Studies on Polypeptides. XLVI. Synthesis of a Stably Labeled, Biologically Active Adrenocorticotropin Fragment[1-3]

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A synthesis of [^{14}C]phenylalanine^{7},glutamine^{5}-β-corticotropin$_{1-20}$ amide is described. The synthetic peptide exhibited 150% of the *in vivo* rat steroidogenic activity of corticotropin A_1 on a weight basis. It is concluded that the γ-carboxyl group of glutamic acid^{5} in β-corticotropin$_{1-20}$ amide is not essential for biological function.

The labeling of ACTH with radioiodine for immunological studies has been reported by several investigators[4-7] but the effect of such labeling on the adrenocorticotropic activity of the hormone has not been assessed adequately. Since the 39 amino acid residue ACTH molecule contains various positions (Tyr-2 and -23, His-6, and Trp-9), which are susceptible to iodination it is difficult to pinpoint the exact position of the iodine in the labeled molecules. Moreover, the relatively short half-life of the available iodine isotopes necessitates frequent relabeling and the labeling pattern of the ensuing products may well vary from batch to batch.

In connection with biochemical studies we required a stably labeled peptide of established structure possessing adrenocorticotropic activity. The synthesis of such a material, *i.e.*, [^{14}C]Phe^{7},Gln^{5}-β-corticotropin$_{1-20}$ amide is described in this communication.

Preparative Aspects.—[^{14}C]Phe^{7},Gln^{5}-β-corticotropin$_{1-20}$ amide (I) was constructed *via* the route shown on Chart I. The Phe residue was selected for labeling since it is located in the very section (active site) of the ACTH molecule which appears to be connected intimately with biological function.[8] The synthesis is patterned according to a scheme which was employed for the preparation of Abut^{4},Gln^{5}-β-corticotropin$_{1-20}$ amide[9] but incorporates several improvements. Steps involving radioactive intermediates were perfected by the use of corresponding unlabeled compounds.

The pentapeptide histidylphenylalanylarginyltryptophylglycine (VI) a structural element which is common to α-MSH, β-MSH, and ACTH from various species[10] has been the subject of a number of synthetic studies.[11-15] Since previous routes to this peptide involved steps which proceeded in poor yields, or approaches in which strong acids were employed to remove protecting groups from intermediates containing the acid-sensitive Trp residue we have now devised an alternative method.

Methyl tryptophylglycinate was acylated with a mixed anhydride of benzyloxycarbonylnitroarginine by the method of Anderson[16] to give methyl benzyloxycarbonylnitroarginyltryptophylglycinate, which was saponified. The ensuing product II was hydrogenated to afford III. Randomly labeled [^{14}C]Phe was carbobenzoxylated and the benzyloxycarbonyl derivative converted into the *N*-hydroxysuccinimido ester (IV).[17] This ester was used to acylate III to give the labeled peptide derivative V in excellent yield. The benzyloxycarbonyl group was removed by hydrogenolysis and the ensuing free tetrapeptide coupled with benzyloxycarbonylhistidine azide.[1] Hydrogenation of the protected pentapeptide intermediate gave VI. The labeled protected decapeptide VIII resulted from the reaction of the azide VII[9] with VI. In order to have available the labeled N-terminal decapeptide section of the ACTH molecule an aliquot of VIII was deprotected.

Our original synthesis[18] of the tetrapeptide amide XI has now been replaced by a modified route which eliminates several steps and proceeds in better yields. For the preparation of valine amide the *N*-hydroxysuccinimido ester of benzyloxycarbonylvaline[17] was treated with NH_3 in $CHCl_3$ and the ensuing benzyloxycarbonylvaline amide was deblocked by hydrogenation. The valine amide was treated with *N*-hydroxysuccinimidobenzyloxycarbonylprolinate[17] to afford benzyloxycarbonylprolylvaline amide, which was converted into IX as described.[18]

For the preparation of X benzyloxycarbonylnitroarginylprolylvaline amide (IX) was deblocked with HBr in AcOH and the ensuing peptide amide coupled with a mixed anhydride of benzyloxycarbonylnitroarginine.[1,16] The arginylarginylprolylvaline amide XI which resulted when X was subjected to hydrogenolysis was used without purification. The coupling of XI

(1) See K. Hofmann, R. Andreatta, H. Bohn, and L. Moroder, *J. Med. Chem.*, **13**, 339 (1970) for paper XLV in this series.

(2) Supported by grants from the U. S. Public Health Service and the Hoffmann-La Roche Foundation.

