Journal of Medicinal Chemistry

VOLUME 14, No. 6 JUNE 1971

Structure and Synthesis of the Thyrotropin-Releasing Hormone†

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Received December 29, 1970

The independent discovery that pyroglutamylhistidylprolinamide (**1**) exhibits hormonal activities closely corresponding to those of the isolated porcine thyrotropin-releasing hormone (TRH) immediately led to additional data showing that the chemical properties and the hormonal activities of synthetic pGlu-His-Pro-NH_2 and porcine TRH are identical. In this manner, the structure of the thyrotropin-releasing hormone was established and 2 syntheses had also been achieved during the structural elucidation. Limited in methodology by the impurities with the isolated hormone, but surmounting these difficulties, it was found that ir, nmr, and mass spectral data, and particularly chromatographic data from 17 diversified systems, electrophoretic mobilities, and gel filtration behavior over Sephadex G-25 all support the conclusion that the porcine thyrotropin-releasing hormone has the structure of pGlu-His-Pro-NH_2.

Five companion papers, which permit brevity, describe interrelated accomplishments on the thyrotropin-releasing hormone (TRH). The first two papers[1,2] describe the details of the structural and synthetic studies, based upon the porcine thyrotropin-releasing hormone, which established the structure and achieved two syntheses of this hormone. The third paper[3] describes clinical studies on both men and women which demonstrated the oral activity of the synthetic hormone as synthesized by procedures described in these papers. The fourth paper[4] describes alternative and very useful steps for the synthesis of TRH, and the fifth paper[5] describes still another synthesis of TRH and the extension of this method to analogs of TRH which contribute to the elucidation of structure–activity relationships; this latter paper also describes an interpretation of the conformational nature of the functional site at which TRH releases TSH.

As a part of the studies on the degradation and structure of the porcine thyrotropin-releasing hormone, it was discovered by Folkers, *et al.*,[6] that synthetic *p*-glutamylhistidylprolinamide (1) exhibited the relative hormonal potencies and activities of the porcine thyrotropin-releasing hormone in 3 biological assay systems. In this synthetic work, pGlu-His-Pro-NH_2[7] was synthesized without the use of Ac_2O or related acylating reagent. The pGlu-His-Pro-NH_2 (1) was obtained synthetically from methylation of Glu-His-Pro (**2**), followed by reaction of the diester **3** with NH_3, and purification by chromatography. It was bioassayed on a critical basis because of the great interest in having a synthetic substance showing the activities of the hormone in the nanogram range.

Background for the synthetic conversion of Glu-His-Pro to pGlu-His-Pro-NH_2 was provided by Coleman[8] on esters of glutamic acid. Beecham[9] showed that γ half-esters of glutamic acid are converted to pyroglutamic acid under mild conditions. Shiba, *et al.*,[10] synthesized L-pyroglutamyl-L-glutaminyl-L-glutamine.

Burgus, *et al.*,[11] also found striking hormonal activities for synthetic reaction products, and their report described TRH-activity at microgram levels for acetylation products of Glu-His-Pro, and they also observed that ovine TRH is active at 1×10^{-3} of the level of these particular synthetic preparations; pGlu-His-Pro-OH was shown to be the major product of this acetylation.

These respective synthetic studies which were reported from one group working on the porcine hor-

† Hypothalamic Hormones. 14.

(1) This paper: see paragraph 1.

(2) J. Bøler, J.-K. Chang, F. Enzmann, and K. Folkers, *J. Med. Chem.*, **14**, 475 (1971).

(3) C. Y. Bowers, A. V. Schally, A. Kastin, A. Arimura, D. S. Schalch, C. Gual, E. Castineda, and K. Folkers, *ibid.*, **14**, 477 (1971).

(4) J. K. Chang, H. Sievertsson, C. Bogentoft, B. Currie, K. Folkers, and G. D. Daves, Jr., *ibid.*, **14**, 481 (1971).

(5) J. K. Chang, H. Sievertsson, B. L. Currie, K. Folkers, and C. Y. Bowers, *ibid.*, **14**, 484 (1971).

(6) K. Folkers, F. Enzmann, J. Bøler, C. Y. Bowers, and A. V. Schally, *Biochem. Biophys. Res. Commun.*, **37**, 123 (1969).

