# Iodination of Luteinizing Hormone–Releasing Hormone†

Shigeyuki Terada,‡ Satoe Hase Nakagawa,§ Dai Chang Yang,‖ Andrew Lipkowski,⊥ and George Flouret*

ABSTRACT: The main products of iodination of the luteinizing hormone–releasing hormone (LH–RH) have been defined. To this effect, [5-iodo-His$^2$]-LH–RH (I), [3-iodo-Tyr$^5$]-LH–RH (II), and [3,5-diiodo-Tyr$^5$]-LH–RH (III) were made unequivocally by total synthesis. Preparation of I was accomplished by coupling <Glu-(5-iodo-His) with the octapeptide LH–RH$^{3-10}$ in the presence of hydroxybenzotriazole (HBT) and dicyclohexylcarbodiimide (DCC). In turn, <Glu-(5-iodo-His) was prepared by iodination of <Glu-His with $I_2$–MeOH (1:1 molar ratio) under basic pH; the substitution of imidazole-5 of histidine by iodine was verified by $^1$H nuclear magnetic resonance spectroscopy. The LH–RH$^{3-10}$ was prepared by the solid-phase method, followed by ammonolysis, deprotection with HF, and purification by gel filtration on Sephadex G-25. Iodination of LH–RH$^{3-10}$ unexpectedly led to substantial destruction of tryptophan. However, iodination of heptapeptide LH–RH$^{4-10}$, synthesized by the solid-phase method, gave the expected [3-iodo-Tyr$^5$]-LH–RH$^{4-10}$ which was coupled to <Glu-His-Trp with DCC and HBT, yielding II; as a byproduct of this iodination, [3,5-diiodo-Tyr$^5$]-LH–RH$^{4-10}$ was obtained, which was also coupled to the above tripeptide, yielding III. All three iodo analogues of LH–RH were extensively purified by gradient elution on carboxymethylcellulose and by partition chromatography and gel filtration on Sephadex G-25. With the availability of genuine samples of iodo derivatives of LH–RH, we studied the direct iodination of the hormone itself employing $I_2$–MeOH (1:1 molar ratio) under basic conditions. The reaction products were analyzed by partition chromatography on Sephadex G-25. The elution profile showed five peaks corresponding to I, II, III, LH–RH, and possibly [5-iodo-His$^2$,3-iodo-Tyr$^5$]-LH–RH, as inferred from the UV spectrum. However, the main products are recovered hormone (34%) and II (23%). Bioassay of these analogues with rat hemipituitaries in vitro showed the following values as percentages of the hormonal values for the release of LH and FSH, respectively: [5-iodo-His$^2$]-LH–RH, 13.3 and 16.9; [3-iodo-Tyr$^5$]-LH–RH (obtained by direct iodination of LH–RH), 3.9 and 3.2; [3-iodo-Tyr$^5$]-LH–RH (obtained by fragment condensation), 3.9 and 2.3; [3,5-diiodo-Tyr$^5$]-LH–RH, 0 and 0. Thus, conclusions in biological studies with [$^{125}$I]LH–RH must take into account its lower biological potency, which may well reflect a decreased affinity for hormonal receptors in the target tissue.

The need for radioactively labeled luteinizing hormone–releasing hormone (LH–RH),[1] which is useful in studying binding to receptors and degradation in various tissues, prompted this study on the iodination of this hormone. Because iodo derivatives of a peptide hormone are analogues, they do not necessarily preserve full biological activity, and, indeed, they may be inactive or even antagonists of the parent hormone, as reported in the case of the neurohypophyseal hormones oxytocin and vasopressin (Flouret et al., 1977); hence, it is advantageous to prepare an iodinated analogue standard, in order to ascertain its physical and biological properties prior to preparing the corresponding radioiodo derivative of the parent hormone. Alternatively, iodo analogues are also useful as intermediates for the preparation of the corresponding [$^3$H]LH–RH analogues by catalytic tritiation.

