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The Relevance of Imidazole Tautomerism for the Hormonal Activity of Histidine-Containing Peptides

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Substitution of 5-nitro-L-histidine for L-histidine is proposed as a useful tool to study the relationships among tautomerism, acid-base properties, and biological activity of peptide hormones. This approach is illustrated by an analog of the tripeptide thyroliberin, [5-nitro-L-histidine]^2-thyroliberin, which has been prepared by solid-phase peptide synthesis. The acid-base properties of the hormone analog and the position of the imidazole ring tautomeric equilibrium have been investigated by spectroscopic methods. Correlation of these properties with the biological activity of the nitrated tripeptide strongly supports the idea that imidazole ring tautomerism is a key factor for hormonal activity and that the N^{τ}-H tautomer must be considered the biologically active form of thyroliberin.

The importance of imidazole ring tautomerism for the biological activity of histidine-containing peptides has been alluded to on several occasions (*1–3*). In our opinion, however, the subject has not received as much attention as it deserves. Seemingly endless discussions about "active conformations" of peptide hormones (i.e., whether they coincide with those determined by NMR or derived from theoretical calculations) contrast with the near indifference to the fact that every histidine-containing peptide may exist, and usually does, as two constitutionally different forms, i.e., both N^{τ}-H and N^{π}-H tautomers in equilibrium (Fig. 1).

^{13}C NMR allowed Reynolds *et al.* (*1*) to conclude for the first time that the N^{τ}-H $\rightleftarrows$ N^{π}-H tautomeric equilibrium of histidine is shifted toward the former tautomer. In the following years this same technique has been applied, at a rather slow pace, to some peptides and proteins. In most cases it has been found that the N^{τ}-H tautomer is predominant (*1, 5*), with a few probably meaningful exceptions (*6*). In any event, and similarly to what happens in the conformation problem, establishing which is the predominant tautomer does not solve the problem of which is the active tautomer of a histidine-containing hormone. In this context, a simple way to approach this question is to use modified peptides in which the tautomeric equilibrium is "frozen" toward one of the two forms. This work presents one such modification, namely the introduction of a nitro group in position 5 of the imidazole ring of histidine.

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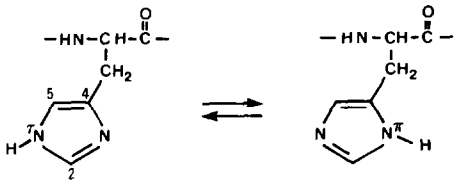


FIG. 1. Tautomeric equilibrium in a histidine residue. Imidazole ring atoms are labeled according to the IUPAC–IUB Commission on Biological Nomenclature (*4*).

The tripeptide thyroliberin (TRH)[1] is perhaps one of the clearest examples of this underrating of the biological significance of histidine tautomerism that we have just mentioned. Rivier *et al.* (*7*) in 1972 described the synthesis of both N^{τ}-methyl and N^{π}-methyl derivatives of thyroliberin. The former was practically inactive whereas the latter surprisingly turned out to be eight times more active than the natural hormone in an *in vivo* assay. After determining the pK_a's of the conjugated acids of all three compounds involved, these investigators concluded that an inverse correlation existed between basicity of imidazole ring and hormonal activity (Fig. 2).

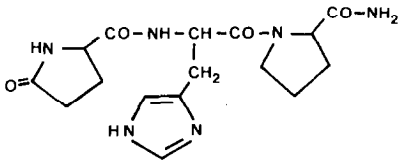
The results of Rivier *et al.*, however, are susceptible of an alternative interpretation. One could view each methyl derivative of thyroliberin as a sort of frozen tautomer of the hormone and consider that the increased activity of the N^{τ}-methyl form was simply due to a complete shift of the tautomeric equilibrium toward the "right" direction. This possibility had already been suggested by Deslauriers *et al.* (*8*) in 1974 in their ^{13}C-NMR study of thyroliberin tautomerism, which showed the N^{τ}-H form to be predominant. The introduction of a nitro group in position 5 of the histidine ring of thyroliberin should in principle decrease the basicity of such ring and predictedly exalt hormonal activity, if indeed a correlation exists between acid-base properties and biological activity. As we see later, the nitro group causes a shift in the tautomeric equilibrium toward the biologically "wrong" N^{π}-H tautomer. Assuming the alternative view that the position of this equilibrium determines biological activity, one could expect a significant loss of potency for this analog. We show that this is in fact the case and, furthermore, we suggest that substitution of 5-nitro-L-histidine for histidine provides a general method to study the relationships among tautomerism, acid-base properties, and biological activity in peptide hormones.