(3) The amino acid residues except glycine are of the L configuration. Abbreviations used are: Boc = *t*-butoxycarbonyl; Z = benzyloxycarbonyl; ONHS = *N*-hydroxysuccinimido; TEA = Et_3N; TFA = F_3CCO_2H; CM-cellulose = carboxymethylcellulose; Bio-Rex 70 = cation-exchange resin (Bio-Rad Co., Richmond, Calif.; Amberlite IRA-400 = anion-exchange resin (Rohm and Haas Co., Philadelphia, Pa.); PPO = 2,5-diphenyloxazole; POPOP = 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene; AP-M = aminopeptidase M. [G. Pfleiderer, P. G. Celliers, M. Stanulovic, E. D. Wachsmuth, H. Determann, and G. Braunitzer, *Biochem. Z.*, **340**, 552 (1964)]; Corticotropin A_1 is column-purified porcine ACTH, which exhibits *in vivo* steroidogenic activity of approximately 100U/mg [H. B. F. Dixon and M. P. Stack-Dunne, *Biochem. J.*, **61**, 483 (1955)].

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(9) K. Hofmann, J. Rosenthaler, R. D. Wells, and H. Yajima, *J. Amer. Chem. Soc.*, **86**, 4991 (1964).

(10) For a review see K. Hofmann, *Annu. Rev. Biochem.*, **31**, 213 (1962).

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(14) R. Geiger, K. Sturm, and W. Siedel, *Chem. Ber.*, **96**, 1080 (1963).

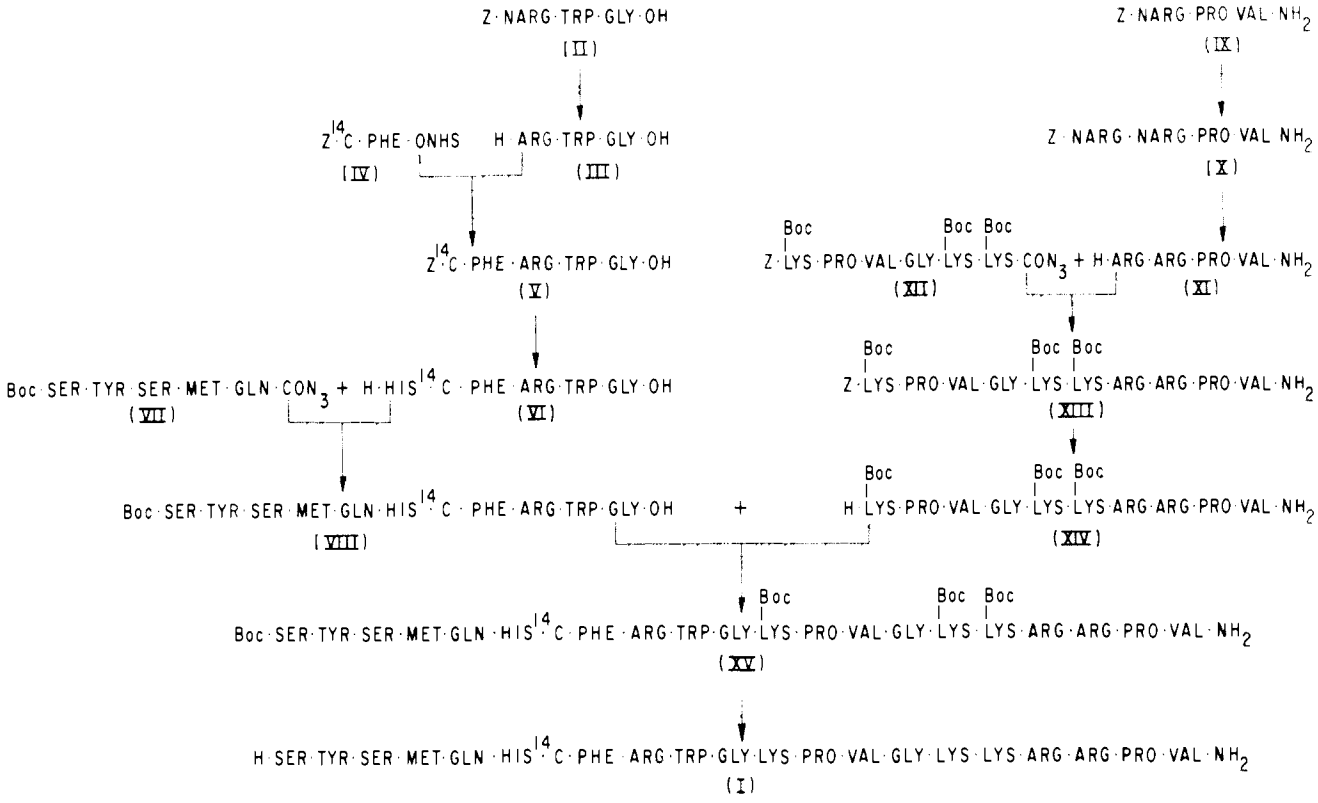
(15) K. Inouye, *Bull. Chem. Soc. Jap.*, **38**, 1148 (1965).