Radioiodinated hormone preparations have been made in several laboratories. Thus, Miyachi et al. (1973a) iodinated LH–RH by a method employing lactoperoxidase and determined that iodination took place on tyrosine; the mono-[$^{125}$I]LH–RH obtained retained LH-releasing activity but had low FSH-releasing potency. Arimura et al. (1973) also prepared [$^{125}$I]LH–RH by the lactoperoxidase method as well as by the chloramine-T method (Greenwood & Hunter, 1963), purified the product by chromatography on carboxymethylcellulose (CMC), and bioassayed an immunoreactive peptide (corresponding to one of three [$^{125}$I]LH–RH fractions) as having 13% of the activity of LH–RH in the release of LH in an in vivo assay. More recently, Marshall & Odell (1975) employed the lactoperoxidase technique and purification on CMC, obtaining an [$^{125}$I]LH–RH which they reported had full LH-releasing activity in an in vitro assay employing cell cultures of rat anterior pituitaries. Such varied findings are not too surprising in view of the use of different chromatographic monitoring techniques, the use of different purification methodologies, the low stability and short half-life of a peptide labeled with $^{125}$I, as well as the different assay systems used. Furthermore, iodination of this hormone can occur on histidine-2 or on tyrosine-5, yielding mono- or polyiodo derivatives (Terada et al., 1979) and this possibility was not adequately explored in the reported iodinations with $^{125}$I. Additionally, if the results of Miyachi et al. (1973a) on [$^{125}$I]LH–RH biological activity were confirmed, the long-sought but hitherto elusive goal of making analogues that exhibit selective release of LH but not FSH would be fulfilled with the preparation of the nonradioactive iodo derivative of LH–RH; furthermore, it would become possible to prepare iodo derivatives of most of the active agonists and strongest antagonists of the latter hormone.

† From the Department of Physiology, Northwestern University, McGaw Medical Center, Chicago, Illinois 60611. *Received December 6, 1979.* This study was supported in part by Grant HD-08869 from the National Institute of Child Health and Human Development.

‡ Present address: Laboratory of Biochemistry, Faculty of Science, Kyushu University, Fukuoka, Japan.

§ Present address: Brain Research Institute, University of Chicago, Chicago, IL 60637.

‖ Present address: Research Department, G. D. Searle and Co., Chicago, IL 60680.

⊥ Present address: Chemistry Department, Warsaw University, Warsaw, Poland.

[1] The abbreviations employed follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (1972). Additionally, the following abbreviations were used: LH–RH, luteinizing hormone–releasing hormone; FSH, follicle-stimulating hormone; CMC, carboxymethylcellulose; DCC, dicyclohexylcarbodiimide; HBT, 1-hydroxybenzotriazole; $Et_3N$, triethylamine; Py, pyridine; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; Tos, tosyl; <Glu, pyroglutamic acid; TLC, thin-layer chromatography; TLE, thin-layer electrophoresis; $Me_2SO$, dimethyl sulfoxide.

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In the present study we describe the unequivocal synthesis, purification, and characterization of [5-iodo-His$^2$]-LH-RH (I), [3-iodo-Tyr$^5$]-LH-RH (II), and [3,5-diiodo-Tyr$^5$]-LH-RH (III). The biological activities of these analogues were evaluated in an in vitro assay measuring the LH and FSH release from rat hemipituitaries and are also reported.

## Experimental Section

Optical rotations were measured with a Rudolph polarimeter (precision ±0.1°). UV spectra were recorded in a Cary 16 recording spectrophotometer. Thin-layer electrophoresis (TLE) was performed on Eastman Chromagram thin-layer sheets with 0.1 M Py-AcOH (pH 5.6) at 500 V for 2–5 h, by means of a Desaga-Brinkman apparatus. Thin-layer chromatography (TLC) was performed on Quanta/Gram silica gel plates. When partition solvent systems were used, the lower phase was sprayed on the plate and development was accomplished with the upper phase. Solvent systems used for column chromatography or TLC were as follows: (A) 15% MeOH-$CHCl_3$; (B) 33% MeOH-$CHCl_3$; (C) *n*-BuOH-AcOH-$H_2O$ (4:1:5); (D) *n*-BuOH-EtOH-Py-1% AcOH (14:2:5:24); (E) *n*-BuOH-AcOH-$H_2O$-EtOAc (1:1:1:1); (F) *n*-BuOH-EtOH-AcOH-$H_2O$ (8:2:1:3). For the detection of peptides, $Cl_2$-tolidine or Ehrlich's or Pauly's reagents were used. For gel permeation (Porath & Flodin, 1959), we used Sephadex G-25 (Pharmacia). Partition chromatography was accomplished on Sephadex G-25 by the method of Yamashiro (1964). Elution profiles were determined by the Folin-Lowry method (Lowry et al., 1951) or by means of a UV monitor (Uvicord, LKB Instruments). For amino acid analyses, peptides were hydrolyzed for 24 h at 110 °C with 6 N HCl and then were analyzed in a JEOL automatic amino acid analzyer (Spackman et al., 1958). Proton magnetic resonance ($^1$H NMR) spectra were recorded at ambient temperature (24 °C) with a Varian CFT-20 spectrophotometer operating at 80 MHz and internally locked on the deuterium signal of dimethyl-$d_6$ sulfoxide, with tetramethylsilane as an internal reference standard. For deprotection of synthetic peptides with liquid HF (Sakakibara & Shimonishi, 1965) an all-Teflon apparatus (Protein Research Foundation, Osaka, Japan) was used. Statistics for the biological assays were computed with the aid of an interactive graphics system terminal connected to the PROPHET system (Raub, 1974).