RESULTS

Synthesis

[5-nitro-L-histidine]^2-Thyroliberin was synthesized following standard solid-phase synthesis procedures on a benzhydrylamine resin prepared from polystyrene via Leuckart reductive formylation (*9*). 5-Nitro-L-histidine was assembled as

[1] Abbreviations used: TRH, tripeptide thyroliberin; TLC, thin-layer chromatography.



Glp — His — Pro-NH_2

	% thyroliberin potency	pKa^{I} (see fig.3)
[N^{π}-methyl-L-histidine]^{2}-thyroliberin	0.04	6.6
Thyroliberin	100	6.25
[N^{τ}-methyl-L-histidine]^{2}-thyroliberin	800	5.95

FIG. 2. Structure of thyroliberin (only the N^{τ}-H tautomer shown). Correlation between *in vivo* ability to induce TSH secretion and acid-base properties of N^{im}-methyl derivatives according to Rivier *et al.* (*7*).

N-t-butoxycarbonyl(Boc) derivative without protection of the imidazole ring due to the expected decrease of nucleophilicity caused by the presence of the nitro group. At the end of the synthesis HF-anisole cleavage of the peptide–resin afforded crude [5-nitro-L-histidine]^{2}-thyroliberin in a 76% yield. Purification by adsorption chromatography on silica gel LC-60 gave a homogeneous peptide in a 69% purification yield. The product was characterized by amino acid analysis, ^{1}H NMR, ^{13}C NMR, TLC, and uv spectroscopy.

The quality of the crude peptide was highly sensitive to the procedure followed for the synthesis of the starting benzhydrylamine resin. Thus, in a preview attempt, a benzhydrylamine resin obtained via $LiAlH_4$ reduction of the benzoylpolystyrene oxime (*9*) was used. In this case after the final HF cleavage a complex mixture of peptides was obtained. After gel filtration on a Sephadex G-10 column further purification was attempted without success either by CM52-carboxymethylcellulose or DEAE-Sephadex A25 chromatography. Finally, silica gel LC-60 chromatography afforded the pure target peptide. Orlowsky and Walter have previously reported similar difficulties in the synthesis of peptides when benzhydrylamine resins synthesized via oxime reduction were used (*10*). Using a modification of the Demjanov reaction, these authors provided some indirect evidence for the formation of secondary amines on the resin due to rearrangements during the metal hydride reduction of the oxime. We have recently reported direct ^{13}C-NMR evidence of the presence of rearranged secondary amines in benzhydrylamine resins obtained via oxime reduction (*11*).

Acid-Base Properties

Acid-base properties of imidazole rings can be conveniently described using two different acidity constants, K_a^{I} and K_a^{II} (Fig. 3). In the case of imidazole pK_a^{I} and pK_a^{II}, take the values of 7.0 and 14.2, respectively.

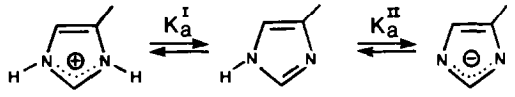


FIG. 3. Protonation equilibria in imidazole derivatives. For simplicity only the N^{τ}-H tautomer present in the equilibrium for the neutral form is represented.

