The potency of oxypressin in relation to the U.S.P. Standard Powder is compared with that of natural oxytocin and vasopressin[4,20,21] in Table I. Of particular interest is the fact that oxypressin possesses the various biological activities of oxytocin and vasopressin in quite different ratios from those existing between these activities in the hormones themselves. The possibility therefore presents itself that by making certain changes in the chemical structure of the natural hormones it may be possible to produce synthetic compounds with highly desirable biological properties.

TABLE I

POTENCY OF OXYPRESSIN AND NATURAL OXYTOCIN AND ARGININE VASOPRESSIN

(All figures are U.S.P. units per mg.[a])

Hormones	Oxytocic (rat uterus[22])	Avian depressor (fowl[15])	Pressor (rat[22])	Ratios of activities (oxytocic: avian depressor: pressor)
Oxypressin	20	45	3	1:2.25:0.15
Oxytocin	500	500	7	1:1:0.014
Vasopressin	30	85	600	1:2.83:20

[a] These assay values are based on an activity of 0.47 unit per mg. for the United States Pharmacopeia Posterior Pituitary Standard Powder. More recently, this same Standard Powder has been assigned a value of 0.40 unit per mg. This would necessarily affect the values given above.

Experimental

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide.—A finely powdered mixture of 180 mg. (0.20 m*M*) of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine[24] and 100 mg. (0.20 m*M*) of S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide[13] was suspended in 0.7 ml. of anhydrous diethyl phosphite and 0.15 ml. of tetraethyl pyrophosphite[14] and heated at 90° for 1 hr. After cooling, the product was precipitated with cold water as a white solid, which was separated by filtration. The crude product was triturated with dilute aqueous $NaHCO_3$ solution, filtered off and washed with water. The wet material was suspended in 20% aqueous ethyl alcohol and stirred for 20 minutes while the *p*H was maintained at approximately 9 by the stepwise addition of triethylamine. The product was then separated by filtration, washed with aqueous ethyl alcohol and dried; wt. 160 mg., m.p. (capillary) 197–201°. Material of this purity was used for further work. For analysis, a sample was triturated repeatedly with aqueous ethyl alcohol plus triethylamine in the manner already described and was finally precipitated from solution in dimethylformamide by addition of ether; m.p. 210–212°; $[\alpha]^{21}D$ −48.1° (*c* 0.8, dimethylformamide).

Anal. Calcd. for $C_{65}H_{84}O_{14}N_{12}S_2$: N, 12.4; S, 4.70. Found: N, 11.9; S, 4.35.

Reduction of Protected Nonapeptide Amide Followed by Oxidation.—The reduction procedure was similar to the one described previously[25,7] for the regeneration of oxytocin from its S,S′-dibenzyl derivative. Two hundred mg. of the protected nonapeptide amide was dissolved in 50 ml. of liquid ammonia in a 250-ml. r.b. flask. The reduction was carried out at the boiling point of the solution by dipping a glass tube containing sodium below the surface of the solution until a permanent blue color was obtained. The blue color was allowed to persist for 2 minutes and was then discharged with the addition of 35 mg. of ammonium chloride. The ammonia was evaporated at atmospheric pressure and the last traces were removed *in vacuo*. The residue was dissolved in 500 ml. of 0.1% acetic acid, the *p*H was adjusted to 6.7 with ammonium hydroxide and the solution was aerated with CO_2-free air for 1 hr. This procedure was repeated several times and the solutions were combined at this point, concentrated in a rotary evaporator[26] and lyo-

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(24) The protected pentapeptide used for this experiment was an amorphous powder (ref. 11), m.p. 208–209° and $[\alpha]^{21}D$ −26° (*c* 1, dimethylformamide). This peptide has recently been crystallized (ref. 12.)

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philized. A total of 1.3 g. of crude protected nonapeptide was reduced in this manner and the product, assayed by the Coon method,[15] possessed an activity of approximately 10,000 avian depressor units.

Purification and Isolation of the Active Product.—The lyophilized crude product was placed in the first 10 tubes of the all-glass automatic countercurrent distribution apparatus[27] and distributed in the system *sec*-butyl alcohol–0.1% acetic acid. The progress of the purification was followed by determining the Folin color[28] and the biological activity of selected tubes. After 900 transfers the activity was concentrated in a single peak (K = 0.48) and was almost completely separated from a slower moving inactive component. The contents of the tubes with the active material were combined, concentrated in a rotary evaporator at a temperature below 30° and lyophilized to give 135 mg. of a white fluffy solid. The biological assays were performed on this solid and the results are given in Table I. The synthetic material possessed the specific rotation $[\alpha]^{22}D$ −33° (c 0.57, 0.1 N acetic acid).

