insol material on silica gel in the BuOH-AcOH-H_2O (4:1:1, v/v) system gave 2 ninhydrin-negative and Cl-positive spots with R_f values of 0.60 and 0.36 which corresponds to peptides II and III.

The MeOH-insol material (270 mg) was dissolved in 20 ml of F_3CCO_2H and stored under N_2 for 60 min. The solvent was removed *in vacuo* and the residue was dissolved in 350 ml of freshly distilled liq NH_3 and treated with small pieces of Na until the blue color persisted for 20 min. NH_3 was evapd and the residue was dissolved in 25 ml of 0.1 *N* AcOH and desalted on an IRC-50 column as described earlier.[18] The crude nonadecapeptide was eluted with pyridine-AcOH-H_2O (30:4:66, v/v) and isolated by lyophilization to yield 146 mg of material. This was further purified by column chromatog on CM-cellulose as previously described.[16] The purified product was rechromatographed on CM-cellulose to yield 32 mg of the nonadecapeptide IV (peptide content based on uv spectra, 80%; 8% yield based on protected nonapeptide).

Peptide IV was found to be homogeneous by electrophoresis (400 v, 5 hr) on paper: at pH 3.7 (pyridine acetate buffer), mobility relative to lysine 0.85; at pH 6.9 (collidine acetate buffer), mobility relative to lysine 0.68. The amino acid composition of an acid hydrolysate of IV as determined by chromatog was in good agreement with theoretically expected values (Table I). A sample of IV was submitted to enzymic digest, first with a mixt of trypsin and chymotrypsin (enzyme/substrate, 1:50; pH 8.5, 37°, 48 hr), and then with leucine aminopeptidase (enzyme/substrate, 1:25; pH 8.5, 37°, 48 hr). The amino acid anal. of the digest gave values (Table I) as expected from the specificity of these enzymes.

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On the Role of the Histidine Moiety in the Structure of the Thyrotropin-Releasing Hormone†

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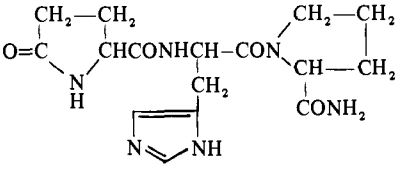
pGlu-Phe-Pro-NH_2 (**2**), an analog of the thyrotropin-releasing hormone (TRH, **1**), where Phe replaces His, has been synthesized and found to have nanogram activity *in vivo* which is up to 10% that of TRH. Characteristic of TRH, pGlu-Phe-Pro-NH_2 is inactivated by serum and inhibited by triiodothyronine; therefore, it closely simulates TRH. pGlu-Phe-Pro-NH_2 is apparently the most potent analog of TRH where one of its natural amino acid moieties is replaced by another common and natural amino acid. It is considered that: (a) both the π electrons and the basicity of His may be functional for ultimate release of thyrotropin; (b) release may consist of both complexing and an ionic mechanism involving a negatively charged group of the receptor site. pGlu-Trp-Pro-NH_2 (**3**) and pGlu-Tyr-Pro-NH_2 (**4**) having both aromaticity and functionality in the second amino acid were also synthesized, but these analogs did not release TSH even at extremely high dose levels. To study possible inhibition of TRH by an analog, pGlu-Phe-3Hyp-NH_2 (**5**) was synthesized. **5** did not inhibit the activity of TRH and neither did the tripeptides **3** and **4**.

Chang, *et al.*,[1] and Bowers, *et al.*,[2] have reported the synthesis and hormonal activities of analogs of the thyrotropin-releasing hormone (TRH), which is pyroglutamylhistidylprolinamide (pGlu-His-Pro-NH_2)[3] (**1**). These analogs consisted of modifications in the proline moiety of TRH, and several showed hormonal activity.

Active analogs were found when the moiety of Pro-NH_2 was modified to Pro-$NHCH_3$ and Pro-OCH_3. Hormonal activity was retained when this Pro-NH_2 moiety was replaced by Ala-NH_2, Abu-NH_2, Val-NH_2, and Leu-NH_2. These latter analogs were considered as "open proline" moieties having 3-6 C atoms, in comparison with the 5 C atoms of proline.