We have previously described the pK_a determination of 5-nitro-L-histidine and related compounds using spectrophotometric methods (*12*). The same experimental approach has now been applied to [5-nitro-L-histidine]^2-thyroliberin. The uv spectra of several aqueous solutions of [5-nitro-L-histidine]^2-thyroliberin, buffered at different pHs with NaOH/glycine, are shown in Fig. 4. pK_a^{II} was calculated using a modification of the Henderson–Hasselbalch equation (*13*)

$$pK_a^{II} = H_0 - \log \frac{A_{A^-} - A}{A - A_{AH}}$$

where H_0 is the Hammet acidity function, which can be assimilated to pH for dilute aqueous solutions; A_{A^-} and A_{AH} are the 353-nm absorbances at extreme pHs and correspond to the absorbance, at this wavelength, of the fully unprotonated and protonated forms, respectively; and A is the absorbance of a solution at any intermediate pH. Following this procedure a value of $pK_a^{II} = 8.50 \pm 0.06$ was obtained.

The pK_a^I of [5-nitro-L-histidine]^2-thyroliberin was determined from the uv absorption at 310 nm of several solutions at different sulfuric acid concentrations in the H_0 range between 0.27 and −0.77. In spite of the presence of four amide bonds, the stability of the peptide against sulfuric acid was shown to be largely sufficient to carry out the determination. Nevertheless, data obtained from the

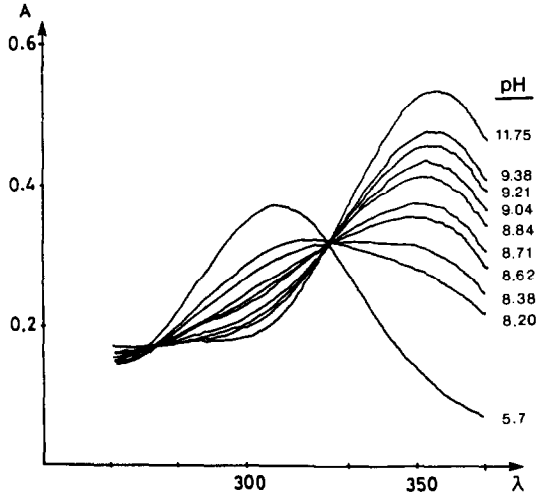
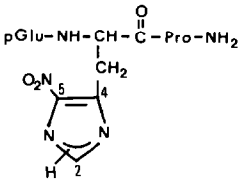
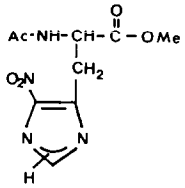


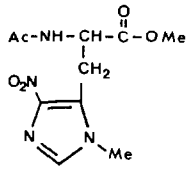
FIG. 4. Absorption spectra of [5-nitro-L-histidine]^2-thyroliberin at different pHs.

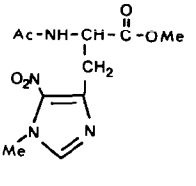
TABLE 1

^{13}C-NMR CHEMICAL SHIFTS (d_6-DIMETHYLSULFOXIDE, 20.12 MHz) OF THE IMIDAZOLE RING CARBON ATOMS OF [5-NITRO-L-HISTIDINE]^2-THYROLIBERIN COMPARED WITH THOSE FROM N^{α}-ACETYL-5-NITRO-L-HISTIDINE METHYL ESTER AND ITS N^{τ}- AND N^{π}-METHYL DERIVATIVES









C_2	C_4	C_5	C_2	C_4	C_5	C_2	C_4	C_5	C_2	C_4	C_5
134.8	130.8	145.0	133.9	130.0	144.1	136.6	131.0	144.6	141.0	142.8	135.5

solutions at the lowest H_0 values were found not to fulfill the Henderson–Hasselbalch equation. pK_a^I was then calculated from the expression

$$A = A_{AH^+} + K_a^I \frac{A_A - A}{h_0}$$

where A_{AH^+} and A_A are the 310-nm absorbances at extreme H_0 values, A is the absorbance at any intermediate pH, and

$$h_0 = \text{antilog } (-H_0).$$

This procedure allows the determination of pK_a^I from the slope of a straight line drawn without using the extreme A_{AH^+} value or other experimental points in the more acidic region that violate linearity. Following this approach a value of $pK_a^I = -0.29 \pm 0.03$ was obtained.