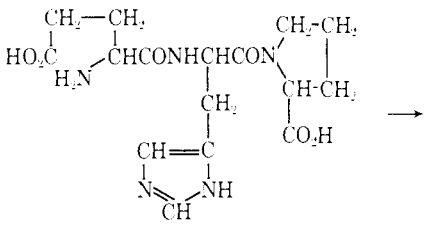
(7) On the basis of reported recommendations, the symbol pGlu- is now used in place of (pyro)Glu- [C. Y. Bowers, A. Weil, J. K. Chang, H. Sievertsson, F. Enzmann, and K. Folkers, *ibid.*, **40**, 683 (1970)].

(8) D. Coleman, *J. Chem. Soc.*, 2294 (1951).

(9) A. F. Beecham, *J. Amer. Chem. Soc.*, **76**, 4615 (1954).

(10) T. Shiba, S. Imai, T. Kaneko, *Bull. Chem. Soc. Jap.*, **31**, 244 (1958).

(11) R. Burgus, T. F. Dunn, D. N. Ward, W. Vale, M. Amoss, and R. Guillemin, *C. R. Acad. Sci.*, **268**, 2116 (1969).



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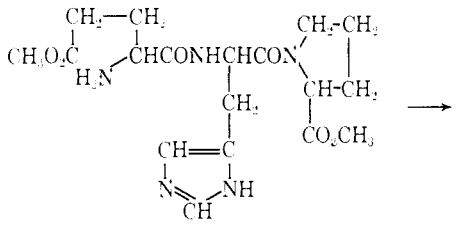

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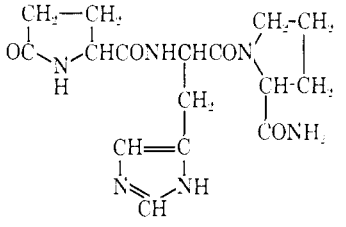

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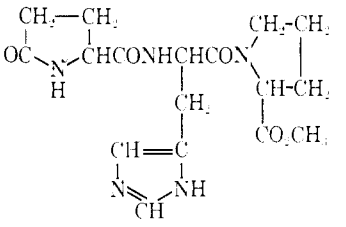

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mone[6] and the other group working on the ovine hormone[11] were mutually independent, and apparently occurred on a concurrent basis. The advent of synthetic compounds having potent TRH activity was of great timely importance.

The startling hormonal activities at nanogram levels of synthetic pGlu-His-Pro-NH_2 led immediately to its synthesis on a larger scale and further characterization, particularly by its nmr spectrum. Bøler, *et al.*, in 1969[12] reported the identity of the chemical properties and hormonal activities of the porcine thyrotropin-releasing hormone and pyroglutamylhistidylprolinamide. Their formulation of the structure, pGlu-His-Pro-NH_2, for TRH represented the elucidation of the first of the hypothalamic hormones that have been the objective of so much research, sometimes controversial, for over a decade.

The details of the research on the structure and synthesis of the thyrotropin-releasing hormone on which two communications[6,12] were based are described herein and in the accompanying paper by Bøler, *et al.*;[2] additional data are included.

General Nature of the Isolated Porcine Hormone.—About 2.8 mg of the porcine hormone had been isolated by Schally, *et al.*,[13] from the hypothalami of about 100,000 pigs, and a similar milligram quantity of the hormone had been isolated from another 165,000 fragments. The value of the isolated material in money and time, in addition to the actual amount available, restricted its use for structural studies. On acid hydrolysis, Schally, *et al.*,[14] had found that such isolated hormone yielded histidine, glutamic acid, and proline in essentially equimolar amounts and that these 3 amino acids accounted for about 30% of the sample which was hydrolyzed. At this stage, it was apparent that if the isolated hormone were 90–100% pure, then a significant proportion of the molecular weight was unaccounted. Alternatively, the yield of 30% of the 3 amino acids could reflect the major part of the molecular weight of the hormone. In any case, it was soon recognized as reported[12] that this isolated porcine hormone contained one or more unsaturated fatty acids, one of which appeared to be myristoleic acid, according to mass spectrometry and gas chromatography. The presence of dioctyl phthalate in one of the isolated hormone preparations was also evident by mass spectrometry. Later,[15] it was estimated that the actual corrected yield of amino acids was about 50–60% for some fractions of the hormone. Therefore, the presence of considerable impurities in the isolated porcine hormone required circumvention. The paucity of samples and the impurities made undesirable the obtaining of classical microanalytical data and obscured the interpretation of spectral data.