(16) G. W. Anderson, F. M. Callahan, and J. E. Zimmerman, *J. Amer. Chem. Soc.*, **89**, 178 (1967).

(17) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *ibid.*, **86**, 1839 (1964).

(18) K. Hofmann, H. Yajima, T-Y. Liu, N. Yanaihara, C. Yanaihara, and J. L. Humes, *ibid.*, **84**, 4481 (1962).

Chart I



and XII and the partial deprotection of the resulting XIII to give XIV is described.[9]

The time-consuming removal of NH_4OAc from CM-cellulose chromatographed peptides by lyophilization to constant weight was replaced by desalting with Bio-Rex 70.[19]

The tosylate salts of VIII and XIV were coupled by the DCI procedure,[1,20] and the ensuing protected peptide XV purified on CM-cellulose. The homogeneous protected peptide XV was deblocked with 90% trifluoroacetic acid, trifluoroacetic acid ions were exchanged for AcO^-, and the peptide was exposed to thioglycolic acid to reduce fully the methionine residue. The amino acid ratios in acid hydrolysates of the final product agreed with those expected by theory.

Experimental Section

General Procedures.—Melting points are uncorrected. Rotations were determined with a Zeiss precision polarimeter. Measurements were carried out with an Hg lamp at 546 and 578 mμ and extrapolated to the 589-mμ Na line. Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.; O values were actually determined and not computed by difference. The amino acid composition of acid and enzymic hydrolysates was determined with a Beckmann-Spinco Model 120 amino acid analyzer.[21] The figures in parentheses are average recoveries of amino acids. Nle and α-amino-β-guanidopropionic acid were used as internal standards. Acid hydrolysates were performed in constant boiling point HCl at 110° for 24 hr in evacuated tubes; values are not corrected for amino acid destruction. AP-M digestions and thioglycolate reductions were carried out as described.[22] See Hofmann, *et al.*,[23] for designation of solvent systems for tlc chromatograms. The designation of the solvent systems used for tlc chromatography in the Experimental Section of paper XLV in this series[1] is incorrect and should read as follows: $R_f^{I} = R_f^{VI}$; $R_f^{III} = R_f^{VII}$ and $R_f^{IV} = R_f^{X}$.

Radioactivity was measured with a Packard Model 574 liquid scintillation spectrometer in a scintillation medium composed of PPO (9.8 g), POPOP (0.2 g) in toluene (2000 ml), and ethylene glycol monomethyl ether (1200 ml). A total of 5000 counts was collected for each sample and converted into dpm in the usual manner, using [^{14}C]toluene as internal standard.

Steroidogenic activity was determined in hypophysectomized or Dexamethasone-blocked rats.[24]

***N*-Hydroxysuccinimidobenzyloxycarbonyl[^{14}C]phenylalaninate (IV).**—Phenylalanine (0.6 g; 3.63 mmoles) and randomly labeled [^{14}C]Phe obtained from International Chemical and Nuclear Corp. (0.5 mCi in 0.01 *N* HCl) were dissolved in 1 *N* NaOH (3.7 ml) and carbobenzoxylated.[25] The oily benzyloxycarbonyl derivative (1.03 g; 3.44 mmoles) was then converted into the *N*-hydroxysuccinimido ester.[17] The ester was crystallized to constant specific radioactivity from $CHCl_3$–Et_2O; yield 1.02 g (75%); mp 140–141°; lit.[17] mp 140.5–141°; specific radioactivity 0.126 mCi/mmole.

Benzyloxycarbonylvaline Amide.—*N*-Hydroxysuccinimidobenzyloxycarbonylvalinate[17] (20 g) was dissolved in $CHCl_3$ (300 ml) and a slow stream of NH_3 was passed through the solution for 2 hr at 0°. The solvent was evaporated and the product was recrystallized three times from MeOH; yield 11.86 g. The combined mother liquors were evaporated to dryness and the residue was distributed between H_2O and EtOAc. The EtOAc layers were evaporated and the residue was recrystallized from MeOH: yield 1.36 g; total yield 13.22 g (92%); mp, 207–208°; $[\alpha]^{29}D$ +21.4° (*c* 1.6, DMF); R_f^{VI} 0.8; lit.[26] mp 206–208°; $[\alpha]^{22}D$ +22° (*c* 2.0, DMF).