The initial finding[2] that pGlu-^{im}Bzl-His-Pro-NH_2 showed about 0.1% of the activity of TRH became very interesting.



1

†Hypothalamic Hormones. 32.

A dose of 10 μg of "^{im}Bzl-TRH" in mice released essentially the same amount of TSH as 9 ng of TRH. The hormonal activity of this benzyl derivative shows that the imidazole NH is not essential for release of TSH, although an intact His moiety is important for potency. ^{im}Bzl-TRH represents a rather large modification of the hormone where not only the His moiety has lost its functionality, but also where the introduced benzyl group might sterically affect the conformation of the hormone.

Hofmann and Bowers[4] reported that pGlu-β-(pyrazolyl-3)-Ala-Pro-NH_2 shows about 5% of the activity of TRH, and that "the acid-base properties of the imidazole ring are not essential for thyrotropin-releasing hormone activity."

Subsequent evidence showing the lack of essentiality of the imidazole NH is that of Rivier, *et al.*,[5] which shows that "1-methyl-TRF" exhibited 0.0004 of the activity of TRF and that "3-methyl-TRF" exhibited about 15 times greater activity than that of TRF.

It appeared of importance to extend the knowledge on the significance of the His-moiety for the structure-release relationships of TRH. We now have replaced His with the 3 aromatic amino acids Phe, Trp, and Tyr. Of the 3 tripeptides, pGlu-Phe-Pro-NH_2 (2), pGlu-Trp-Pro-NH_2 (3), and pGlu-Tyr-Pro-NH_2 (4), hormonal activity was only observed for pGlu-Phe-Pro-NH_2 (2) (Table I).

Hormonal Activity. pGlu-Phe-Pro-NH_2 (2) represents a relatively potent analog of TRH and a dose of 100 ng releases essentially the same amount of TSH as does 3-9 ng of synthetic TRH, *e.g.*, 2 has up to 10% of the activity of TRH. pGlu-Phe-Pro-NH_2 (2) is the most potent analog of TRH when one amino acid moiety of TRH has been replaced by another common and natural amino acid. The 3-methyl-TRF of Rivier, *et al.*,[5] appears to be the most potent structural modification of TRH.

The release of TSH from 2 indicates that availability of π electrons in the second amino acid of a tripeptide may be important for hormonal activity. The basicity of His in TRH also appears to be important for activity. The greater activity of the 3-methyl-TRF of Rivier, *et al.*,[5] in comparison with TRH could be due to the inductive effect of the methyl group and, if so, supports consideration of the importance of the basicity of His. The relative inactivity of 1-methyl-TRF[5] might be explained by specificity of structural requirements for the tripeptide of the site.

The activity of the Phe analog 2 and that of TRH may indicate separate functionalities for the π electrons and the ionic charge of the basic group of His in the mechanism which ultimately releases TSH. The basicity of His could indicate the participation of a negatively charged group at the receptor site in an ionic mechanism.

Other studies reviewed by McCann[9] in the mechanism of action of releasing factors have implicated Ca^{2+}, K^+, and cyclic AMP in the cellular processes. The participation of ionic mechanisms in the overall release processes is seemingly compatible with the knowledge that the pituitary hormone is evident within seconds and minutes after injection of a releasing hormone.

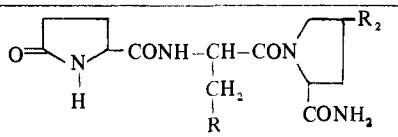
The activity of 2 supports all the diverse data stemming from ^{im}Bzl-TRH[2] that the imidazole NH is not necessary for release of TSH.

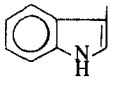
Replacement of His by Trp and Tyr gave inactive compounds. pGlu-Trp-Pro-NH_2 (3) and pGlu-Tyr-Pro-NH_2 (4) showed no hormonal activity even at doses up to 50 μg, or at nearly 5000 times an effective dose of TRH (Table I). The inactivity of pGlu-Tyr-Pro-NH_2 (4) in comparison with pGlu-Phe-Pro-NH_2, which differ by the OH group in 4, may again support the concept of the role of the basic function of His in TRH. The Trp moiety is sterically different from His.