Condensation of Spectral Data.—Schally, *et al.*,[16] had found that the 3 amino acids existed in the sequence Glu-His-Pro and presumed this sequence to be a part of the molecule of the porcine hormone. The discovery[6] of the hormonal activity of synthetic pGlu-His-Pro-NH_2 and the hormonal inactivity of derivatives of altered sequence, confirmed the assignment of the Glu-His-Pro sequence to the structure of TRH, and subsequent spectral data obtained on the isolated hormone could be interpreted on this basis.

Ir Data.—The ir spectrum of the isolated porcine hormone in MeOH showed a C=O stretching band at 1695 cm^{-1} which is characteristic of the C=O lactam band of synthetic pyroglutamic acid in MeOH solution.

Nmr Spectrum.—The nmr spectrum of the isolated porcine hormone in MeOH-d_4–D_2O did not reveal any meaningful characteristics such as aromatic protons or olefinic protons, separate from those of the histidine moiety, which could indicate a nonpeptide moiety of the molecule. The principle peaks of the spectrum appeared to correspond mainly to those which could be assigned to the structure of pGlu-His-Pro-NH_2. Although the nmr data from the hormone were compared with the nmr spectrum of synthetic pGlu-His-Pro-NH_2, the comparison could not be as definitive as desired. Nmr spectra of the isolated hormone showed other absorptions which were presumed to be associated with impurities, because of their lower intensities when compared with those due to the tripeptide struc-

(12) J. Bøler, F. Enzmann, K. Folkers, C. Y. Bowers, and A. V. Schally, *Biochem. Biophys. Res. Commun.*, **37**, 705 (1969).

(13) A. V. Schally, C. Y. Bowers, T. W. Redding, and J. F. Barrett, *ibid.*, **25**, 165 (1966).

(14) A. V. Schally, K. Folkers, C. Y. Bowers, F. Enzmann, J. Bøler, R. M. G. Nair, and J. F. Barrett, "Hypophysiotropic Hormones of the Hypothalamus: Assay and Chemistry," J. Meites, Ed., Williams and Wilkins Co., Baltimore, Md., 1970, p 226.

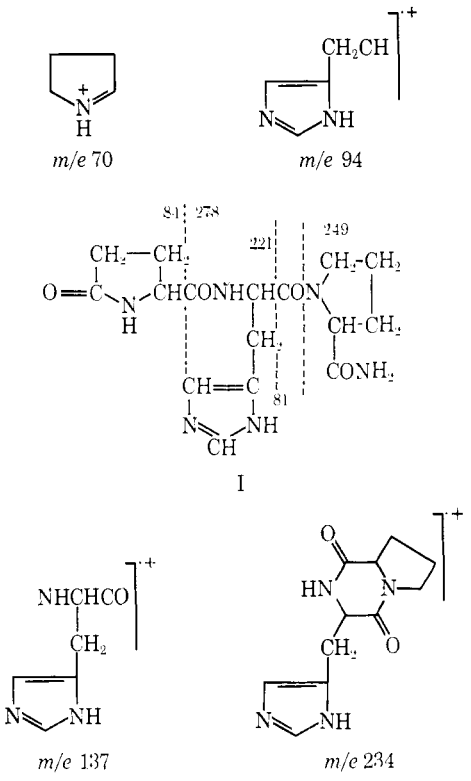
(15) R. M. G. Nair, J. F. Barrett, C. Y. Bowers, and A. V. Schally, *Biochemistry*, **9**, 1103 (1970).

(16) A. V. Schally, T. W. Redding, C. Y. Bowers, and J. F. Barrett, *J. Biol. Chem.*, **244**, 4077 (1969).

ture. Therefore, the nmr spectra of the isolated porcine hormone and synthetic pGlu-His-Pro-NH_2 were compatible.

Mass Spectral Data.—Ions are depicted in Chart I.

CHART I



These mass spectra obtained by the direct introduction of the porcine hormone into the ion source at 160° revealed the following ions: *m/e* 84, the pyrrolidone fragment corresponding to glutamic acid derivatives; *m/e* 81, the imidazolylmethylene ion; *m/e* 70, the amine fragment from the proline moiety.

At 200°, a new ion appeared which is apparently the result of a thermal reaction. Its mass was determined to be 234.1129. This mass corresponds to $C_{11}H_{14}N_4O_2$ which has a theoretical mass of 234.1117. This ion is assigned to the diketopiperazine formed from the histidyl–proline sequence of the tripeptide. The thermal formation of this diketopiperazine only confirms that histidine is linked to proline, but does not independently prove whether the dipeptide moiety is histidylproline or prolylhistidine in the hormone molecule. The spectra obtained at 200° also revealed an ion, *m/e* 154, which may correspond to the diketopiperazine which has lost the imidazolyl CH_2 fragment, with simultaneous H transfer to the charged fragment.