Benzyloxycarbonylprolylvaline Amide.—Benzyloxycarbonylvaline amide (11.0 g; 43.95 mmoles) was hydrogenated over Pd in MeOH (120 ml) and 20% AcOH (13.5 ml). The ensuing valine amide acetate was dissolved in DMF (150 ml), *N*-hydroxysuccinimido benzyloxycarbonylprolinate[17] (15.22 g; 43.94 mmoles)

(19) K. Hofmann and H. Bohn, *J. Amer. Chem. Soc.*, **88**, 5914 (1966).
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(26) M. Bodanszky and N. J. Williams, *J. Amer. Chem. Soc.*, **89**, 685 (1967).

and TEA (6.27 ml) were added and the mixture was kept at room temperature for 12 hr. The solution was evaporated to dryness and the residue was crystallized first from MeOH–H_2O then from MeOH–Et_2O: yield 11.78 g (77.1%); mp 205–207°; $[\alpha]^{29}D$ −80.4° (*c* 0.964, AcOH); R_f^{VI} 0.8; lit.[11] mp 201–203°; $[\alpha]^{23}D$ −80.2° (*c* 1.14, AcOH).

Benzyloxycarbonylnitroarginylnitroarginylprolylvaline Amide·MeOH Solvate (X).—Benzyloxycarbonylnitroarginylprolylvaline amide[18] (IX) (2.3 g; 4.19 mmoles) was dissolved in AcOH (6 ml), HBr in AcOH (3.3 *N*) (20 ml) was added, and the solution was kept at room temperature for 1 hr. The bulk of the solvent was removed, the product was precipitated by addition of Et_2O and was reprecipitated three times from MeOH with Et_2O and dried over KOH. The hydrobromide was dissolved in DMF (25 ml) and TEA was added to a pH of 8 (approximately 0.74 ml required). To this solution was added at 0° a solution containing a mixed anhydride prepared from benzyloxycarbonylnitroarginine (2.04 g; 5.77 mmoles) in THF (40 ml) with *N*-methylmorpholine (0.644 ml; 5.77 mmoles) and isobutyl chloroformate (0.68 ml; 5.19 mmoles).[16]

The reaction mixture was stirred for 10 min at 0°, the temperature was raised slowly to 45°, then the bulk of the solvents was evaporated, and the product precipitated by addition of EtOH. For purification the material was distributed between *n*-BuOH and 10% AcOH, the *n*-BuOH layers were washed with 5% $NaHCO_3$ and H_2O and were concentrated to a small volume. The product was precipitated by addition of EtOAc; yield 2.71 g (83%); a sample for analysis was precipitated once from MeOH with EtOAc and once from MeOH with Et_2O: mp 128–130°; $[\alpha]^{28}D$ −31.3° (*c* 1.09, DMF); $[\alpha]^{29}D$ −54.6° (*c* 1.0 MeOH). *Anal.* ($C_{30}H_{47}N_{13}O_{10}\cdot CH_3OH$): C, H, N, O.

Arginylarginylprolylvaline Amide Triacetate Tetrahydrate (XI).—Compound X (3.25 g) was hydrogenated for 24 hr over Pd in MeOH (50 ml) and 10% AcOH (100 ml). The product was lyophilized several times from dioxane–H_2O (1:1): yield 3.13 g (97%); $[\alpha]^{28}D$, −44.2° (*c* 1.0, 10% AcOH); lit.[18] $[\alpha]^{31}D$ −40.2° (*c* 1.1, 10% AcOH); R_f^{VI} origin; R_f^{VII} 0.4; amino acid ratios in AP-M digest, $Arg_{1.9}Pro_{1.1}Val_{1.0}$ (88%).

Benzyloxycarbonylnitroarginyltryptophylglycine (II).—Methyl tryptophylglycinate hydrochloride hemihydrate[12] (2.12 g; 6.8 mmoles) in THF (120 ml) and TEA (0.95 ml; 6.81 mmoles) was acylated with a mixed anhydride prepared from benzyloxycarbonylnitroarginine (4.24 g; 12 mmoles).[16] The reaction mixture was evaporated to dryness, the residue was dissolved in a mixture of EtOAc (450 ml) and MeOH (50 ml), and the solution was washed in the usual manner with 1 *N* HCl-saturated $NaHCO_3$ and H_2O. The dried organic phases were evaporated and the residue was triturated first with Et_2O then with boiling EtOAc: yield 3.9 g (94%); mp 117–118°; $[\alpha]^{20}D$ −20.5° (*c* 1.33, MeOH); R_f^{VI} 0.8; R_f^{VII} 0.8; lit.[14] mp 126–128°; $[\alpha]^{22}D$ −25.8° (*c* 1.3, MeOH). This material (3.4 g; 5.57 mmoles) was dissolved in dioxane (20 ml) and 1 *N* NaOH (6.5 ml; 6.5 mmoles) was added. The mixture was kept at room temperature for 45 min, the organic solvent was evaporated, 1.5% NH_4OH (80 ml) was added to the residue, and the solution was extracted with EtOAc. The organic layers were washed with three 40-ml portions of 3% NH_4OH and the pooled aqueous phases were cooled at 0° and acidified with 1 *N* HCl. The precipitate was collected, washed with H_2O, and dried over P_2O_5 and KOH: yield 2.83 g (85%); mp 211–213°; $[\alpha]^{28}D$ −13.2° (*c* 1.5, DMF); R_f^{VI} 0.7; R_f^{VII} 0.6. *Anal.* ($C_{27}H_{32}N_8O_8$) C, H, N, O.