*Solid-Phase Synthesis of Peptides.* Boc-Gly-resin (0.7 mmol of Gly/g) prepared as previously described (Flouret et al., 1973) was taken manually through the required number of coupling cycles by the solid-phase method (Merrifield, 1963) as modified in a previous synthesis of LH-RH (Flouret et al., 1973). In each cycle, the Boc group was removed with 25% trifluoroacetic acid in $CH_2Cl_2$ and, after neutralization of the resin with triethylamine ($Et_3N$), coupling was performed with a 4-mol excess of the desired amino acid and dicyclohexylcarbodiimide (DCC). For the protection of side-chain functionalities, Boc-Ser(Bzl), Boc-Tyr(Bzl), and Boc-Arg(Tos) were used. Completion of coupling was monitored by means of the ninhydrin test (Kaiser et al., 1970). The final peptide was removed by ammonolysis for 2 days in MeOH-DMF (1:1) saturated with $NH_3$, and it was purified by dissolving in MeOH and precipitating from solution by addition to EtOAc. The homogeneity of the final product (50–60% overall yields from Boc-Gly-resin) was monitored by TLC with solvent systems A and B.

*Ser-Tyr-Gly-Leu-Arg-Pro-Gly-$NH_2$ (LH-RH$^{4-10}$).* Boc-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly-$NH_2$ (750 mg, 0.63 mmol), prepared by the solid-phase method, was deprotected by treatment with anhydrous HF (30 mL) and anisole (3 mL) for 60 min at 0 °C. The HF was removed in vacuo, and the residue was distributed between EtOAc (30 mL) and 0.1 M AcOH (30 mL). The aqueous extract was filtered through an AGl-X2 ($AcO^-$) column (1.5 × 19 cm), and the filtrate and washings were lyophilized, yielding 590 mg of product. This crude peptide was purified by partition chromatography on a Sephadex G-25 column (2.4 × 116 cm) with solvent system A. The major peak ($R_f$ 0.16) yielded 283 mg of product, which was subjected to gel permeation on a Sephadex G-25 column (2.4 × 117 cm) with 1% AcOH (elution volume 422–473 mL), yielding 265 mg (43%) of heptapeptide: $[\alpha]^{24}_D$ −46° (*c* 1, 1 M AcOH). The product was homogeneous on TLE (4 h) and on TLC: $R_f^C$ 0.24; $R_f^D$ 0.51; $R_f^E$ 0.43; $R_f^F$ 0.26. Amino acid analysis of heptapeptide gave the following molar ratios with Gly taken as 2.00: $Ser_{0.94}Tyr_{1.02}Gly_{2.00}Leu_{0.94}Arg_{0.97}Pro_{0.98}$. Anal. Calcd for $C_{33}H_{53}N_{11}O_9 \cdot 3C_2H_4O_2$: C, 49.5; H, 7.14; N, 16.3. Found: C, 49.5; H, 6.90; N, 16.6.

*Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-$NH_2$ (LH-RH$^{3-10}$).* Boc-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly-$NH_2$ (230 mg, 0.17 mmol), prepared by the solid-phase method, was deprotected with HF as described for the heptapeptide. The product was purified by gel filtration on a Sephadex G-25 column (2.4 × 114 cm) with 1% AcOH. The main peptide peak was lyophilized, yielding 101 mg of product which was homogeneous on TLE (3 h) and on TLC: $R_f^C$ 0.29; $R_f^D$ 0.56; $R_f^E$ 0.51; $R_f^F$ 0.34; $[\alpha]^{24}_D$ −33° (*c* 1, 1 M AcOH). An amino acid analysis gave the following molar ratios with Gly taken as 2.00: $Arg_{0.92}Ser_{0.98}Pro_{0.88}Gly_{2.00}Leu_{0.93}Tyr_{0.99}Trp_{0.54}$ (partially destroyed under the conditions of hydrolysis employing 6 N HCl).

For use in peptide sysnthesis with DCC, this octapeptide (probably a diacetate) was converted to the hydrochloride by adding 2 equiv of 1 N HCl and lyophilizing the solution.

*<Glu-His-OMe.* The following is a simpler method that produces higher yields than published ones. His-OMe·2HCl (11.6 g, 48 mmol) was converted to the free amino acid ester (Bailey, 1950) by treatment with 2% $NH_3$ in $CHCl_3$ (108 mL) for 15 min, followed by filtration to remove $NH_4Cl$ (quantitative yield), drying ($Na_2SO_4$), and evaporation to dryness. A solution of this free ester in $CH_2Cl_2$ (40 mL) was treated with <Glu-pentachlorophenyl ester (15.1 g, 48 mmol). After 2 h, the solvent was removed in vacuo, and the residue was suspended in $Et_2O$ and filtered. The product was crystallized from MeOH-$Et_2O$ yielding 10.82 g (97%): mp 212–214 °C; $[\alpha]^{26}_D$ −53.4° (*c* 1, AcOH) [lit. (Flouret et al., 1972): mp 211–213 °C; $[\alpha]^{25}_D$ −53.5° (*c* 1, AcOH)].