Treatment of the porcine hormone under conditions of methanolysis for conversion of an amide to an ester, even in low yield, leads to a mass spectrum showing a molecular ion at *m/e* 377 which corresponds to pGlu-His-Pro-OCH_3. Further, an ion, *m/e* 249, was observed which corresponds to the loss of the fragment of proline methyl ester having a mass of 128. A molecular ion, *m/e* 409, was not observed as expected which could correspond to OCH_3-Glu-His-Pro-OCH_3.

All these characteristic ions in the mass spectra related to the porcine hormone are compatible with the structure of pGlu-His-Pro-NH_2 for the porcine hormone.

Synthesis of pGlu-His-Pro-NH_2 from Glu-His-Pro.

A. Methods.—Commercial abs MeOH was dried before use by treating it with Mg turnings and refluxing for 2 hr followed by distn. Anhyd HCl was bubbled through the dried anhyd MeOH until the mixt contd 5 g of HCl per 100 ml of MeOH (1.37 mmoles of HCl per ml).

In 1966, Dr. Frederick W. Holly of Merck Sharp and Dohme Research Laboratories, Rahway, N. J., kindly provided gift samples on a milligram basis of tripeptides containing Pro, Glu, and His, and including the sequence Glu-His-Pro. At the time of the gift of these several tripeptides, the data on the sequence of the three amino acids had not yet been obtained.

Glu-His-Pro (1) (2 mg, 5 μmoles) was dissolved in 2 ml of the dried anhyd MeOH contg 1.37 mmoles/ml of HCl and the mixt was allowed to stand in a sealed screw cap vial at room temp for 90 min. The MeOH and HCl were then removed at room temp under vacuum and over NaOH. The residue was redissolved in anhyd MeOH and the solvent was removed under vacuum. This residue was then redissolved in anhyd MeOH and the solvent was removed under vacuum several times in order to remove the excess HCl.

The dimethyl ester of Glu-His-Pro·HCl was then dissolved in 1 ml of MeOH and the mixt was transferred to a screw cap vial. The MeOH was removed under vacuum and the residue was then dissolved in 2 ml of anhyd MeOH satd with NH_3 at −5°. The mixt was allowed to stand at room temp for 24 hr in a sealed screw cap vial, and, after the excess MeOH and NH_3 were removed under vacuum at room temp in a desiccator over H_2SO_4, pGlu-His-Pro-NH_2 was obtained. Characterization data are in Table I.

B. Discussion of Results.—The product, pGlu-His-Pro-NH_2, exhibited a positive reaction with the Pauly reagent revealing the presence of the His moiety. It exhibited no reaction with the ninhydrin reagent showing that the NH_2 group of the glutamic acid moiety was cyclized to the lactam form.

The data in Table I were obtained for the R_f values in 4 diversified chromatographic systems which characterize and define the chemical mobility of the pGlu-His-Pro-NH_2 according to the Pauly reagent.

In Table I, the R_f values for the Pauly-spot characterization of the porcine hormone are included. It is evident that the synthetic pGlu-His-Pro-NH_2 and the porcine hormone are indistinguishable by the Pauly reagent in these 4 diversified chromatographic systems.

The possible appearance of pGlu-His-Pro-OCH_3 (**4**) in the chromatography, which would also have been ninhydrin negative but revealed by the Pauly reagent, was not observed during the chromatography after the reaction of the Me ester with NH_3. A sample of the separately characterized pGlu-His-Pro-OCH_3, described in a companion paper,[5] exhibited an R_f value of 84 in comparison with 68 for pGlu-His-Pro-NH_2 in system 3 of Table I. Consequently, any concern[17] over confusion between pGlu-His-Pro-NH_2 (**1**) and pGlu-His-Pro-OCH_3 is not applicable for the experiments which we conducted, since the latter compound was absent.

(17) R. Burgus and R. Guillemin, *Annu. Rev. Biochem.*, **39**, 499 (1970).