Tautomerism

As already pointed out in the introduction, ^{13}C NMR is probably the method of choice to study the position of the tautomeric equilibrium in imidazole compounds when derivatives representing the frozen forms of the two tautomeric possibilities are not available. We have recently reported a study of the position of the imidazole tautomeric equilibrium in N^{α}-acetyl-5-nitrohistidine methyl ester (*12*). Following different independent criteria the tautomeric equilibrium in this nitrohistidine derivative was shown to be unequivocally shifted to the N^{π}-H tautomer (ca. 98%). The ^{13}C-NMR imidazole ring chemical shifts of N^{α}-acetyl-5-nitrohistidine methyl ester are shown in Table 1 and compared with those of its two frozen tautomeric forms (N^{τ}-methyl and N^{π}-methyl) and those of [5-nitro-L-histidine]^2-thyroliberin. The excellent matching of the chemical shifts of [5-nitro-L-histidine]^2-thyroliberin with those of N^{α}-acetyl-5-nitrohistidine methyl ester and its N^{π}-methyl derivative shows without ambiguity that the tautomeric equilibrium in the peptide is also strongly shifted to the N^{π}-H tautomer.

Biological Activity

The ability of [5-nitro-L-histidine]^2-thyroliberin to stimulate TSH release from the pituitary was assayed in mice by monitoring the release of ^{125}I-labeled T_3 and T_4 from thyroid (*14*). At normal doses, [5-nitro-L-histidine]^2-thyroliberin does not stimulate the secretion of TSH (even a decrease in the blood sample's radioactivity was observed). At high doses (250 μg/mice), an increase in radioactivity was measured which amounted to 0.096% of the activity of native thyroliberin (i.e., a similar response could be obtained with a ca. 1000 times lower dose of thyroliberin).

DISCUSSION

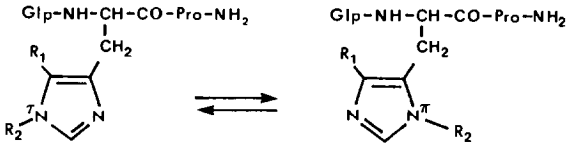
As described above, the pK_a^I and pK_a^{II} values determined for [5-nitro-L-histidine]^2-thyroliberin are -0.29 and 8.50, respectively. That means that at a physiological pH of 7 the imidazole neutral form predominates (ca. 97%) whereas small amounts of anionic form (ca. 3%) and no cationic form (less than 10^{-5}%) are present in the equilibrium. From the reported pK_a values of thyroliberin and its N^τ-methyl and N^π-methyl derivatives it can be easily calculated that the native hormone exists at pH 7 as 85% in neutral form (together with 15% in cationic form), the hyperactive N^τ-methyl analog exists as 92% in neutral form, whereas the inactive N^π-methyl derivative exists as 72% in neutral form. The lack of hormonal activity found in our study when [5-nitro-L-histidine]^2-thyroliberin was administered in doses at which thryoliberin provokes a strong increase of thyrotropin release in mice represents a breaking point in the correlation between the acid-base properties of thyroliberin analogs (pK_a and/or the population of the neutral imidazole form) and their hormonal activity.