*<Glu-His.* A solution of the above dipeptide ester (15.6 g, 56 mmol) in 2 N NaOH (33 mL) was allowed to stand at room temperature. After 1 h, the reaction mixture was neutralized by the addition of 2 N HCl (33 mL) and the solution was evaporated to dryness in vacuo. Residual water was removed by repeated addition and evaporation of EtOH. The dry residue was digested with AcOH (150 mL), the suspension was filtered to remove undissolved salts, and the filtrate was evaporate to dryness in vacuo. The residue was crystallized by dissolving in a mininum amount of $H_2O$ and adding EtOH, yielding 13.5 g (86%): mp 167–170 °C; $[\alpha]^{26}_D$ +10.3° (*c* 2, AcOH) [lit. (Flouret et al., 1972): mp 169–171 °C; $[\alpha]^{25}_D$ +10.4° (*c* 2, AcOH)].

*<Glu-(5-iodo-His).* To a solution of <Glu-His monohydrate (142 mg, 0.5 mmol) in 0.2 N NaOH (5 mL) was added 0.2 M $I_2$ in MeOH (2.6 mL, 0.52 mmol) at 0 °C, maintaining the pH at 7.5–8.0 by the addition of 0.2 N NaOH (0.7 mL). After 30 min the reaction MeOH was partially

removed in vacuo by evaporation of the solution to about half the initial volume, and the solution was applied to an AGl-X2 ($AcO^-$) column (0.9 × 12 cm) previously equilibrated with 0.05 M Py–AcOH (pH 6.0). Elution was accomplished with the same buffer at pH 6.0 (500 mL) and then at pH 4.8 (200 mL). The latter buffer eluted the product, which, after lyophilization, gave 78 mg of monoiodopeptide homogeneous on TLC: $R_f^C$ 0.25; $[\alpha]^{24}_D$ −25.2° (*c* 1, AcOH); $^1H$ NMR ($Me_2SO$-$d_6$) shows im-$CH_2$-2 at 7.61 ppm and the absence of im-$CH_2$-5 at 6.82 ppm. Anal. Calcd for $C_{11}H_{13}N_4O_4I \cdot 0.5C_2H_4O_2 \cdot 0.5H_2O$: C, 33.4; H, 3.73; N, 13.0. Found: C, 33.3; H, 3.59; N, 13.2.

*[5-Iodo-His²]-LH–RH (I).* To <Glu-(5-iodo-His) (20.1 mg, 50 μmol) and 1-hydroxybenzotriazole (HBT) (8.4 mg, 55 μmol) in DMF (2 mL) was added the octapeptide hydrochloride LH–$RH^{3-10}$, and to the resulting solution was added $Et_3N$ (7 μL, 50 μmol) and DCC (10.3 mg, 50 μmol). The reaction mixture was stirred at 3 °C for 4 days, the dicyclohexylurea was then removed by filtration, and the filtrate was evaporated to dryness. The residue was dissolved in MeOH (1 mL) and 0.002 M $NH_4OAc$ (pH 4.6, 2 mL), and the solution was chromatographed on a carboxymethylcellulose (CMC) column (1.5 × 89 cm, 0.68 mequiv/g) equilibrated with 0.002 M $NH_4OAc$ (pH 4.6). Elution was accomplished with the same buffer (200 mL) and then by a linear gradient from 0.002 M $NH_4OAc$ (pH 4.6) to 0.125 M $NH_4OAc$ (pH 7.0), 450 mL. The desired product was eluted as a single peak which, after lyophilization, gave 62 mg of product, which was purified further by partition chromatography on Sephadex G-25 with solvent system C, yielding 48 mg of material. The latter was purified once more by gel permeation on Sephadex G-25, yielding, after lyophilization, 43 mg of product homogeneous on TLE (4 h) and on TLC: $R_f^C$ 0.31; $R_f^D$ 0.67; $R_f^E$ 0.49; $R_f^F$ 0.38; $[\alpha]^{24}_D$ −36° (*c* 1, 1 M AcOH). An amino acid analysis of this analogue showed the following molar ratios with Gly taken as 2.00: $Arg_{0.98}Ser_{0.88}Glu_{1.05}Gly_{2.00}Pro_{0.96}Leu_{0.99}Tyr_{0.96}Trp_{0.54}$ (Trp is partially destroyed during hydrolysis with 6 N HCl). Anal. Calcd for $C_{55}H_{74}N_{17}O_{13}I \cdot 2C_2H_4O_2 \cdot 2H_2O$: C, 48.4; H, 5.92; N, 16.3. Found: C, 48.1; H, 5.63; N, 16.1.