TABLE I

R_f VALUES OF pGLU-HIS-PRO-NH_2 FROM GLU-HIS-PRO AND OF TRH

No.	Chromatographic solvent systems	Volume ratio	Adsorbents	$100 \times R_f$ values	$100 \times R_f$ values for porcine hormone
1	$CHCl_3$–MeOH–AcOH (38°)	60:40:20	Silica gel G	52	52
2	$CHCl_3$–MeOH–AcOH (38°)	60:40:20	Cellulose F	56	56
3	$CHCl_3$–MeOH–coned NH_3	60:45:20	Silica gel G	68	68
4	$CHCl_3$–MeOH–coned NH_3	60:45:20	Cellulose F	75	75

TABLE II

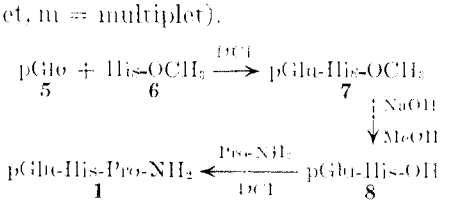
R_f VALUES OF TRH AND pGLU-HIS-PRO-NH_2 (1) FROM 2ND SYNTHESIS

No.	Solvent systems	Volume ratio	Adsorbents, paper and sheet	$100 \times R_f$ values TRH	1
1	$CHCl_3$–MeOH–AcOH (38°)	60:40:20	Polyamide[b]	73	73
2	$CHCl_3$–MeOH–AcOH (38°)	60:40:20	Chromar 500	51	51
3	MeOH–$CHCl_3$	60:30	Silica gel G	15	15
4	MeOH–$CHCl_3$	60:30	Polyamide	15	15
5	Me_2CO–H_2O	80:40	Cellulose	30	30
6	Me_2CO–H_2O	80:20	Chromar 500	56	56
7	*n*-BuOH–EtOAc–AcOH–H_2O	1:1:1:1	Silica gel G	24	24
8	*n*-BuOH–EtOAc–AcOH–H_2O	1:1:1:1	Cellulose F	53	53
9	EtOAc–pyridine–AcOH–H_2O	60:20:6:11	Aluminum oxide	23	23
10	$CHCl_3$–MeOH–AcOH (38°)	60:40:20	Whatman paper No. 54	52	52
11	*n*-BuOH–H_2O–coned NH_3	86:9:5	Whatman paper No. 54	15	15
12	Coned HCl–*i*-PrOH–H_2O	82:325:84	Cellulose F	52	52
13	Pyridine–EtOH–Et_2NH–H_2O	44:20:0.2:16	Silica gel G	64	64

[a] Cellulose and silica G precoated plates from E. Merck. [b] MN Polyamide-U_{254} (Brinkmann).

Synthesis of pGlu-His-Pro-NH_2 from the Amino Acids.—Since the pGlu-His-Pro-NH_2 which had been synthesized from Glu-His-Pro had exhibited the hormonal activities of TRH on a similar nanogram basis and since the 4 Pauly R_f values for the synthetic and isolated porcine hormone preparations were indistinguishable and since much more synthetic pGlu-His-Pro-NH_2 was needed than could be obtained from the generous gift samples of the tripeptide, another independent synthesis was urgently needed in order to characterize further the synthetic pGlu-His-Pro-NH_2 and to permit many more comparisons of it with the porcine hormone, both chemically and biologically. Consequently, a new and second synthesis was performed as described in detail in an accompanying paper;[2] it was as follows.

L-Pyroglutamic acid (**5**) and L-histidine Me ester (**6**) were coupled by *N,N'*-dicyclohexylcarbodiimide (DCI) and the resulting L-pyroglutamylhistidine Me ester (**7**) was hydrolyzed by NaOH in abs MeOH to pyroglutamylhistidine (**8**). The latter dipeptide was coupled with prolinamide by DCI to give pGlu-His-Pro-NH_2 (**1**). Its nmr spectrum (MeOH-d_4, τ values relative to TMS) revealed the following absorptions: 2-H His at τ 2.39 (bs), 4-H at 3.10 (bs), α-H His at 5.55 (m), α-H pGlu at 5.80 (m), α-H Pro at 6.35 (m), CH_2-His at 7.0 (m), 5-CH_2 Pro at 7.0 (m), CH_2CH_2-pGlu at 7.7 (m), CH_2CH_2-Pro at 8.1 (bm) (b = broad, s = singlet, m = multiplet).



Chromatographic Identity of pGlu-His-Pro-NH_2 and Porcine TRH.—The pGlu-His-Pro-NH_2 which was obtained by this second synthesis and on a larger scale, was found to have the same R_f values in the same 4 solvent systems described in Table I for the pGlu-His-Pro-NH_2 which was derived directly from Glu-His-Pro. Consequently, the pGlu-His-Pro-NH_2 obtained from both syntheses is identical.