Arginyltryptophylglycine (III).—Benzyloxycarbonylnitroarginyltryptophylglycine (II) (3.2 g) was hydrogenated for 24 hr over Pd in MeOH (80 ml) and 10% AcOH (80 ml). The crude material was applied to a CM-cellulose column (3 × 25 cm) in 500 ml of H_2O and the column was eluted with pH 6.8 NH_4OAc, 0.005 *M* (500 ml) and 0.015 *M* (3000 ml). Sakaguchi-positive 0.015 *M* eluates were pooled, the solution was concentrated to approximately 300 ml, and Bio-Rex 70 (H^+ form) was added until the supernatant was Sakaguchi negative. The resin was collected and was washed with H_2O until the washings failed to give a Nessler test. The washed resin was then suspended in ice-cold, freshly prepared 4 *N* NH_4OH (150 ml) and the suspension was stirred for 30 min. The resin was collected and eluted in the same manner with three additional portions of NH_4OH. The combined eluates were evaporated to a small volume, and the solution was lyophilized: yield 1.99 g (89%); $[\alpha]^{28}D$ +3.9° (*c* 1.01, H_2O); R_f^{VI} 0.1; R_f^{VII} 0.4 single Cl-, Sakaguchi-, Ehrlich-, and ninhydrin-positive spot; amino acid ratios in AP-M digest, $Arg_{1.0}Trp_{1.1}Gly_{1.0}$ (95%). A sample was converted into the acetate by lyophilization from 5% AcOH: $[\alpha]^{29}D$, +26.3° (*c* 0.98,) H_2O).

Benzyloxycarbonyl[^{14}C]phenylalanylarginyltryptophylglycine Monohydrate (V).—Compound IV (1.014 g; 2.56 mmoles) was added to a solution of arginyltryptophylglycine (1.175 g; 2.81 mmoles) in DMF (40 ml), H_2O (2 ml), and TEA (0.36 ml; 2.56 mmoles) and the mixture was kept at room temperature for 12 hr. The bulk of the solvent was removed and the product was precipitated by addition of H_2O. For purification the material was twice precipitated from DMF with H_2O: yield 1.7 g (84%); $[\alpha]^{27}D$ −12.9° (*c* 0.924, DMF); R_f^{VI} 0.5; R_f^{VII} 0.7. *Anal.* ($C_{36}H_{42}N_8O_6\cdot H_2O$) C, H, N, O.

Histidyl[^{14}C]phenylalanylarginyltryptophylglycine Diacetate Dihydrate (VI).—The monohydrate V (1.695 g; 2.364 mmoles) was hydrogenated for 24 hr over Pd in DMF (100 ml) and the product was isolated in the usual manner and precipitated from MeOH with Et_2O: $[\alpha]^{28}D$, −4.0° (*c* 1.5, 1 *N* HCl); lit.[12] $[\alpha]^{28}D$, −4.1° (*c* 1.0, 1 *N* HCl). A solution of this product in DMF (30 ml) was added at −60° to an azide solution prepared from benzyloxycarbonylhistidine hydrazide[27] (1.434 g; 4.728 mmoles) in the manner described previously.[1] The reaction mixture was stirred at 5° for 24 hr, the bulk of the solvent was evaporated, and the product was precipitated by addition of H_2O saturated with EtOAc. The protected peptide was decarbobenzoxylated by hydrogenation over Pd in 50% aq MeOH (100 ml) and DMF (20 ml) and the product was lyophilized from 10% AcOH: yield 1.92 g (94%); $[\alpha]^{27}D$ −12.0° (*c* 0.855, 1 *N* HCl); lit.[12] $[\alpha]^{28}D$ −12.0° (*c* 1.0, 1 *N* HCl); R_f^{VI} 0.1; R_f^{VII} 0.4.