The hormonal activity of pGlu-Phe-Pro-NH_2 (2) was abolished by treatment with serum (Table II), and the activity was inhibited by triiodothyronine and shows that pGlu-Phe-Pro-NH_2 (2) closely simulates TRH (Table III). These biological criteria of inactivation and inhibition are characteristics of the hormone.[7] In contrast, several of the active analogs of TRH which we have reported earlier were not inactivated by serum.[2]

An inhibitor to TRH could be a peptide with an affinity for the "release site" of TRH, but which did not release TSH. As pGlu-Phe-Pro-NH_2 (2) is now known to be acceptable at the release site because it does release TSH just like TRH, it seemed that a slight modification of this peptide could possibly possess some TRH-inhibiting activity. As a modification possessing another functional group, the moiety of 3-hydroxyproline (3Hyp), pGlu-Phe-3Hyp-NH_2 (5) was synthesized and bioassayed for both TRH and TRH-inhibiting activities.

Table I. Assay of Tripeptides by the T_3 Method in Mice for Release of Thyrotropin[a]



No.	Tripeptide	R_1	R_2	Dose, ng	Δcpm ^{125}I[b]
1	pGlu-His-Pro-NH_2 (TRH)		H	9	5612
2	pGlu-Phe-Pro-NH_2	C_6H_5	H	100	3534
3	pGlu-Trp-Pro-NH_2		H	50,000	204
4	pGlu-Tyr-Pro-NH_2	C_6H_4OH-*p*	H	50,000	100
5	pGlu-Phe-3Hyp-NH_2	C_6H_5	OH	50,000	88

[a]The assay was conducted as described by Bowers *et al.*[6-8] [b]Each result is the mean obtained from 5 mice.

Table II. Effect of Inactivation of TRH and pGlu-Phe-Pro-NH_2 (2) by Serum

Tripeptide	Dose, μg	Serum inactivation[a] Δcpm ^{125}I 0	+
pGlu-His-Pro-NH_2 (TRH) (1)	0.003	2985	238
pGlu-Phe-Pro-NH_2 (2)	0.30	2970	177

[a]Serum was obtd from a normal adult male and treated with TRH and with 2. The mixt was assayed at zero time and after (+) incubation for 30 min at 37°. 0 = zero time and + = after 30 min. Each result is the mean obtd from 5 mice.

Table III. Inhibition of TRH and pGlu-Phe-Pro-NH_2 by Triiodothyronine (T_3) in Mice[a]

Tripeptide	Dose, μg/ml	T_3 Δcpm ^{125}I 0	+
pGlu-His-Pro-NH_2 (TRH) (1)	0.009	4061	1077
pGlu-Phe-Pro-NH_2 (2)	1.0	5042	296

[a]T_3 was administered sc to the mice 2 hr before the iv administration of TRH. Each result is the mean obtd from 5 mice. 0 = zero times and + = after 15 min.

Table IV. Assays in Mice of Tripeptides for Inhibition of Activity of TRH

Tripeptide	Dose level of tripeptide, μg[a]	Δcpm ^{125}I
pGlu-Trp-Pro-NH_2	10	6008
	50	6110
pGlu-Tyr-Pro-NH_2	50	5545
pGlu-Phe-3Hyp-NH_2	50	5552
Saline		5612

[a]Each tripeptide was injected with 0.009 μg of synthetic TRH; each result is the mean obtd from 5 mice.

As seen in Table I, pGlu-Phe-3Hyp-NH_2 (**5**) did not release TSH at dose levels up to 50 μg. When 50 μg of **5** (*ca.* 5000-fold/wt) and 9 ng of synthetic TRH were jointly administered to the mice, there was no inhibition of the hormonal activity. This result shows that a peptide like pGlu-Phe-3Hyp-NH_2 (**5**) does not compete with TRH (Table IV) under these conditions. The data in Table IV also show that neither pGlu-Trp-Pro-NH_2 (3) nor pGlu-Tyr-Pro-NH_2 (**4**) inhibited the activity of TRH under the same conditions.