It was evident that R_f values could uniquely serve the purpose of an extended comparison of synthetic pGlu-His-Pro-NH_2 and the isolated porcine hormone. The traditional comparisons of synthetic and natural products were handicapped, because of the nature of the isolated hormone. Consequently, 13 additional chromatographic systems were devised to represent as great a diversity as feasible for both the solvent systems and the absorbents and were then used to compare the synthetic and natural products. The 13 R_f values of Table II for the pGlu-His-Pro-NH_2 from the second synthesis and the isolated porcine hormone are indistinguishable in all of the 13 systems. Combining these values with those in Table I, it is evident that the synthetic and natural products are indistinguishable in all 17 systems or far more chromatographic systems than usually employed in such research.

Chromatographic techniques in addition to those in Tables I and II were also employed. When equal amounts of synthetic pGlu-His-Pro-NH_2 and the isolated porcine hormone were both dissolved in MeOH and spotted on a silica gel plate and developed two dimensionally in pyridine–EtOH–Et_2NH–H_2O (44:20:0.2:16) and then with BuOH–EtOAc–0.2 *N* aq NH_4OH (1:1:2), the Pauly reagent revealed only one spot which again showed identity.

The Pauly derivatives of the spots were developed

in a system consisting of PhOH–HCOOH–H_2O (500:13:167); there was no difference in the chromatographic behavior of the Pauly derivatives of the synthetic and natural products; they were identical.

Altogether, 17 R_f values and an 18th tlc two-dimensional chromatographic technique show that the synthetic pGlu-His-Pro-NH_2 in the isolated porcine hormone are identical.

Electrophoretic Identity of pGlu-His-Pro-NH_2 and Porcine TRH.—The De Saga–Brinkmann electrophoretic apparatus was used for comparison of synthetic and natural samples by tlc. The buffers were as follows: pH 4.45, pyridine acetate buffer, 700 V, 3.5 mA, 65 min; pH 6.5, pyridine acetate buffer, 400 V, 10.5 mA, 100 min; pH 8.6, Et_3N carbonate buffer, 1st run, 300 V, 7.5 mA, 2.5 hr, 2nd run, 450 V, 7.0 mA, 2.5 hr.

The tl electrophoretic mobilities (U) of the synthetic and natural porcine TRH were found to be as follows: pH 4.45, layer of cellulose 300 HR (Brinkmann), 250 μ thick, $\tau = 7°$, $U = -7.1$ cm^2 V^{-1} sec^{-1} $\times 10^{-5}$; pH 6.5, Brinkmann microcrystalline cellulose, 250 μ thick, $\tau = 7°$, $U = -9.2$ cm^2 V^{-1} sec^{-1} $\times 10^{-5}$; pH 8.6, layer same as for pH 4.45, $\tau = 7°$, $U = -3.1$ cm^2 V^{-1} sec^{-1} $\times 10^{-5}$.

The electrophoretic mobilities of synthetic pGlu-His-Pro-NH_2 and the isolated porcine hormone in the 3 different systems were identical, and both the natural and synthetic products moved toward the cathode. The mobilities are relative and do not take into consideration the effects of electroosmosis, diffusion, etc.

Identity of Behavior of pGlu-His-Pro-NH_2 and Porcine TRH Over Sephadex G-25.—A column of Sephadex G-25 (fine beads), 1.2 × 123 cm, was used. The solvent was 0.2 M AcOH and the hold-up vol (calibrated with blue dextran) was 50 ml. The fraction size was 1.6 ml and the flow rate was 9 ml/hr. Samples of 120 μg of the isolated porcine hormone and 200 μg of synthetic pGlu-His-Pro-NH_2 were used. Each product was applied in 300 μl of solvent.

The R_f value was calcd according to Porath and Schally[18] and was 50/(59 × 1.6) = 50/94 = 0.53. Previously, Schally, *et al.*,[16] had reported 0.51.

The identity of the gel filtration behavior of synthetic pGlu-His-Pro-NH_2 and isolated porcine TRH on Sephadex G-25 is recorded in Figure 1.

Densitometric Determination of TRH.—To compare critically and quantitatively the biological activity of synthetic and porcine TRH, a densitometric method for quantitating the weight of small amts of this hormone was used,[19] so that equal amts of the prepns could be assayed. As little as 0.5 μg of these prepns could be estimated (±10% error) with this densitometric method by photometry of the charred spots developed after chromatography on glass-paper impregnated with silica gel. After chromatography in $CHCl_3$–MeOH–conc'd NH_3 (60:5:0.5), the chromatograms were sprayed with H_2SO_4–dichromate soln and the charred spots were developed by heating. The regression for synthetic and porcine TRH by this method was linear between 0.5 and 1.5 μg.