*Iodination of LH–$RH^{4-10}$.* To a stirred solution of LH–$RH^{4-10}$ (222 mg, 0.235 mmol) in a mixture of $H_2O$ (1.25 mL), MeOH (1.25 mL), and 2 N $NH_4OH$ (0.625 mL, 1.25 mmol) was added a freshly prepared 0.2 M iodine solution in MeOH (1.375 mL, 0.275 mmol) at room temperature, and the pH of the solution was adjusted to 8.5 with 2 N $NH_4OH$. After 5 min, β-mercaptoethanol (0.02 mL) was added to stop the reaction. The mixture was immediately applied to an AGl-X2 ($AcO^-$) column (0.9 × 12 cm), and the column was washed with $H_2O$ and the filtrate and washings were lyophilized. The crude product thus obtained was purified by partition chromatography on a Sephadex G-25 column (2.4 × 119 cm) with solvent system D, yielding the following fractions: A, $R_f$ 0.54, 80 mg; B, $R_f$ 0.43, 90 mg; C, $R_f$ 0.26, 54 mg. Each of these fractions was separately subjected to gel filtration on a Sephadex G-25 column (2.4 × 118 cm) with 1% AcOH as the eluant.

*Fraction A* (elution volume 504–554 mL) gave 72 mg (25%) corresponding to [3,5-diiodo-Tyr⁵]-LH–$RH^{4-10}$, homogeneous on TLE and on TLC: $R_f^C$ 0.28; $R_f^D$ 0.53; $R_f^E$ 0.50; $R_f^F$ 0.35; $[\alpha]^{24}_D$ −40° (*c* 1, 1 M AcOH); UV spectrum $\lambda_{max}^{H_2O}$ 286 nm (ε 2250), $\lambda_{max}^{OH^-}$ 311 nm (ε 5300). Anal. Calcd for $C_{33}H_{51}N_{11}O_9I_2 \cdot 3C_2H_4O_2 \cdot H_2O$: C, 39.1; H, 5.47; N, 12.9. Found: C, 38.7; H, 5.52; N, 12.7. Amino acid analysis showed the following molar ratios with Gly taken as 2.00: $Arg_{0.97}Ser_{0.85}Pro_{0.98}Gly_{2.00}Leu_{0.94}Tyr_{1.02}$.

*Fraction B* (elution volume 460–503 mL) gave 83 mg (33%) corresponding to [3-iodo-Tyr⁵]-LH–$RH^{4-10}$, homogeneous on TLE and on TLC: $R_f^C$ 0.26; $R_f^D$ 0.52; $R_f^E$ 0.49; $R_f^F$ 0.30; $[\alpha]^{24}_D$ −40.3° (*c* 1, 1 M AcOH); UV spectrum $\lambda_{max}^{H_2O}$ 282 nm (ε 2300), $\lambda_{max}^{OH^-}$ 305 nm (ε 4100). Amino acid analysis gave the following amino acid molar ratios with Gly taken as 2.00: $Arg_{0.98}Ser_{0.85}Pro_{0.99}Gly_{2.00}Leu_{0.96}Tyr_{0.98}$. Anal. Calcd for $C_{33}H_{52}N_{11}O_9I \cdot 3C_2H_4O_2 \cdot H_2O$: C, 43.7; H, 6.21; N, 14.4. Found: C, 43.3; H, 6.18; N, 14.6.

*Fraction C* (elution volume 403–459 mL) gave 51 mg (23%) of starting material LH–$RH^{4-10}$ as determined by TLC.

For the purpose of coupling with DCC, both iodopeptide triacetates were converted to the hydrochlorides by adding a slight excess of 3 molar equivalents of 1 N HCl to their respective aqueous solutions and then lyophilizing.

