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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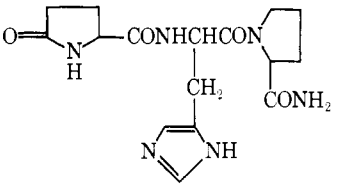
The synthesis of pyroglutamylhistidylprolineamide was effected by coupling pentachlorophenyl pyroglutamate with histidylprolineamide. The dipeptide was made by condensation of *t*-butyloxycarbonylhistidine with prolineamide mediated by dicyclohexylcarbodiimide, removal of the blocking group in acid, 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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with Et_3N and coupled with *t*-butyloxycarbonyl (BOC)-histidine by means of dicyclohexylcarbodiimide.[13] The amorphous product was purified by chromatography on a silica gel column with combinations of MeOH–$CHCl_3$ as the eluent. The purity of BOC-histidylprolineamide (Ia) was determined by tlc in several solvent systems, and by electrophoresis. Removal of the BOC group, the only blocking group used in this synthesis, was easily accomplished by treatment of Ia with HCl in dioxane. The free H_2O-soluble dipeptide amide was obtained in nonaqueous medium by neutralizing the MeOH solution of the hydrochloride with the ion-exchange resin Rexyn 201 (OH^-). Finally, coupling of pentachlorophenyl pyroglutamate with histidylprolineamide led to the desired tripeptide pyroglutamylhistidylprolineamide (IIa), in amorphous form. Purification of this synthetic material was accomplished by column chromatography on silica gel with MeOH–$CHCl_3$ as the eluent. The purified synthetic material was homogeneous by the criteria of tlc (5 different solvent systems), electrophoresis, and paper chromatography. The nmr spectrum of the sample was consistent with the molecular structure. In the mass spectral study of the product, a molecular ion peak (*m/e* 362) was present when the sample was vaporized at 40 eV, but not when the sample was vaporized at 70 eV.

The synthetic sample IIa was subjected to biological tests for hormonal activity by the T_3-TRH method in mice.[8] The procedure is based on the ability of TRH to release TSH from the anterior pituitary. The elevation of endogenous TSH increases the rate of release of labeled thyroid hormone from the thyroid gland. Therefore, TRH activity is measured principally by the release of ^{131}I or ^{125}I from the thyroid glands into the blood. The rise in blood radioactivity is determined as cpm 2 hr after the iv injection of TRH and the synthetic preparation.

Levels of 1–9 ng of the synthetic IIa increased the count of radioactive I in a range of 100–6400, cpm compared with 100 cpm for acid–saline controls. Levels of 1–9 ng of the porcine TRH usually increase radioactive iodine count[8] in a range of 100–3500 cpm. Thus, the activity of the synthetic preparation compares very favorably with the activity of the natural preparation.[14] A direct comparison of the two preparations is difficult to quantitize at present on account of the difficulty in weighing an exact amount of pure natural TRH.

The overall yield of tripeptide amide from prolineamide hydrochloride was 45–50%. Thus, this method appears to be suitable for the preparation of a substantial amount of the synthetic hormone.

The Merrifield method of solid phase synthesis was also tried and found convenient for a fast synthesis of moderate amounts of the tripeptide amide. Starting with BOC–proline–resin,[15] the usual steps of deprotection, neutralization, and coupling[15] were accomplished employing BOC-histidine and pyroglutamic acid at the appropriate coupling steps. Removal of di- or tripeptide from the resin was accomplished by ammonolysis. Purification was effected for both peptide derivatives by chromatography on a silica gel column with methanol chloroform as the eluent. By this procedure the dipeptide intermediate, BOC-histidylprolineamide (IB), was obtained in yields varying from 48 to 61%. The tripeptide amide, pyroglutamylhistidylprolineamide (IIb) was obtained in 15 to 30% yield. Identification of both intermediates was facilitated by the availability of samples by two independent routes.

Attempts to crystallize pyroglutamylhistidylprolineamide are in progress.