***N*-*t*-Butoxycarbonylseryltyrosylserylmethionylglutaminylhistidyl[^{14}C]phenylalanylarginyltryptophylglycine Acetate Dihydrate (VIII).**—To a stirred solution cooled at −10° of *N*-*t*-butoxycarbonylseryltyrosylserylmethionylglutamine hydrazide monohydrate[1] (1.324 g; 1.773 mmoles) in DMF (9 ml) and H_2O (0.75 ml) 1 *N* HCl (3.6 ml) was added followed by $NaNO_2$ (0.135 g; 1.96 mmoles) in 1.5 ml of H_2O. The solution was stirred at −10° for 30 min, then TEA (10% in DMF 2.5 ml; 1.80 mmoles) was added. To this solution was added a solution, precooled at 0°, of VI (0.949 g; 1.110 mmoles) in DMF (10 ml), H_2O (2 ml), and TEA (10% in DMF 1.55 ml; 1.18 mmoles). The mixture was stirred at 5° for 72 hr, the pH being maintained at 7.5–8.0 by addition of TEA. H_2O (150 ml) was added and the mixture was kept in a refrigerator for 12 hr. The gelatinous precipitate was collected and dissolved in 20% AcOH and the solution was lyophilized: yield 1.36 g (82%); $[\alpha]^{28}D$ −14.5° (*c* 0.6, AcOH); lit.[28] $[\alpha]^{25}D$ −21.8° (*c* 0.321, AcOH); R_f^{VI} 0.3; R_f^{VII} 0.6; amino acid ratios in acid hydrolysate: $Ser_{1.9}Tyr_{1.0}Met_{1.0}Glu_{1.1}His_{1.0}$-$Phe_{1.0}Arg_{1.0}Gly_{1.0}$ (97%). *Anal.* ($C_{64}H_{87}N_{17}O_{17}\cdot AcOH\cdot 2H_2O$ C, H, N.

Tosylate Salt.—To an ice-cold solution of VIII (106 mg; 71 μmoles) in 50% aq pyridine (4 ml) Ts monohydrate (14 mg; 71 μmoles) in H_2O (0.5 ml) was added. The solution was lyophilized and the residue dried over KOH.

Seryltyrosylserylmethionylglutaminylhistidyl[^{14}C]phenylalanylarginyltryptophylglycine Acetate Hydrate.—Compound VIII (68 mg) was dissolved in 90% TFA (2 ml) and the solution was kept at room temperature for 30 min. The bulk of the TFA was evaporated, H_2O (5 ml) was added, and the solution was lyophilized. TFA ions were exchanged for AcO^- on a column (1 × 5 cm) of IRA-400 and the eluate was lyophilized. The resulting fluffy colorless material dissolved in H_2O (100 ml) was added to a CM-cellulose column (1 × 10 cm) which was eluted with 60 ml of H_2O and the following NH_4OAc solutions: 0.01 *M* (50 ml); 0.025 *M* (50 ml); 0.035 *M* (50 ml); and 0.05 *M* (50 ml). Absorbancy measurements at 280 mμ located the desired compound in the 0.05 *M* eluates. These fractions were pooled and added to a column (1 × 2 cm) of Bio-Rex 70. The column was washed with H_2O (100 ml), then the desired material was eluted with 30% AcOH. Radioactive fractions were pooled, evaporated to a small volume and lyophilized; yield 37 mg: $[\alpha]^{25}D$ −29.3° (*c* 0.27, 1% AcOH); R_f^{VI} 0.2; R_f^{VII} 0.5; amino acid ratios in acid hydrolysate $Ser_{1.7}Tyr_{1.0}Met_{1.1}Glu_{1.0}His_{1.0}$-$Phe_{1.0}Arg_{1.0}Gly_{1.0}$ (81%); specific radioactivity 0.117 mCi/mmole.

Seryltyrosylserylmethionylglutaminylhistidyl[^{14}C]phenylalanylarginyltryptophylglycylprolylvalylglycyllysyllysylarginylarginylprolylvaline Amide Acetate Hydrate (I).—The dried tosylate salts derived from VIII (500 mg; 0.31 mmole) and (XIV)[9] (550 mg; 0.27 mmole) were dissolved in DMF (24 ml), pyridine (12

(27) R. W. Holley and E. Sondheimer, *J. Amer. Chem Soc.*, **76**, 1326 (1954).
(28) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **44**, 1997 (1961).

ml), and 10% TEA in DMF (0.447 ml) and DCI (four 130-mg portions) was added over a period of 48 hr at room temperature. EtOAc (500 ml) was added and the precipitate was collected, washed with EtOAc, and dried. The solid was dissolved in 50% MeOH (50 ml) and the solution was passed through a column (2 × 5 cm) of IRA-400 which was washed with 50% MeOH until the eluate was free of radioactivity (approximately 250 ml required). DMF (15 ml) was added, and the solution was concentrated to a volume of approximately 150 ml. This solution was added to a CM-cellulose column (2.5 × 15 cm) which was eluted with 10% aq DMF (75 ml) followed by the following solutions of NH_4OAc in 1:9 DMF–H_2O: 0.025 *M* (400 ml); 0.05 *M* (600 ml); 0.075 *M* (300 ml); 0.1 *M* (200 ml); and 0.125 *M* (900 ml). Fractions (20 ml each) were collected at a flow rate of 160 ml/hr. Absorbancy measurements at 280 mμ and tlc of individual fractions located the desired material in the 0.1 *M* and 0.125 *M* eluates. The desired tubes were pooled and the solution was passed through a Bio-Rex 70 column (2 × 5 cm) which was eluted with water (100 ml), 5% AcOH (50 ml), and 50% AcOH (300 ml). The latter eluates were concentrated to a small volume and were lyophilized: yield 367 mg; $[\alpha]^{28}D$ −52.3° (*c* 0.53, 10% AcOH); amino acid ratios in acid hydrolysate, $Ser_{2.6}$-$Tyr_{1.0}Met_{1.0}Glu_{1.2}His_{1.1}Phe_{1.0}Arg_{2.6}Gly_{2.0}Lys_{3.2}Pro_{1.0}Val_{2.0}$ (83%).