For a given compound, using well-established procedures from heterocyclic chemistry, one can infer the position of a tautomeric equilibrium from the experimental values of its pK_a and the pK_as of suitable models (*15*). Such a calculation gives a K_T value of ca. 0.22 for the N_π-H/N_τ-H tautomeric equilibrium of thyroliberin. Similar values can be obtained by comparison of the ^{13}C-NMR resonances of imidazole carbon atoms of thyroliberin with those of its N^{im}-methyl derivatives (*8*). As shown in Table 2, a correlation can be built between the biological activity of thyroliberin and that of its methyl analogs in the sense that the only active tautomer of the native hormone would be the N^τ-H whereas the N^π-H tautomer would be virtually inactive. As demonstrated in the Results section, the tautomeric equilibrium in [5-nitro-L-histidine]^2-thyroliberin is nearly completely shifted toward the N^π-H tautomer. The lack of activity of the nitrated analog of thyroliberin correlates well with the hypothesis that tautomerism and not acid-base properties is the key factor to interpreting the differences in hormonal activity observed between thyroliberin and its N^{im}-methyl derivatives. Therefore we submit that the N^τ-H tautomer of the native hormone is solely responsible for its ability to stimulate thyrotropin release.

Two main objections can be made against the use of [5-nitro-L-histidine]^2-thyroliberin as a model compound for one of the tautomeric forms of thyroliberin. First,

TABLE 2

RELATIONSHIP BETWEEN TAUTOMERISM AND BIOLOGICAL ACTIVITY OF [5-NITRO-L-HISTIDINE]^2-THYROLIBERIN, NATIVE THYROLIBERIN, N^{τ}-METHYL, AND N^{π}-METHYL THYROLIBERIN



R_1	R_2			Biological activity
H	H	82%	18%	100
H	$^{\tau}N$–CH_3	100%	—	800
H	$^{\pi}N$–CH_3	—	100%	0.04
NO_2	H	ca. 0%	ca. 100%	0.01

the physiological behavior of the nitrated analog could differ from that of the native hormone and this could be the cause of its lack of activity. This is, in fact, a very general limitation which applies to all *in vivo* assays using synthetic model compounds and which includes the studies reported by Rivier *et al.* using the N^{τ}- and N^{π}-methyl derivatives of thyroliberin.

The second factor to be taken into account is the possibility of the nitro group provoking strong changes in the conformation of thyroliberin which could cause a drop in biological activity. The ^{1}H NMR (250 MHz) of [5-nitro-L-histidine]^2-thyroliberin in d_6-dimethylsulfoxide allows the determination of the $J_{\alpha\beta}$ and $J_{\alpha\beta'}$ coupling constants in the nitrohistidine side chain by double resonance techniques. The population analysis for the rotamers of the histidine side chain of [5-nitro-L-histidine]^2-thyroliberin can be performed from these values (Table 3) assuming that the *pro-R* protons resonate at lower field than the *pro-S* (*16*) and taking the values of 13.56 and 2.60 Hz as coupling constants between two protons in pure antiperiplanar and synclinal relative positions (*17*). Results are shown in Table 3 and compared with those resulting from NMR data previously reported for native thyroliberin (*18*). From this data any severe modification in the $^{\alpha}C$-$^{\beta}C$ torsion angle in the histidine side-chain can be excluded. This $^{\alpha}C$-$^{\beta}C$ bond is probably one of the most sensitive to conformational changes induced by the presence of the nitro group. Energy calculations of the most stable conformations of [5-nitro-L-histidine]^2-thyroliberin are currently under way in our group and will provide complementary information about conformational changes induced by the nitro group in any part of the molecule.

EXPERIMENTAL

Dichloromethane was dried over anhydrous potassium carbonate and distilled over it immediately before use. Dimethylformamide was dried over 4 Å molecular

TABLE 3

POPULATION ANALYSIS OF HISTIDINE SIDE-CHAIN ROTAMERS IN [5-NITRO-L-HISTIDINE]²-THYROLIBERIN

	-sc	*ap*	*-sc*
[5-Nitro-L-histidine]²-Thyroliberin	47%	29%	24%
Thyroliberin (from Ref. 18)	46%	25%	29%

Note. Nomenclature of the conformers follows the IUPAC rules, i.e., reference groups are the amino group in the α-carbon and imidazole group in the β-carbon.