*[3-Iodo-Tyr⁵]-LH–RH (II).* [3-Iodo-Tyr⁵]-LH–$RH^{4-10}$ (40 mg, 38 μmol) and <Glu-His-Trp (22.6 mg, 50 μmol), HBT (7.7 mg, 50 μmol), and $Et_3N$ (5.6 μL, 40 μmol) in DMF (0.6 mL) were treated with DCC (9.3 mg, 45 μmol) at 20 °C. The reaction mixture was stirred for 4 days at 4 °C and then diluted with $H_2O$ (10 mL) and applied to a CMC ($H^+$) column (1.5 × 10 cm, 20 mL). After washing with $H_2O$ (50 mL), fractions containing product were eluted with 1 M AcOH (15–45 mL) as a major peak (UV) and were pooled and evaporated in vacuo and lyophilized yielding 41 mg of product. The latter was purified one more time by partition chromatography on a Sephadex G-25 column (2.0 × 42 cm) with solvent system C. Fractions from the main components, $R_f$ 0.21, gave 34 mg of product after lyophilization. Further purification was accomplished by gel permeation on Sephadex G-25 with 1% AcOH yielding the final product, 22 mg (38%), homogeneous on TLE and on TLC: $R_f^C$ 0.22; $R_f^D$ 0.53; $R_f^E$ 0.49; $R_f^F$ 0.27; $[\alpha]^{24}_D$ −39° (*c* 1, 1 M AcOH). Amino acid analysis gave the following molar ratios with Gly taken as 2.00: $His_{0.95}Arg_{0.95}Ser_{0.89}Glu_{1.0}Pro_{1.0}Gly_{2.00}Leu_{0.92}Tyr_{1.00}Trp_{0.08}$ (Trp is largely destroyed under acid conditions). Anal. Calcd for $C_{55}H_{74}N_{17}O_{13}I \cdot 3C_2H_4O_2 \cdot H_2O$: C, 48.6; H, 5.89; N, 15.8; I, 8.42. Found: C, 48.5; H, 5.74; N, 15.7; I, 8.63.

*[3,5-Diiodo-Tyr⁵]-LH–RH (III).* A solution of [3,5-diiodo-Tyr⁵]-LH–$RH^{4-10}$ (45 mg, 37 μmol), HBT (27 mg, 50 μmol), and $Et_3N$ (5.6 μL, 40 μmol) in DMF (1.1 mL) was treated with DCC (9.3 mg, 45 μmol) at −20 °C. The mixture was stirred at 4 °C for 5 days and then diluted with $H_2O$ (10 mL), the solution was applied to a CMC ($H^+$) column (1.5 × 10 cm, 20 mL), and the column was washed with $H_2O$ (50 mL); the product was eluted with 1 M AcOH, and the appropriate fractions were pooled and lyophilized. The product thus obtained was subjected to partition chromatography on a Sephadex G-25 column (2.0 × 92 cm) with solvent system C. The major peak, $R_f$ 0.34, gave, after lyophilization, 28.4 mg of product which was purified further by gel permeation on a Sephadex G-25 column (2.4 × 119 cm) with 1% AcOH (filtration volume 519–639 mL) yielding 35 mg (58%) of III homogeneous on TLE and on TLC: $R_f^C$ 0.23; $R_f^D$ 0.59; $R_f^E$ 0.50; $R_f^F$ 0.29; $[\alpha]^{24}_D$ −32° (*c* 1, 1 M AcOH). Amino acid analysis gave the following molar ratios with Glu taken as 1.00: $Glu_{1.00}His_{0.93}Ser_{0.83}Tyr_{1.04}Gly_{1.99}Leu_{0.93}Arg_{0.96}Pro_{0.98}Trp_{0.23}$ (Trp is extensively destroyed under the conditions of acid hydrolysis used). Anal. Calcd for $C_{55}H_{73}N_{17}O_{13}I_2 \cdot 3C_2H_4O_2 \cdot H_2O$: C, 44.9; H, 5.37; N, 14.6; I, 15.6. Found: C, 44.9; H, 5.15; N, 14.4; I, 15.2.

*Direct Iodination of LH–RH.* Synthetic LH–RH (27 mg, 20 μmol), prepared by the method of Flouret et al. (1973), was dissolved in a mixture of $H_2O$ (0.2 mL), MeOH (0.2 mL),

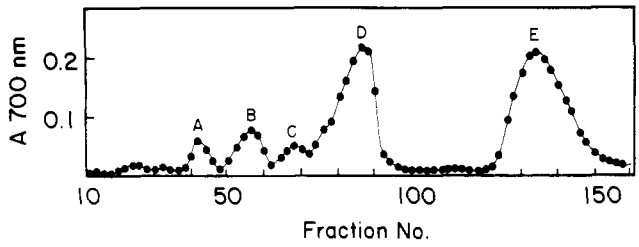


FIGURE 1: Separation of the products of iodination of LH-RH. The products of LH-RH (27 mg) iodination were separated by partition chromatography on a Sephadex G-25 column (1.5 × 72 cm) with the solvent system *n*-BuOH-AcOH-$H_2O$ (4:1:5), fraction size 2.4 mL/tube, flow rate 0.5 mL/min. Aliquots of 0.1 mL were used for Folin-Lowry determination. The products eluted were designated A to E.