The protected eicosapeptide amide XV (367 mg) was deblocked with 90% TFA in the usual manner, TFA ions were exchanged for AcO^- ions on IRA-400, and the product was isolated by lyophilization. The peptide was then incubated at 37° for 24 hr with freshly distilled thioglycolic acid.[22] The final product was lyophilized and dried over P_2O_5 at room temperature: yield 239 mg; R_f^{VI} 0.1; R_f^{VII} 0.3; $[\alpha]^{25}D$ −89.4° (*c* 0.175, 10% AcOH based on actual peptide content); amino acid ratios in acid hydrolysate, $Ser_{2.6}Tyr_{1.0}Met_{1.0}Glu_{1.1}His_{0.8}Phe_{1.2}Arg_{2.8}Gly_{2.1}Lys_{2.9}$-$Pro_{2.3}Val_{2.6}$ (80%); amino acid ratios in AP-M digest, Ser + $Glu_{2.7}Tyr_{1.0}Met_{0.9}His_{1.0}Phe_{1.0}Arg_{3.0}Trp_{0.8}Gly_{2.1}Lys_{3.2}Pro_{2.1}Val_{2.4}$ (80%); specific radioactivity 0.126 mCi/mmole.

Discussion

The key feature of our synthesis of β-corticotropin$_{1-20}$ amide and its analogs involves the assembly of 4 fragments, *i.e.*, VI, VII, XI, and XII or analogs thereof. Since the final coupling steps are little affected by amino acid variations within each fragment it is a simple matter to produce analogs once conditions for the synthesis and purification of the complete molecule are known. It is thus possible to stockpile essential building blocks and to assemble them in a reproducible manner for syntheses of analogs. The variations of the amino acid sequence in each of these small building blocks has become routine. For example, the recently described substitutions of His[6] and Trp[9] (ref 1) and the present incorporation of [^{14}C]Phe[7] into Gln[5]-β-corticotropin$_{1-20}$ amide involved resynthesis of only the center portion VI of the total molecule. Of particular importance is the fact that available analytical tools allow the critical characterization of small peptides with satisfactory accuracy and that larger molecules which are assembled from fragments of established homogeneity are less likely to contain "failure sequences."[29] These advantages are not realized when complex polypeptides are assembled by the solid phase method.[30]

Several solid phase synthetic peptides which, following purification by sophisticated procedures, were shown to be indistinguishable from their natural counterparts by available physical and chemical techniques possessed but a fraction of the expected biological activity.[31–35] Thus, this method does not appear adequate to establish meaningful relations between structure and biological activity with complex physiologically active peptides.

A major difficulty, when working with complex biologically active polypeptides, is the determination of the actual peptide content of a given sample. This situation arises from the tendency of these materials to solvate and to bind various counter ions. Since many biologically interesting polypeptides cannot be obtained in the form of well-defined crystals the degree of hydration may vary significantly as a function of the surrounding atmosphere. Drying to constant weight is not suitable for determination of water content since some peptides associate very firmly with the solvating agent and undergo marked alterations when dried *in vacuo* at elevated temperatures. The average amino acid recovery in acid or enzymic hydrolysates provides a satisfactory measure of the actual peptide content of a given preparation.[23] This situation is simplified when working with radioactively labeled peptides when one amino acid containing the label is employed to introduce the isotope. For example, in the present investigation the [^{14}C]Phe was incorporated into I in the form of the crystalline benzyloxycarbonyl-[^{14}C]Phe *N*-hydroxysuccinimido ester whose specific radioactivity was established accurately. The specific radioactivity of the homogeneous final product (peptide I) must be identical within the limits of error to that of the crystalline derivative which was used to incorporate the label. Thus, the radioactivity of a solution of I will provide an accurate measure of actual peptide concentration irrespective of the degree of hydration or the number of counter ions which may be present in the sample.