Indistinguishable Biological Activities of pGlu-His-Pro-NH_2 and Porcine TRH.—The T_3-TRH method in mice of Bowers, *et al.*,[20–22] was used to compare the synthetic pGlu-His-Pro-NH_2 and the isolated porcine TRH. In this methodology, the biological activity is quant measured by the change, Δcpm, in ^{125}I in the samples of blood which are taken before and 2 hr after iv injection of the sample. The blood levels of ^{125}I are proportional to the amount of induced release of TSH from the pituitary gland. Each result (Δcpm) is the mean average of the changes in the blood levels of ^{125}I for 8 mice. The comparative data for the synthetic and natural products are in Table III. Extensive testing, as represented by the data in Table III showed that graded responses were obtained as the dose levels of the synthetic and natural products were increased. There was no significant difference in the hormonal potency and activity of the synthetic and natural products according to this method in mice.

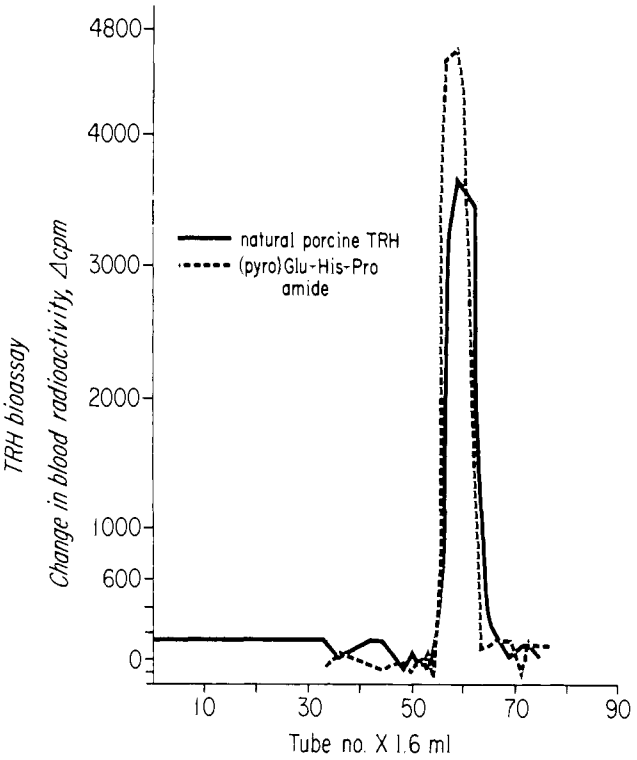


Figure 1.—Behavior on Sephadex G-25.

TABLE III

BIOLOGICAL COMPARISON OF PORCINE TRH AND SYNTHETIC pGLU-HIS-PRO-NH_2 BY THE T_3-TRH METHOD IN MICE

Dose, ng	^{125}I Δcpm TRH	^{125}I Δcpm synth	p value	Dose, ng	^{125}I Δcpm TRH	^{125}I Δcpm synth	p value
Control	24			Control	280	320	ns
3	2834	2746	ns	6	4011	4243	ns

By the method of Bowers, *et al.*,[23] in an assay of TRH in rats, the synthetic and natural products (50 ng of each) were comparably effective in elevating the blood levels of TSH 10 and 15 min after iv injection of

(18) J. Porath and A. V. Schally, *Endocrinology*, **70**, 738 (1962).

(19) C. Y. Bowers, J. G. Hamilton, J. E. Muldrey, W. T. Miyamaeu, G. A. Reynolds, and A. V. Schally, *J. Amer. Oil Chem. Soc.*, **43**, 1 (1966).

(20) C. Y. Bowers, A. V. Schally, G. A. Reynolds, and W. D. Hawley, *Endocrinology*, **81**, 741 (1967).

(21) C. Y. Bowers and A. V. Schally, "Hypophysiotropic Hormones of the Hypothalamus: Assay and Chemistry," J. Meites, Ed., Williams and Wilkins Co., Baltimore, Md., 1970, p 74.

(22) C. Y. Bowers, A. V. Schally, F. Enzmann, J. Bøler, and K. Folkers, *Endocrinology*, **86**, 1143 (1970).

(23) C. Y. Bowers, T. W. Redding, and A. V. Schally, *ibid.*, **77**, 609 (1965).

the samples into 250-g male rats, which were anesthetized with urethane.

