THE IDENTITY OF CHEMICAL AND HORMONAL PROPERTIES OF THE THYROTROPIN RELEASING HORMONE AND PYROGLUTAMYL-HISTIDYL-PROLINE AMIDE

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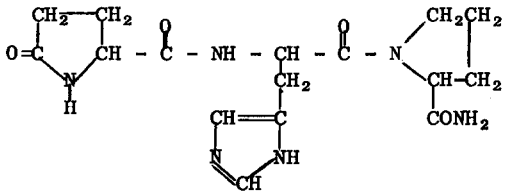
Summary. The structure of L-pyroglutamyl-L-histidyl-L-proline amide (I) is in agreement with all of the known chemical and hormonal properties of the thyrotropin releasing hormone (TRH) from porcine hypothalami. This structural interpretation is probably applicable to the TRH of other mammalian species, and is chemically based on the chromatographic identity in seventeen diversified systems and is biologically based on a quantitative comparison of the hormonal activities of the natural and synthetic products. This formulation of structure represents the elucidation of the first of the hypothalamic hormones that have been sought for so long.



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The thyrotropin releasing factor or hormone which was isolated from porcine hypothalami by Schally et al. in 1966-'69 (1,2) was "nearly pure". The enormous effort and cost associated with the attainment of only a few milligrams of the hormone from about one-quarter million animals restricts the stepwise experimentation to elucidate its chemical structure. Two or three samples of the hormone appeared to be homogeneous by TLC, electrophoresis and paper chromatography. The component(s) in such samples on chromatography is revealed by a reagent or a physical characteristic, but other components can be present in such natural materials which are not normally revealed. For

example, one of these hormone samples yielded an unsaturated fatty acid which appeared to be myristoleic acid, according to mass spectrometry and gas-chromatography. The presence of the insidious dioctyl phthalate was seen by mass spectrometry. The presence of such components with the hormone obscures the interpretation of spectral data. Myristoleic acid and related components could conceivably be related to the hormone. Structural elucidation on a microgram-basis and further purification are alternated so that each experiment could be significant towards the objective. On this basis, we now describe new data which contribute to the structure and synthesis of the thyrotropin releasing hormone from porcine hypothalami.

A significant degradation by acid hydrolysis was reported by Schally et al. in 1966 (1) which yielded three amino acids, histidine, glutamic acid and proline. These three amino acids were obtained in essentially equimolar amounts, and it was found by Schally et al., in 1969 (2) that these three amino acids were from the structural sequence of Glu-His-Pro as a probable moiety of TRH. The tripeptide, Glu-His-Pro, as well as several alternative sequences of the three amino acids showed no hormonal activity of TRH according to Schally et al., 1968 (3). Since TRH does not have a free amino or carboxyl group, synthetic experiments were carried out on Glu-His-Pro to modify both the amino and carboxyl groups, and biological assays on the preparations were carried out under the diverse conditions which characterize the hormonal activities of TRH. It was discovered by Folkers et al., in 1969 (4) that a synthetic preparation, presumably (pyro)Glu-His-Pro(NH_2) , resulting from the methylation and ammonation of the tripeptide, exhibited hormonal activities, at nanogram-dose levels in vivo and at picogram-levels in vitro, which were qualitatively indistinguishable from those of TRH.

(pyro)Glu-His-Pro(NH_2) (I) has now been characterized by its hydrolysis to the three amino acids. In its nuclear magnetic spectrum, (CH_3OH-d_4, τ-values relative to TMS) the following absorptions can be distinguished: 2-H His at τ=2.39 (bs); 4-H His at τ=3.10 (bs); α-H His at τ=5.55 (m); α-H (pyro)Glu at τ=5.80 (m); α-H Pro at τ=6.35 (m); $-CH_2$-His at τ=7.0 (m); 5-CH_2 Pro at τ=7.0 (m); $-CH_2CH_2$-(pyro)Glu at τ=7.7 (m); $-CH_2CH_2$-Pro at τ=8.1 (bm). (b=broad; s=singlet; m=multiplet)

(pyro)Glu-His-Pro(NH_2) is also characterized by the seventeen R_f-values in Table I, which represent diversified solvent systems and chromatographic techniques. For comparison, the R_f-values of TRH in each of the systems are included. The presence of other components in the hormone samples is not always a difficulty; it is strategically useful that there is only one component which reacts with the Pauly reagent and this component is believed to be

Table I. R_f-Values of TRH and (Pyro)Glu-His-Pro(NH_2) (I) in Chromatography

No.	Solvent Systems and Ratio		Adsorbents Paper and TLC[2]	100x R_f Values TRH	I
1	Chloroform/methanol/acetic acid (38%).	60:40:20	Silica Gel G	52	52
2	———— " ————	— " —	Cellulose F	56	56
3	———— " ————	— " —	Polyamide[1]	73	73
4	———— " ————	— " —	Chromar 500	51	51
5	Chloroform/methanol/conc. ammonia.	60:45:20	Silica Gel G	68	68
6	———— " ————	— " —	Cellulose F	75	75
7	Methanol/chloroform.	60:30	Silica Gel G	15	15
8	———— " ————	— " —	Polyamide	15	15
9	Acetone/water.	80:40	Cellulose	31	30
10	Acetone/water.	80:20	Chromar 500	56	55
11	n-Butanol/ethyl acetate/ acetic acid/water.	1:1:1:1	Silica Gel G	21	21
12	———— " ————	— " —	Cellulose F	53	53
13	Ethyl acetate/pyridine/ acetic acid/water.	60:20:6:11	Aluminumoxide	23	23
14	Chloroform/methanol/acetic acid (38%).	60:40:20	Whatman paper No. 54	52	52
15	n-Butanol/water/conc. ammonia.	96:9:5	Whatman paper No. 54	15	15
16	Conc. HCl/isopropanol/water.	92:325:83	Cellulose F	52	52
17	Pyridine/ethanol/diethyl-amine/water.	44:20:0.2:16	Silica Gel G	64	64

[1] MN Polyamide-U_{254} (Brinkmann).
[2] Cellulose and Silica G precoated plates from E. Merck.