sieves and freed of amines by nitrogen bubbling until a negative 1-fluoro-2,4-dinitrobenzene test was observed. Pyroglutamic acid, L-histidine, and Boc-L-proline were from Fluka and its purity was checked by TLC. Two different batches of benzhydrylamine resin have been used in this work. In both cases the starting material was copoly(styrene-1%-divinylbenzene) beads (Bio-Beads SX1) purchased from Bio–Rad Laboratories and thoroughly washed before use with benzene, methanol, water, methanol, boiling 1 N sodium hydroxide, boiling 1 N hydrochloric acid, water, methanol, dimethylformamide at 80°C, and methanol. Derivatization via $LiAlH_4$ reduction of phenylketoximylpolystyrene following the procedure described by Pietta *et al.* (*9*) gave a benzhydrylamine resin (batch **1**) with an amine content of 0.25 mmol/g (determined by hydrolysis and amino acid analysis after incorporation of one amino acid on the resin). Alternatively, benzhydrylamine resin (batch **2**, 0.62 mmol NH_2/g) was obtained via Leuckart reaction following the synthetic protocol described by Albericio *et al.* (*19*). Hydrolysates for amino acid analyses from peptide or peptide–resin samples were prepared by treatment with 6 N hydrocholoric acid for 24 h or with 12 N hydrochloric acid/glacial acetic acid (1 : 1) for 48 h in vacuum-degassed sealed tubes at 110°C with norleucine added as internal standard. Amino acid analyses were obtained in a Beckman 119-C autoanalyzer equipped with an AA20 resin (*20*). TLC was performed on precoated silica gel plates (Merck, 0.2 mm). NMR spectra were recorded on Perkin–Elmer R-24 (^{1}H-60 MHz), Cameca (^{1}H-250 MHz), and Varian FT-80A (^{13}C-20.12 MHz) spectrometers. Ultraviolet spectra were recorded on a Perkin–Elmer 124 spectrophotometer.

N^{α}-Boc-5-nitro-L-histidine

5-Nitro-L-histidine was synthesized by nitration of L-histidine following the procedure described by Tautz *et al.* (*21*). We have previously demonstrated that this reaction takes place without racemization (*22*). 5-Nitro-L-histidine (6.5 g, 0.033 mol) was suspended in 30 ml dioxane/water (1 : 1) in a pH-state vessel. The pH was adjusted to 10 with 4 N sodium hydroxide, Boc-azide (5.1 g, 0.036 mol) was added, and the mixture was mechanically stirred keeping the pH at a constant value of 10 by automatic addition of 4 N sodium hydroxide. When the reaction was complete (usually after 7 h) the solution was extracted thrice with ether and the aqueous phase was acidified to pH 2 with 2 N hydrochloric acid at 5°C. The

solution was extracted with ether and the organic phase was dried and evaporated. After crystalyzation in absolute ethanol 5.1 g (52% yield) of colorless crystalline N^{α}-Boc-5-nitro-L-histidine was obtained.

Anal. Calcd for $C_{11}H_{16}N_4O_6$: C, 44.0%; H, 5.37%; N, 18.66%. Found: C, 43.92%; H, 5.58%; N, 18.38. $[\alpha]_D^{20} = -24.2°$ (*c*1, methanol). ^{1}H NMR (d_6-dimethylsulfoxide, 60 MHz) 7.75 (s, C_2-imidazole), 1.31 (s, CH_3). ir(KBr) 3420, 3140, 2995, 1730, 1680, 1520, 1430, 1575 cm^{-1}. TLC: single spot (R_f 0.72) in ethanol/water (1 : 1).