Experimental Section[16]

BOC-Histidylprolineamide (Ia).—A solution of 1.275 g of BOC-histidine[1] (5 mmoles) and 0.831 g of prolineamide·HCl (5 mmoles) in 20 ml of DMF was treated with 0.7 ml (5 mmoles) of Et_3N. To the cold (0°) solution was added 1.032 g of DCI (5 mmoles). After 16 hr, the dicyclohexylurea was removed by filtration and the filtrate evaporated *in vacuo* at 40°. The residual oil was taken up in a small volume of 5% MeOH in $CHCl_3$ and passed through a 20 × 2.5 cm column packed with 20 g of silica gel (E. Merck A. G., Darmstadt, Germany, 70–325 mesh activity acc. to Brockmann and Schodder 2–3) in $CHCl_3$. Elution was performed with 5% MeOH in $CHCl_3$, 5-ml fractions being collected. The fractions obtained were analyzed by tlc (A system, ninhydrin color reaction). The early fractions carried a faster-moving component, characterized as a yellow spot on tlc. When most of the by-product had been removed, elution was continued with 15% MeOH–$CHCl_3$. The selected fractions (R_f 0.3, on tlc) were pooled and evaporated to a foamy solid, 1.047 g (58%), which could not be crystallized from organic solvents. This intermediate was homogeneous on tlc (system A, Pauly, ninhydrin, and Cl_2–tolidine color reactions, R_f 0.3); electrophoresis (same color reactions as for tlc) showed BOC-histidylprolineamide to travel toward the cathode as a single spot; the nmr spectrum (MeOH-d_4, δ values relative to TMS) shows: CH_3-(BOC-group), 1.38 (s); Im-2-H-histidine, 7.53 (s); Im-4-H-histidine, 6.92 (s); α-CH-histidine, 3.6 (m); CH_2-histidine, 2.98 (m); α-CH-proline, 4.48 (m); 5-CH_2-proline, 3.30 (m); CH_2CH_2-proline, 2.0 (m) (b = broad; s = singlet; m = multiplet); $[\alpha]^{25}D$ −46° (c 1, H_2O).

Elemental analysis of this material showed the presence of Cl^-.[17] For the removal of Cl^-, a MeOH solution of the dipeptide amide was treated with Rexyn 201 (OH^-) and filtered. The filtrate was evaporated to dryness *in vacuo;* the film obtained was dissolved in H_2O and the solution lyophilized; *Anal.* ($C_{16}H_{25}N_5O_4$) C,H,N.

Pentachlorophenyl Pyroglutamate.—A solution of 1.291 g of pyroglutamic acid (10 mmoles) and 2.633 g of pentachlorophenol (10 mmoles) in 20 ml of DMF was treated in the cold (0°) with 2.063 g (10 mmoles) of DCI. After stirring for 16 hr the suspension was filtered to remove dicyclohexylurea and the filtrate was

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

(14) The author is indebted to Dr. Schally and his staff at the Endocrine and Polypeptide Laboratories, Veteran Administration Hospital, and Department of Medicine, Tulane University School of Medicine, New Orleans, for the determination of the level of activity of this synthetic preparation.

(15) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis," W. H. Freeman and Co., San Francisco, Calif., 1969.

(16) A Thomas-Hoover apparatus was used for melting point determinations in capillary tubes. Where analyses are indicated only by symbols of the elements, analytical results were obtained for the elements within ±0.4% of the theoretical value. Where tlc was used to determine purity of intermediates and products, silica gel G plates were used and were developed with one of the following solvent combinations: (A) 15% MeOH in $CHCl_3$, (B) MeOH–$CHCl_3$ (2:3), (C) MeOH–$CHCl_3$ (2:1), (D) $CHCl_3$–MeOH–38% AcOH (3:2:1), (E) $CHCl_3$–MeOH–NH_4OH (60:45:20), (F) BuOH–EtOAc–AcOH–H_2O (1:1:1:1). Where the ninhydrin color reaction was the detection method of choice, the BOC intermediate was exposed to HCl vapors in a chromatographic chamber for 10 min prior to spraying. For electrophoresis, Brinkmann precoated thin-layer cellulose sheets were employed with pyridine–AcOH buffer, pH 6.3 (25–45 min). The nmr spectrum of the dipeptide Ia was obtained at 60 MHz using a Varian Associates A-60 spectrometer. Chemical shifts are reported in parts per million from internal TMS (O.S.). Coupling constants are reported in Hz. The nmr spectrum of IIa was obtained at 100 MHz using a Varian Associates HA-100 spectrometer. Pr assignments were confirmed by appropriate frequency sweep spin decoupling experiments using a Hewlitt-Packard audio oscillator, Model 200AB or by comparison with the spectra of the appropriate amino acid. Mass spectra were recorded on an AEI MS-902 mass spectrometer.