TRH, because of abundant data from three years of experimentation with the highly purified TRH which showed that the Pauly-reactive component is always associated with the hormonal activity. Consequently, the seventeen R_f-values for TRH are based upon the Pauly-zone. For development, the ascending technique at chamber saturation and at room temperature was generally used. Samples in the range of 1-3 μg. were applied.

The chromatographic data in Table I show that the R_f-values of TRH and those of the synthetic (pyro)Glu-His-Pro(NH_2) are indistinguishable in all of

the seventeen systems. The diversity of the systems provides additional credence on the possible identity of the natural and synthetic products.

(pyro)Glu-His-Pro(NH_2) has been critically and quantitatively examined for the characteristic hormonal activities in comparison with natural porcine TRH. In Table II, are the data on the comparison on the biological activities and potencies of porcine TRH and the synthetic (pyro)Glu-His-Pro(NH_2) by the T_3-TRH method in mice of Bowers et al. (5,6,7). In this method, the biological response is quantitatively measured by the change, as Δcpm, in I^{125} in the blood samples before and two hours after the intravenous injection of the sample to be assayed. The levels of I^{125} in the blood are proportional to the amount of the induced release of TSH from the pituitary. Each result (Δcpm) is the mean average of the changes in the levels of I^{125} in the blood from eight mice.

The data in Table II show that graded responses were obtained as the dose levels of TRH and (pyro)Glu-His-Pro(NH_2) were increased. No significant difference in the biological activity and potency of the natural and synthetic products was observed by this method in mice.

Fifty but not twenty-five nanograms of both TRH and (pyro)Glu-His-Pro (NH_2) were effective in elevating the blood levels of TSH ten and fifteen minutes after intravenous injection in male rats weighing 250g. which were anesthetized with urethane. By this method of Bowers et al. (5) in rats, the natural and synthetic products showed essentially equivalent potencies.

Both the natural and synthetic products were indistinguishable by two important biological characteristics of TRH: (a) both were inactivated after incubation in human serum at 37°C for fifteen minutes; (b) the degree of the biological response in mice depended upon the amount of T_3 injected. The

Table II. Data on the Biological Comparison of Procine TRH and (pyro)Glu-His-Pro(NH_2) by the T_3-TRH Method in Mice.

Dose nanograms	I^{125} Δcpm		p-value	Dose nanograms	I^{125} Δcpm		p-value
	TRH	I*			TRH	I*	
Control	24	-		Control	280	320	ns
1	582	825	ns	2	1450	1605	ns
3	2834	2746	ns	6	4011	4243	ns
9	4015	4664	ns	18	5401	5205	ns

* (pyro)Glu-His-Pro(NH_2)

synthetic product was indistinguishable, in vitro, from TRH by the method of Bowers et al. (5); this response could be partially or completely inhibited by the addition of T_3 not only in vitro but also when T_3 was given in vivo. The plasma levels of TSH increased within two minutes after the intravenous injection of both products in mice and in rats. The duration of the elevated levels of TSH depended upon the dosage.

The synthetic and natural products are indistinguishable in seventeen chromatographic systems, and in hormonal activity on a quantitative basis. Neither the synthetic (pyro)Glu-His-Pro(NH_2) nor the porcine TRH under conditions of mass spectrometry give a molecular ion. On the basis of the structure of (pyro)Glu-His-Pro(NH_2) for TRH, then the samples of TRH from porcine hypothalami have been approximately 30-35% pure (2). This approximation of purity is important, because it reflects upon the interpretation of spectral and other data, and provides guidelines for other structural experiments on TRH.

If the structure of TRH is not that of (pyro)Glu-His-Pro(NH_2), then certain possibilities are evident: (a) substitution of pyro-Glu or a Gln-moiety by hydrolyzable groups attached to the nitrogen atom(s); (b) modification of the proline-amide group; (c) replacement of amidic proton(s) by a group(s) which would permit acid hydrolysis to the three amino acids; (d) attachment of a group to the nuclear imino group of histidine is ruled out, because of the positive Pauly-reaction. Any structural modification of (pyro)Glu-His-Pro(NH_2) would necessitate another compound having identical R_f-values to those of (pyro)Glu-His-Pro(NH_2) in all of the seventeen systems. There would also be a certain lack of structural specificity and potency for the hormonal activity of TRH, because then (pyro)Glu-His-Pro(NH_2) would have comparable activity and potency to that of a structurally different TRH.

Burgus et al. (8) have described the TRH activity at microgram levels of the acetylation products of Glu-His-Pro. Burgus et al. (9) have currently reported (pyro)Glu-His-Pro(NH_2) to show TRH-activity in vivo at about 50 ng./dose which was one-third to one-fifth that of ovine TRH, and stated that the structure of ovine TRH is not that of (pyro)Glu-His-Pro(NH_2) and that a secondary or tertiary amide modification is not excluded.

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