Both the synthetic and natural products were inactivated after incubation with human serum at 37° for 15 min and the degree of the biological activity which was exhibited in mice for both products was shown to depend upon the amount of T_3 which was injected. These biological characteristics are identical for the two products.

The synthetic and biological products were indistinguishable, *in vitro*, by the method of Bowers, *et al.*[23] This *in vitro* response to both the synthetic and natural products could be partially or completely inhibited by the addition of T_3. The same inhibition by T_3 was also observed, *in vivo*, for both products.

It was observed that the plasma levels of TSH increased within 2 min after iv injection of both the synthetic and natural products in mice and in rats. The duration of the elevated levels of TSH was dependent upon dosage.

By all these diversified biological assays and responses which have been generally accepted for TRH for some time, the synthetic pGlu-His-Pro-NH_2 and the isolated porcine hormone are identical.

Discussion

If the isolated porcine TRH had been 100% pure, then acid hydrolysis of it would give a theoretical yield of the combined 3 amino acids amounting to about 110% of the weight of the pure TRH. On the basis of the structure of TRH, the recognized presence of certain impurities, and the previously reported 50–60% actual yield (corr) of combined amino acids from the acid hydrolysis of porcine TRH, it is evident that the actual purity of the isolated hormone was a little less than 50–60%. In this situation, this range of actual purity is of importance only to show the unfeasibility of certain traditional chemical comparisons for the identity of synthetic and natural products. The achievement of even 50–60% purity for the isolation of the porcine hormone and about 80% purity for the ovine hormone[24] were results of prodigious and highly successful efforts.

The important point about the comparison of the synthetic pGlu-His-Pro-NH_2 and the isolated TRH showing identity is not so much how the comparisons were performed under these exceptional circumstances of structural elucidation, but rather the validity of the assignment of structure to the natural product.

It was stated[12] that if the structure of porcine TRH is not that of pGlu-His-Pro-NH_2, then any structural modification of pGlu-His-Pro-NH_2 would necessitate another compound having identical R_f values to those of pGlu-His-Pro-NH_2 in all of the 17 systems. Since such modification seemed untenable, it was concluded in the summary that TRH is pGlu-His-Pro-NH_2 and that this formulation represents the elucidation of the first of the hypothalamic hormones that had been sought for so many years.

Then the new report appeared by Burgus, *et al.*,[25] on their concomitant structural and synthetic studies on their isolated ovine TRH which led them to state that "Quoique la molécule de l'hormone hypothalamique TRF d'origine ovine ne corresponde pas á la séquence Pyroglu-His-Pro-NH_2, la possibilité n'est pas exclue d'une structure avec un groupement amidé secondaire ou tertiarie" and "a structure such as R-Glu-His-Pro-R' where R- designates a blocked N-terminus (most likely as PCA) may represent the minimal active core of the ovine hypothalamic hypophysiotropic hormone TRF";[26] a subsequent addendum was added to this paper[26] which reconciled their structural and synthetic studies with the conclusion that ovine TRH also has the structure pGlu-His-Pro-NH_2, and acknowledged our two communications.[6,12] Burgus, *et al.*,[27] reported mass spectra data for their ovine TRH which was in agreement with pGlu-His-Pro-NH_2.

As the final stages of the structural elucidation and synthesis of porcine and ovine TRH were being completed with the emerging knowledge that TRH of both mammalian species are identical, there was also a reorientation of the implications stemming from the knowledge that a hypothalamic hormone such as TRH could be as small as just a tripeptide, pGlu-His-Pro-NH_2.

Besides the publications on the structure and synthesis of porcine TRH[6,12] and on ovine TRH,[27] several important contributions to the chemistry of TRH have also appeared. These included additional data on the structure of porcine TRH by Nair, *et al.*,[15] the synthesis of the thyrotropin-releasing hormone and related peptides by Gillesen, *et al.*,[28] and the synthesis of the thyrotropin-releasing hormone by classical and solid phase methods by Flouret.[29]

Acknowledgments.—Appreciation is expressed (K. F.) to Dr. Sheldon J. Segal for his early interest and to the Population Council for support, and to Dr. David Isaksson of Aktiebolaget/Kabi, Stockholm, Sweden, for his interest and support, and to Dr. Conrad Cone of the University of Texas at Austin, Austin, Texas, and Dr. G. Doyle Daves, Jr., of the Oregon Graduate Center, Portland, Oregon, for their cooperation on mass spectral data. The biological aspects (C. Y. B.) were supported in part by National Institutes of Health Grant AM 06164-08.

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