In addition to providing a valuable tool for the investigation of various aspects of the mode of action of ACTH, [^{14}C]Phe[7],Gln[5]-β-corticotropin$_{1-20}$ amide (I) has made it possible to assess the importance of the free γ-carboxyl group in β-corticotropin$_{1-20}$ amide for steroidogenic activity. Lebovitz and Engel[24] compared the *in vivo* and *in vitro* steroidogenic potencies of β-corticotropin$_{1-20}$ amide[18] with that of corticotropin A_1 in the rat and found that the synthetic compound exhibited 181% the steroidogenic potency of the natural material on a weight basis. Since the molecular weight of the synthetic hormone is approximately 50% that of corticotropin A_1 the synthetic product has approximately the same molar potency as A_1. Under identical conditions peptide I assayed 150% of A_1 on a weight basis and, thus, possessed approximately 80% the potency of β-corticotropin$_{1-20}$ amide. In *in vitro* assays using quartered adrenals[36] the biological activity of β-corticotropin$_{1-20}$ amide was only 78% that of A_1 on a weight basis. The radioactive peptide I was 93% as active as A_1 under the same conditions. In this experimental design the compounds are approximately equipotent but are considerably less effective than the intact natural hormone. Greater instability of the shorter-chain synthetic peptides under the *in vitro* conditions of assay may explain this behavior which has been observed with other analogs of β-corticotropin$_{1-20}$ amide.

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These biological results demonstrate that the free γ-carboxyl group of the glutamic acid residue in β-corticotropin$_{1-20}$ amide is not essential for function; it appears very likely that a similar situation pertains in the intact ACTH molecule.

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Synthesis of Pyroglutamylhistidylprolineamide[1] by Classical and Solid Phase Methods

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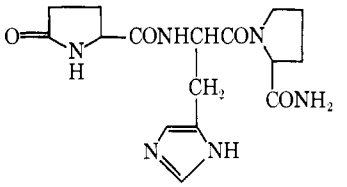
The synthesis of pyroglutamylhistidylprolineamide was effected by coupling pentachlorophenyl pyroglutamate with histidylprolineamide. The dipeptide was made by condensation of *t*-butyloxycarbonylhistidine with prolineamide mediated by dicyclohexylcarbodiimide, removal of the blocking group in acid, and treatment of the peptide hydrochloride with a basic ion-exchange resin. Peptide intermediates were purified by chromatography on silica gel. The Merrifield method of solid phase peptide synthesis was used to obtain a comparative sample.

The thyrotropin-releasing factor or hormone (TRF or TRH) was isolated in a state of high purity from ovine hypothalami by Guillemin, *et al.*,[2] in 1965 and from porcine hypothalami by Schally, *et al.*,[3] in 1966.

During attempts to elucidate the structure of TRH, a sample of the porcine hormone was subjected to acid hydrolysis and amino acid analysis, yielding the amino acids histidine, glutamic acid, and proline in an equimolar ratio,[3] and accounting for 30% of the dry weight of the active material. In the case of ovine TRH, 80% of the sample weight could be accounted for by the presence of the same amino acids.[4]

It has been reported[4,5] that of the six isomers possible for a tripeptide containing histidine, proline, and glutamic acid, none had TRH activity. In pursuing this work, Burgus, *et al.*, showed that the acetylation products of glutamylhistidylproline gave some of the activity typical of TRH.[4] One of the acetylation products, pyroglutamylhistidylproline, accounted for the TRH activity of the mixture, and the methyl ester prepared from it was also active.[6] The derivative pyroglutamylhistidylprolineamide, prepared from the Me ester by ammonolysis, showed very high TRH activity. However, since the ir and nmr spectra of this derivative were not identical with those of the highly purified natural material it was concluded that the structure of the hormone was not that of pyroglutamylhistidylprolineamide.[6] More recently Burgus, *et al.*, studied mass spectra of purer preparations of natural and synthetic TRH and concluded that pyroglutamylhistidylprolineamide is indeed the true structure of the naturally occurring ovine hormone.[7]

Independently, Schally established more directly the amino acid sequence of the "peptide portion" of TRH by degradation to be glutamylhistidylproline.[8] Subsequently, strong evidence was advanced that the structure of TRH corresponds to that of pyroglutamylhistidylprolineamide (II) made synthetically.[9] The



II

reported method of synthesis[10] employed the tripeptide glutamylhistidylproline (80% pure) as the starting material and the reactions were carried out with milligram amounts. The synthetic and natural materials were indistinguishable in many chromatographic systems and also in biological activity. In a systematic investigation of the porcine TRH, Nair, *et al.*, have now shown that the porcine hormone has the structure pyroglutamylhistidylprolineamide.[11]

Since the yields from batches of 100,000 and 165,000 porcine hypothalami were reported to be 2.8 mg and 4.4 mg, respectively,[8] it follows that a practical laboratory chemical synthesis of this tripeptide amide becomes of extreme importance for studying its chemical, physical, and biological properties. A direct and practical synthesis of pyroglutamylhistidylprolineamide by classical methods of peptide synthesis is reported here, as well as a quick synthesis by the solid phase method of Merrifield[12] to provide a small-scale reference sample.

For the synthesis by classical methods, the starting material prolineamide hydrochloride was neutralized

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