Two mg. of the synthetic product and a similar amount of natural oxytocin were applied separately in pyridine–acetic acid buffer at pH 5.6 to a strip of Whatman No. 1 filter paper for electrophoresis, and 400 v. was applied for 24 hours at 5°. The material was stained on the paper by the method of Durrum[29] in which the dye, brom phenol blue, in ethyl alcohol saturated with mercuric chloride is employed. Both compounds, oxypressin and oxytocin, gave a single spot and exhibited the same mobility.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, ILLINOIS INSTITUTE OF TECHNOLOGY]

Oxygen-18 Studies of the Mechanism of the α-Chymotrypsin-catalyzed Hydrolysis of Esters[1]

BY MYRON L. BENDER AND KENNETH C. KEMP[2]

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An investigation of the mechanism of the α-chymotrypsin-catalyzed hydrolysis of esters has been made utilizing oxygen-18 techniques. The carbonyl oxygen exchange accompanying hydrolysis, the mode of fission and the alcoholysis accompanying hydrolysis were studied. The α-chymotrypsin-catalyzed hydrolysis of esters was found to differ from non-enzymatic (alkaline) hydrolysis with respect to carbonyl oxygen exchange during the hydrolytic process. Such oxygen exchange was found to occur during the alkaline hydrolysis of methyl β-phenylpropionate-*carbonyl*-O^{18} and benzoyl-L-phenylalanine ethyl ester-*carbonyl*-O^{18}, but not during the α-chymotrypsin-catalyzed hydrolysis of these esters. However, both the α-chymotrypsin-catalyzed and base-catalyzed hydrolytic reactions occur by means of acyl–oxygen fission. This was demonstrated in the enzymatic case by means of the hydrolysis of methyl β-phenylpropionate-*alkoxyl*-O^{18}. Both methyl β-phenylpropionate-*alkoxyl*-O^{18} and benzoyl-L-phenylalanine ethyl ester underwent simultaneous hydrolysis and methanolysis in the presence of α-chymotrypsin, the rate of methanolysis being greater than that of hydrolysis. The alcoholysis may be considered another example of an enzymatic exchange reaction. The above results are consistent with a mechanism of α-chymotrypsin action involving an acyl–enzyme intermediate.

Introduction

The report is the third in a series of investigations designed to explore the mechanism of action of a typical endopeptidase, α-chymotrypsin.[3,4] These studies are concerned not with the elucidation of multi-enzymatic systems, but rather with the determination of the detailed mechanism of individual acts by a hydrolytic enzyme, including a description of all intermediates in the process with the twin aims of elucidating the structure of the "active site" on the enzyme and of attempting the synthesis of a compound capable of producing enzymatic hydrolysis. The general approach of these investigations is to compare enzymatic and non-enzymatic hydrolyses from a mechanistic viewpoint. Following this procedure, it is hoped that the methods and results of studies of the mechanism of non-enzymatic hydrolysis, which are relatively complete,[5] can be applied to their enzymatic counterparts. The assumption is made here, of course, that enzymatic catalysis is explicable in chemical terms and that non-enzymatic hydrolysis is the proper point of departure for such an explanation. The methods that have yielded the most information in elucidating the mechanism of non-enzymatic hydrolysis have been kinetic studies, the effect of structure on reactivity and isotopic studies involving oxygen-18 for cleavage and exchange experiments. This report concerns an investigation of the mechanism of the α-chymotrypsin-catalyzed hydrolysis of esters utilizing oxygen-18 techniques. The carbonyl oxygen exchange accompanying hydrolysis, the mode of fission and the alcoholysis accompanying hydrolysis have been studied.

(1) This investigation was aided by research grant G-3787 from the National Institutes of Health, U. S. Public Health Service.

(2) From the Ph.D. thesis of K. C. K.

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Experimental

Materials.—α-Chymotrypsin was an Armour and Co., salt-free preparation. The concentration of α-chymotrypsin was determined by measuring the optical density at 282 mμ in a Beckman DU spectrophotometer. A standard calibration curve was obtained by determining the enzyme concentration by a micro-Kjeldahl procedure.[6] Methanol (Baker and Adamson Co.) was distilled, b.p. 64°. The oxygen-18 labeled esters listed in Table I were prepared by conventional means.

Kinetics of Carbonyl Oxygen Exchange during Alkaline Hydrolysis.—The rates of the alkaline hydrolysis of methyl and ethyl β-phenylpropionate-*carbonyl*-O^{18} and benzoyl-L-

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