and 2 N $NH_4OH$ (0.02 mL). To the reaction mixture was added a solution of 0.2 M $I_2$ in MeOH (0.11 mL, 22 $\mu$mol) with stirring at room temperature; the pH of the solution was adjusted to 8.5 by the addition of 2 N $NH_4OH$ (10 $\mu$L). After 5 min, the reaction mixture was treated with AcOH (50 $\mu$L) and $H_2O$ (3 mL), and the resulting solution was immediately applied to an AGl-X2 ($AcO^-$) column (0.9 × 6 cm), and the column was eluted with $H_2O$ (10 mL). The eluate was lyophilized yielding 30 mg of product which was subjected to partition chromatography on a Sephadex G-25 column (1.5 × 72 cm) with solvent system C. The elution profile, detected by the Folin-Lowry method (Figure 1), demonstrated the presence of five products designated A-E. Fractions corresponding to each of these products were pooled and lyophilized yielding the following: A, $R_f$ 0.45, 1.0 mg; B, $R_f$ 0.34, 1.82 mg; C, $R_f$ 0.28, 0.9 mg; D, $R_f$ 0.21, 7.0 mg; E, $R_f$ 0.14, 9.1 mg. TLE (5 h) of products A-E and of standards of I, II, III, and LH-RH showed the following identities (mobilities): B = I (4 cm); C = III (8 cm); D = II (11 cm); E = LH-RH (14.5 cm); the identities were corroborated in part on TLC systems. Additionally, the UV spectra of B, C, D, and E were identical with those of standards of I, III, II, and LH-RH, respectively. Product A had a UV spectrum corresponding to a 3-iodotyrosyl peptide, and, because of its high lipophilicity, it should have at least one additional iodine atom on histidine; hence, A has been tentatively characterized as [5-iodo-$His^2$,3-iodo-$Tyr^5$]-LH-RH. For bioassay, analogue II was purified once more by gel permeation on Sephadex G-25, in order to ensure complete removal of any traces of parent hormone.

*Biological Assay Procedure.* In vitro bioassay procedures for agonistic activity were described previously (White et al., 1973). Usually the analogues were assayed at two dose levels in comparison to hormone standard (kindly supplied by Dr. Jean Rivier, Salk Institute, La Jolla, CA) at two dose levels, for the release of LH and FSH from male rat hemipituitaries. Media were sampled 6 h later and assayed for LH and FSH by radioimmunoassay.

## Results and Discussion

The aim of our study was to obtain unequivocally monoiodohistidyl and monoiodotyrosyl analogues of LH-RH; thus, we devised synthetic schemes by which appropriately iodinated fragments of LH-RH would be condensed with suitable peptide fragments, yielding the desired iodo analogues devoid of possible contamination with the parent hormone.

Following a synthetic scheme for the synthesis of [5-iodo-$His^2$]-LH-RH (I) free of iodotyrosyl byproducts, we sought to make <Glu-(5-iodo-His) and LH-$RH^{3-10}$. To this effect, we prepared <Glu-His (Flouret et al., 1972) by an improved, high-yield method; the useful innovation introduced was the process of desalting <Glu-His, which was obtained by saponification of its methyl ester with NaOH followed by neutralization with HCl, by dissolving the peptide selectively in glacial acetic acid. We have recently reported on a similar desalting procedure in connection with the synthesis of neurohypophyseal hormones (Flouret et al., 1979). Iodination of <Glu-His yielded <Glu-(5-iodo-His), as inferred from $^1H$ NMR data; coupling of this iodopeptide to LH-$RH^{3-10}$, obtained by the solid-phase method of peptide synthesis, yielded the desired iodohistidyl analogue I.

In another attempt to devise an unequivocal synthesis of [3-iodo-$Tyr^5$]-LH-RH free of iodohistidyl byproducts, we iodinated LH-$RH^{3-10}$ in the hope of obtaining [3-iodo-$Tyr^5$]-LH-$RH^{3-10}$ which was to be coupled with <Glu-His; the iodination failed, unexpectedly, as extensive degradation of the tryptophyl residue took place yielding a complex mixture. Such susceptibility of a tryptophyl residue to oxidation during iodination has been reported in the case of lysosyme, in which iodination degrades tryptophan-105, but does not affect five other tryptophyl residues (Hartdegen & Rupley, 1967). In a more successful approach, we iodinated LH-$RH^{4-10}$, also prepared by the solid-phase method, obtaining the expected [3-iodotyrosyl]-LH-$RH^{4-10}$ as well as the byproduct [3,5-diiodo-$Tyr^5$]-LH-$RH^{4-10}$. Both of these iodopeptides were coupled with <Glu-His-Trp, yielding the desired analogues [3-iodo-$Tyr^5$]-LH-RH (II) and [3,5-diiodo-$Tyr^5$]-LH-RH (III).

All three analogues, I, II, and III, were purified by a sequence of chromatographic steps on columns, including gradient elution on CMC, partition chromatography on Sephadex G-25, and gel permeation chromatography on Sephadex G-25; the highly purified analogues obtained were characterized by the criteria of homogeneity on TLE and on TLC with several solvent systems, optical rotations, elemental and amino acid analyses, and UV spectra.