[*5-Nitro-L-histidine*]^2-*Thyroliberin*

Benzyhydrylamine resin (3 g from batch **1** or 1.5 g from batch **2**) was placed in the reaction vessel of a custom-made peptide synthesizer (*23*) and submitted to the following synthetic protocol for the incorporation of each amino acid residue: (1) 4 × 2 min dichloromethane; (2) 1 × 2 min 30% trifluoroacetic acid-dichloromethane; (3) 1 × 30 min 30% trifluoroacetic acid-dichloromethane; (4) 4 × 2 min dichloromethane; (5) 1 × 2 min 5% diisopropylethylamine-dichloromethane; (6) 1 × 30 min 5% diisopropylethylamine-dichloromethane; (7) 4 × 2 min dichloromethane; (8) shake with 2.5 equivalent of Boc-amino acid for 10 min; (9) without filtering, add 2.5 equivalent of dicyclohexylcarbodiimide and shake for 120 min; (10) 4 × 2 min dichloromethane; (11) 4 × 2 min dimethylformamide; (12) repeat from step (4) to step (7); (13) 1 × 2 min dimethylformamide; (14) repeat from step (8) to step (11). After each synthetic cycle the completeness of the amino acid incorporation was checked using the ninhydrin test (*24*). At the end of the synthesis the peptide resin was treated with anhydrous HF-anisole (10 : 1) at 0°C for 1 h to give crude [5-nitro-L-histidine]^2-thyroliberin (240 mg from batch **1** and 289 mg from batch **2**). Crude peptide (283 mg from batch **2**) was dissolved in a minimal amount of chloroform/methanol 2 : 1 and loaded on a 40 × 2 cm silica gel column (Merck LC-60, 40–60 μm) eluted with chloroform/methanol 1 : 2 (36 ml/h). From the uv absorbance of the fractions the major peak was defined. After evaporation and liophylization of the pooled fractions (eluted between 180 and 360 ml) 195 mg of pure [5-nitro-L-histidine]^2-thyroliberin was obtained. Crude peptide (230 mg) from HF cleavage of batch **1** resin was chromatographed on a Sephadex G-10 column (70 × 2 cm eluted with 0.01 N hydrochloric acid at 15 ml/h). The major peak (216 mg) was shown to be not homogeneous by TLC and amino acid analysis. Pure peptide (56% purification yield) could be obtained by silica gel LC-60 chromatography performed as described above.

Amino acid anal. Glu (0.96), *Pro* (1.04), *His(5-nitro)* (1.00). $[\alpha]_D^{20} = -1.39$ (*c*1, 1% acetic acid). ^{1}H NMR (d_6-dimethylsulfoxide, 250 MHz) 8.08 (d, NH His(5-nitro)), 7.82 (s, carboxamide *trans*), 7.60 (s, C_2-H imidazole), 7.55 (s, NH Glp), 7.10 (s, carboxamide *cis*), 5.02 (m, $C^{\alpha}H$ His(5-nitro)), 4.30 (m, CH Pro), 3.99 (m, CHGlp), 3.2–3.7 (m, $C^{\gamma}H_2$Glp–$C^{\beta}H_2$His(5-nitro)–$C^{\delta}H_2$Pro), 1.6–2.3 (m, $C^{\beta}H_2$Glp–$C^{\beta}H_2$Pro–$C^{\gamma}H_2$Pro). ^{13}C NMR (d_6-dimethylsulfoxide, 20.12 MHz) 178.4 (C^{δ}OGlp), 174.7 (CO Pro), 172.6 (CO His(5-nitro), 169.0 (CO Glp), 145.0 (C_5 imidazole), 134.8 (C_2 imidazole), 130.8 (C_4 imidazole), 60.2 (CH Pro), 56.1 (CH Glp), 49.6 (CH His(5-nitro)), 47.4 ($C^{\delta}H_2$Pro), 29.8–29.4–29.3 ($C^{\beta}H_2$Glp–

TABLE 4

UV ABSORPTIONS (353 nm) OF [5-NITRO-L-HISTIDINE]^2-THYROLIBERIN IN SODIUM HYDROXYDE–GLYCINE BUFFER SOLUTIONS

pH	A	$\log \frac{A_{A^-} - A}{A - A_{AH}}$	pK_a^{II}
5.7	0.065		
8.20	0.22	0.236	8.44
8.38	0.255	0.086	8.47
8.62	0.3	−0.099	8.52
8.71	0.322	−0.192	8.52
8.84	0.36	−0.366	8.47
9.04	0.383	−0.485	8.55
9.21	0.406	−0.624	8.59
9.38	0.428	−0.789	8.59
11.75	0.487		