Chemistry. The tripeptides, pGlu-Phe-Pro-NH_2 (2) and pGlu-Trp-Pro-NH_2 (3), were synthesized by coupling their respective dipeptides pGlu-X (X = Phe and Trp) with Pro-NH_2 mediated by dicyclohexylcarbodiimide (DCI); in these coupling reactions, 1-hydroxybenzotriazole was used, as reported by König and Geiger.[10] pGlu-Phe-Pro-NH_2 (**2**) was purified by column chromatography on silica gel and the product was eluted with MeOH-$CHCl_3$ (1:20 v/v). pGlu-Trp-Pro-NH_2 (**3**) was purified by repeated recrystallization of the product from the reaction mixture.

pGlu-Tyr-Pro-NH_2 (**4**) was prepared by coupling pGlu-Tyr(Bzl) with Pro-NH_2 using the DCI method, followed by removal of the Bzl group by catalytic reduction using 10% Pd/C to yield **4**. In the synthesis of pGlu-Tyr-Pro-NH_2 (**4**), the intermediate, pGlu-Tyr(Bzl)-Pro-NH_2 was purified by preparative tlc (silica gel G), and **4** was obtained in pure form by recrystn.

pGlu-Phe-3Hyp-NH_2 (**5**) was prepared by coupling pGlu-Phe with 3Hyp-OCH_3 by the DCI method. The pGlu-Phe-3Hyp-OCH_3 (**6**) was treated with MeOH saturated with NH_3 to give **5**. pGlu-Phe-3Hyp-OCH_3 (**6**) was purified by preparative tlc (silica gel G), and pGlu-Phe-3Hyp-NH_2 (**5**) was purified by recrystn.

The tripeptides were found to be homogenous when tested for purity on tlc (silica gel G) in 3 different solvent systems (acidic, neutral, and basic).

The dipeptides, pGlu-Phe, pGlu-Trp, and pGlu-Tyr(Bzl), were prepared as described by Sievertsson, *et al.*[11]

Experimental Section

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Microanalyses were performed by the Mikroanalytisches Laboratorium, Bonn, West Germany. Where analyses are indicated by symbols of the elements, analytical results obtained for those elements were within ±0.4% of theoretical values. On tlc (silica gel G, E. Merck) R_f^1, R_f^2, and R_f^3 refer to the systems of *n*-BuOH-glacial HOAc-EtOAc-H_2O (1:1:1); $CHCl_3$-MeOH-concd NH_4OH (60:45:20), and EtOH-H_2O (7:3), respectively. The nmr spectra were measured on a Varian A-60 spectrometer (Me_4Si) as internal standard). The optical rotations were measured on a Perkin-Elmer Model 141 digital readout polarimeter using a microcell. All of the amino acids which were used as starting materials were purchased as the L isomers. Silica gel for column chromatography was purchased from J. T. Baker Chemical Co., Cat. No. 3405.

The 3 dipeptides pGlu-Phe, pGlu-Trp, and pGlu-Tyr(Bzl) were prepared as described[11] and their reported physical properties were again observed. TRH was synthesized as described by Chang, *et al.*[12]

pGlu-Phe-Pro-NH_2 (2). pGlu-Phe (828 mg), 1-hydroxybenzotriazole[10] (432 mg), and DCI (640 mg) were mixed in DMF (10 ml) and the mixt was stirred at 0° for 1 hr, and then stirred at room temp for 1 hr. Pro-NH_2 (342 mg) was added at 0°, and the reaction mixt was left at room temp for 10 hr. After cooling at 4° for 1 hr, filtering, and evaporating of the solvent *in vacuo* (40°), the residue was purified by column chromatography (silica gel). Starting with $CHCl_3$ as elution solvent, and increasing the polarity of the solvent using MeOH, 2 was eluted with MeOH-$CHCl_3$ (1:20 v/v). After recrystallization from MeOH-Et_2O, pure pGlu-Phe-Pro-NH_2 (**2**) was obtained; 340 mg, yield 30%, mp 194–197° dec, $[\alpha]^{22}D$ −44.0 (*c* 1.56 MeOH), R_f^1 0.66, R_f^2 0.64, R_f^3 0.84, positive to Cl-tolidine reagent. *Anal.* ($C_{19}H_{24}N_4O_4 \cdot 0.5H_2O$) C, H, N. Hydrolysis in 6 *N* HCl at 110° for 24 hr in a sealed ampule yielded Glu, Phe, Pro.