(17) The presence of Cl^- in the peptide intermediates purified in MeOH–$CHCl_3$ may arise from the presence of traces of HCl in the $CHCl_3$.

evaporated *in vacuo* at 40°. The solid residue was treated with cold EtOAc and filtered, yielding 2.2 g of white solid, mp 180–184°. Recrystallization from EtOH afforded 2.0 g (54%): mp 196–199°; $[\alpha]^{26}D$ +21° (*c* 2, DMF); the nmr spectrum (DMSO-d_6, δ values with TMS reference standard) shows: N-H, 8.33 (s); α-CH, 4.67 (m); CH_2CH_2-, 2.45 (m); *Anal.* ($C_{11}H_6Cl_5NO_3$) C,H,Cl,N.

Pyroglutamylhistidylprolineamide (IIa).—A solution of 0.447 g of BOC-histidylprolineamide in 4 ml of dioxane was treated with 12 ml of 4 *M* HCl in dioxane at room temperature. A white solid separated immediately. After 30 min 100 ml of Et_2O was added, the supernatant liquid decanted, and the solid material washed by decantation with 4 additional 150-ml portions of Et_2O. The solid residue was dried *in vacuo* over P_2O_5 and KOH and dissolved in MeOH and the solution treated with ion-exchange resin Rexyn 201 (OH^-). The solvent was removed under reduced pressure and dried (desiccator, P_2O_5). The free dipeptide, obtained as a glassy film, was dissolved in 2 ml of DMF and 0.481 g of pentachlorophenyl pyroglutamate was added. The resulting solution was allowed to stand for 16 hr. The disappearance of free dipeptide was verified by tlc (system B, ninhydrin color reaction). The solution was evaporated to an oil which was subjected to purification by column chromatography on a 20 × 2.5 cm column packed with 20 g of silica gel. The sample was applied as a solution in MeOH–$CHCl_3$, 1:2 ratio, and elution was accomplished with MeOH–$CHCl_3$, 2:1 ratio with 5-ml fractions being collected. All fractions were analyzed by tlc (System C, Pauly and Cl_2-tolidine color reaction). The suitable fractions (R_f 0.3 on tlc) were pooled and evaporated to dryness. After drying over P_2O_5 *in vacuo* the solid white residue weighed 358 mg (78%). At this stage of purification, elemental analysis showed 1–1.5 moles of Cl^- per mole of peptide. To remove Cl^-, a MeOH solution of the tripeptide amide was treated with Rexyn 201(OH^-) and filtered. The filtrate was evaporated to dryness *in vacuo*, the solid film obtained was dissolved in H_2O, and the resulting solution was lyophilized. *Anal.* ($C_{16}H_{22}N_6O_4 \cdot H_2O$)C, H,N,O. This intermediate was homogeneous on paper chromatography (system D), electrophoresis, and tlc (systems C, D, E, and F, with Pauly and Cl_2–tolidine color reactions). An authentic synthetic sample supplied by Dr. Andrew Schally was identical with this synthetic material as ascertained by tlc (system C, D, E, and F, Pauly color reaction) and electrophoresis (same color reaction). Mass spectrometry (sample dissolved in MeOH-d_4) at 70 eV did not reveal a molecular ion. However at 40 eV a molecular ion was detected with *m/e* 362; $[\alpha]^{25}D$ −65.5° (*c* 1, H_2O).