$C^{\beta}H_2$His(5-nitro)–$C^{\gamma}H_2$Pro), 25.4–25.0 ($C^{\beta}H_2$Pro–$C^{\gamma}H_2$Glp). TLC: single spot in pyridine/ethanol/diethylamine/water 44 : 20 : 0.2 : 16 (R_f 0.50), chloroform/methanol/ammonium hydroxyde 60 : 45 : 10 (R_f 0.38), and butanol/ethyl acetate/acetic acid/water 1 : 1 : 1 : 1 (R_f 0.34). uv λ_{max} 310 nm (ε = 8240) in water, pH 7; λ_{max} 353 nm (ε = 12,400) in water, pH 12; λ_{max} 278 nm (ε = 7870) in 1.9 N sulfuric acid.

Determination of Acidity Functions

Aliquots (1 ml) of a stock solution of [5-nitro-L-histidine]^2-thyroliberin (3 mg) in water (25 ml) were taken and diluted with 2 ml each of different glycine–sodium

TABLE 5

UV ABSORPTIONS (310 nm) OF [5-NITRO-L-HISTIDINE]^2-THYROLIBERIN IN SULFURIC ACID SOLUTIONS

pH	A	$10^2 (A_A - A)/h_0$
5.0	0.725	
0.27	0.65	14.0
0.09	0.625	12.3
−0.05	0.594	11.6
−0.18	0.573	10.0
−0.18	0.578	9.7
−0.27	0.555	9.1
−0.36	0.543	7.9
−0.60	0.496	5.7
−0.77	0.455	4.6
−1.75	0.330	0.7

TABLE 6

THS-RELEASING ABILITY OF TRH AND [5-NITRO-L-HISTIDINE]^2-THYROLIBERIN

Treatment	TSH released[a]
15 ng thyroliberin	0.152
30 ng thyroliberin	0.266
60 ng thyroliberin	0.588
50 ng [5-nitro-L-histidine]^2-thyroliberin	−0.136
500 ng [5-nitro-L-histidine]^2-thyroliberin	−0.249
5 μg [5-nitro-L-histidine]^2-thyroliberin	−0.278
250 μg [5-nitro-L-histidine]^2-thyroliberin	1.04

[a] Expressed as the mean of the increase of the radioactivity level (cpm) of the second blood sample relative to the first blood sample. Each treatment was given to six mice.

hydroxide buffer solutions (*25*). Observed absorbances as well as the resulting pK_a^{II} values are listed in Table 4.

Aliquots (1 ml) of the above stock solution were taken and diluted with 2 ml of standard solutions of sulfuric acid at different concentrations. For each concentration of acid, the spectrophotometer was adjusted with a solution prepared by mixing 1 ml of water and 2 ml of the acid solution, and then the spectrum was recorded (Table 5).

Bioassay

In vivo assays for TSH-releasing ability were carried out following the method described by Vale *et al.* (*14*) with minor modifications. Male mice, body weight 15–20 g when received in the laboratory, were kept for 2 weeks in a constant-temperature room (27°C) on a low-iodine diet (Nutritional Biochemical Co.) and given one ip injection of ^{125}I 10 μCi, carrier free, and simultaneously 1.2 μg of thyroxine dissolved in 1% bovine seroalbumine in 0.01 N sodium hydroxyde. 48 h later one blood sample (ca. 50 μl) was withdrawn from the retro-orbital sinus and the material to be tested was immediately injected ip (0.5 ml saline). A second blood sample (50 μl) was taken 2 h later. Total blood radioactivity was measured in a well scintillation counter on both samples (Table 6).

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