pGlu-Trp-Pro-NH_2 (3). pGlu-Trp (1.87 g), 1-hydroxybenzotriazole[10] (500 mg), DCI (742 mg), and Pro-NH_2 (410 mg) in DMF (15 ml) were allowed to react as described for 2. After filtering and evaporating of the solvent, the product was repeatedly recrystallized from EtOH-EtOAc to afford pure **3** (780 mg, yield 54%). The compd showed no sharp melting point and completely decomposed at 170°; $[\alpha]^{22}D$ −23.1 (*c* 1.48 MeOH), R_f^1 0.68, R_f^2 0.64, R_f^3 0.82, positive to Ehrlich and Cl-tolidine reagents. *Anal.* ($C_{21}H_{25}N_5O_4 \cdot 0.5H_2O$) C, H, N. Hydrolysis, as described for 2, yielded Glu and Pro, and hydrolysis in 15% NaOH at 110° yielded Glu and Trp.

pGlu-Tyr-Pro-NH_2 (4). pGlu-Tyr(Bzl) (382 mg) and Pro-NH_2 (114 mg) were mixed in DMF (20 ml), and DCI (220 mg) in DMF (10 ml) was then added at 0°. After stirring at room temp for 36 hr, the soln was evaporated *in vacuo* (40°), and the residue was purified by preparative tlc (silica gel G) using MeOH-$CHCl_3$ (3:7 v/v) as developing solvent. This procedure afforded pure pGlu-Tyr(Bzl)-Pro-NH_2; 120 mg, yield 25%, R_f^1 0.79, R_f^3 0.67, single spot positive to Cl-tolidine reagent. This protected tripeptide in EtOH (20 ml) was hydrogenated for 3 hr at room temp and atmospheric pressure using 10% Pd/C catalyst. The reaction mixt was filtered and evaporated, to give **4**; 75 mg, yield 77%, no sharp mp, $[\alpha]^{22}D$ −31.5° (*c* 1.0 MeOH), R_f^1 0.72, R_f^2 0.80, R_f^3 0.65, single spot to Pauly and Cl-tolidine reagents. *Anal.* ($C_{19}H_{24}N_4O_5 \cdot 1.5H_2O$) C, H, N. Hydrolysis, as described for 2, yielded Glu, Tyr, and Pro.

pGlu-Phe-3Hyp-OCH_3 (6). pGlu-Phe (276 mg), 3Hyp-OCH_3 (182 mg), and DCI (206 mg) were mixed in DMF as described for 4. The product was purified by preparative tlc (silica gel G) using H_2O-EtOH (3:7 v/v) as developing solvent. This procedure gave, after pptn from MeOH-Et_2O, pure **6**; 70 mg, yield 17%, mp 130–132°, nmr (MeOH-d_4) 2.72 (ArH, 5 H), 6.28 (OCH_3, 3 H), 7.60–8.10 (CH_2 protons).

pGlu-Phe-3Hyp-NH_2 (5). Compd **6** (50 mg) was treated for 48 hr at room temp with MeOH saturated with NH_3. After evaporation of the solvent *in vacuo*, the residue was recrystallized from MeOH-Et_2O to give **5**; 34 mg, yield 71%, mp 169–171° dec, $[\alpha]^{22}D$ −12.3 (*c* 0.6 DMF), single spot to Cl-tolidine reagent. *Anal.* ($C_{19}H_{24}N_4O_5 \cdot 0.25H_2O$). Hydrolysis, as described for 2, yielded Glu, Phe, and Hyp.

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