The nmr spectrum (pyridine-d_5, δ values from internal TMS) shows: Im-2-H-His, 7.81; Im-4-H-His, 7.14 ($J_{2,4}$ = 1.0 Hz); α-H-His, 5.26 ($J_{\alpha,\beta}$ = 8.8 Hz, $J_{\alpha,\beta'}$ = 4.5 Hz, $J_{\alpha,NH}$ = 8.0 Hz); α-H-prolineamide, 4.88 ($J_{\alpha,\beta}$ = 4.2 Hz, $J_{\alpha,\beta'}$ = 7.9 Hz);[18] α-H pyroglutamic acid, 4.51 ($W_{1/2}$ = 13 Hz);[19] δ-CH_2-prolineamide, 3.69 (m); β-CH_2-His, 3.49; β'-CH_2-His, 3.21 ($J_{\beta,\beta'}$ = 14.2 Hz); β-CH_2-prolineamide, 3.35 (m); β'-CH_2-prolineamide, about 2.2 (m); γ,γ'-CH_2-prolineamide, 1.5 to 2.2 (m); β,β'-CH_2- and γ,γ'-CH_2- of pyroglutamic acid, about 2.4 (m); NH one at 9.26, one at 9.05, one at 7.29, α-NH of His 9.06.

Solid Phase Synthesis. BOC-Histidylproline Polymer.—BOC-Proline polymer (8 g, 0.40 mmole of proline/g of resin), made by the usual methods,[15] was placed in a Merrifield reaction vessel. The following steps of deprotection and neutralization were carried out: (1) four washings with 40-ml portions of dioxane; (2) cleavage of the BOC group by treatment with 4 *M* HCl in dioxane for 30 min at room temperature; (3) four washings with 40-ml portions of dioxane; (4) four washings with 40-ml portions of EtOH; (5) four washings with 40-ml portions of $CHCl_3$; (6) neutralization of the hydrochloride with three 40-ml portions of 10% Et_3N in $CHCl_3$ for a total time of 10 min; (7) four washings with 40-ml portions of $CHCl_3$; (8) six washings with 40-ml portions of CH_2Cl_2; (9) addition of 30 mmoles of BOC-His dissolved in 40 ml of DMF and mixing for 10 min; (10) addition of 30 mmoles of DCI in 5 ml of DMF, followed by a reaction period of 5 hr; (11) four washings with 40-ml portions of MeOH–$CHCl_3$, 1:2 ratio, to dissolve and remove dicyclohexylurea; (12) four washings with 40-ml portions of abs EtOH. The weight of the resin after drying over KOH pellets and P_2O_5 *in vacuo* was 8.4 g.

BOC-Histidylprolineamide (Ib).—An aliquot of 1.05 g (0.4 mmole) of the preceding protected peptide polymer was suspended in 20 ml of MeOH–DMF, 1:1 ratio, and saturated with NH_3 at 0°. After 24 hr the suspension was filtered and the collected resin was washed with additional MeOH and DMF. The filtrate and washings were pooled and evaporated to an oil. Purification by column chromatography as described for Ia afforded, in two separate runs, 68 and 85 mg (48–61%) of BOC-histidylprolineamide Ib. Preparations Ia and Ib were identical as ascertained by electrophoresis and tlc (system A, Cl_2-tolidine, ninhydrin, Pauly color reactions).

Pyroglutamylhistidylprolineamide. (IIb).—An aliquot of 1.05 g (0.4 mmole) of BOC-histidylproline polymer was deprotected and neutralized as described through step 8 of the preparation of Ib. In step 9 pyroglutamic acid (6 mmoles), dissolved in 10 ml of DMF, was added. In step 10 DCI (6 mmoles), dissolved in 2 ml of DMF, was added. Steps 11 and 12 were the same as described in the previous experiment. The final resin, 1.07 g, was suspended in 10 ml of MeOH and 10 ml of DMF and the suspension was saturated with NH_3 at 0°. After 24 hr the resin was removed by filtration and the solvents were evaporated under reduced pressure at 0°. The resulting oil was purified as described for IIa. There was obtained 21 mg and 42 mg (15–30%) of pyroglutamylhistidylprolineamide in two separate runs. Comparison of IIb with IIa by electrophoresis and tlc (systems C, D, E, and F, with Pauly and Cl_2–tolidine sprays) showed identical mobilities.

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(18) These values represent measured coupling constants obtained from first order analysis of the ABX system. Additional broadening due to virtual coupling was observed.

(19) Couplings were not determined due to significant virtual coupling.