The direct iodination of the hormone with 1 mol equiv of $I_2$ was performed, and the products obtained were analyzed by partition chromatography on Sephadex G-25 (Figure 1). The identity of products was determined from UV spectra and TLE mobilities, in comparison to those of the genuine standards described above. The main products obtained were the monoiodotyrosyl analogue II and recovered hormone, suggesting that direct iodination of LH-RH is the most convenient and direct route for the preparation of II. From the low level of monoiodohistidyl analogue I, it follows that iodination under these conditions occurs more rapidly on tyrosine-5 than on histidine-2. Nevertheless, the presence of diiodo derivatives is detectable, and there is no doubt that higher proportions of iodine would likely lead to higher proportions of di, tri-, and even tetraiodo derivatives. The methods used to prepare [$^{125}$I]LH-RH appear to have used an excess of $^{125}$I and led to several major peaks of iodo-LH-RH. Thus, the oligomers reportedly obtained on polyacrylamide gel electrophoresis (Miyachi et al., 1973a) could have been [monoiodo-$His^2$,monoiodo-$Tyr^5$]-LH-RH and [diiodo-$His^2$,monoiodo-$Tyr^5$]-LH-RH, as no evidence was sought in those studies for histidine iodination. Indeed, our analogues I and III are slower than LH-RH or II on TLE, suggesting lower charge or higher molecular weight than one might predict.

Bioassay of the iodo analogues I, II, and III was performed in vitro, with male rat hemipituitaries, measuring the release of LH and FSH by radioimmunoassay. The results obtained (Table I) show that analogue I retains substantial biological activity in the release of LH (13.3%) and FSH (16.9%) and

Table I: Potency of Iodo Derivatives of LH-RH for the Release of LH and FSH from Male Rat Hemipituitaries in Vitro

| compound | % LH release | % FSH release |
|---|---|---|
| LH-RH | 100 | 100 |
| [5-iodo-His$^2$]-LH-RH (I) | 13.3 | 16.9 |
| [3-iodo-Tyr$^5$]-LH-RH (II, total synthesis) | 3.9 | 2.3 |
| [3-iodo-Tyr$^5$]-LH-RH (II, direct iodination of LH-RH) | 3.9 | 3.2 |
| [3,5-diiodo-Tyr$^5$]-LH-RH (III) | 0 | 0 |

that analogue II, prepared either by direct iodination of LH-RH or by the fragment condensation method, retains low but equivalent activity in the release of either LH (3.9%) or FSH (2.3–3.2%). Thus, we find very low potency for II, whereas Marshall & Odell (1975), in sharp discrepancy, had found their sample of [$^{125}$I]LH-RH to have full biological activity for the release of LH. One plausible explanation is that their single purification step for [$^{125}$I]LH-RH on a CMC column is insufficient to attain homogeneity and, hence, constant biological activity. Indeed, their elution profile shows that both compounds emerge from the column with inadequately close retention volume; consequently, one purification step should have been insufficient to quantitatively remove LH-RH which would normally "tail" (observable in their figure) under the [$^{125}$I]LH-RH peak. In support of this view, we note that Arimura et al. (1973) described a second purification of [$^{125}$I]LH-RH on their CMC column, which led to a product with a LH-releasing potency of 13%. We can only wonder if a third purification on CMC would have brought the potency of the latter sample still further down to our presently reported values. Although we cannot explain the results of Miyachi et al. (1973a), who found high LH-releasing but no FSH-releasing activity for [$^{125}$I]LH-RH, we suspect that their preparation may have lacked constant biological activity, as they performed only one purification step.

Our present findings suggest that iodo-LH-RH analogues have no significant selectivity for the release of pituitary LH or FSH. Additionally, the relatively low level of biological activity of II suggests that [$^{125}$I]LH-RH, in spite of higher specific radioactivity attainable, offers no great advantage over the fully potent [$^3$H]LH-RH for use in binding studies; furthermore, its use in metabolic studies (e.g., Miyachi et al., 1973b) is injudicious, since iodo derivatives are analogues of the hormone, and the iodine atom may affect enzymatic attack in imponderable fashion.

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References

Arimura, A., Sato, H., Kumasaka, T., Worobec, R. B., Debeljuk, L., Dunn, J., & Schally, A. V. (1973) *Endocrinology 93*, 1092.

Bailey, J. L. (1950) *J. Chem. Soc.*, 3461.

Flouret, G., Alter, A., & Gendrich, R. (1972) *J. Labelled Compd. 8*, 53.

Flouret, G., Arnold, W. H., Cole, J. W., Morgan, R. L., White, W. F., Hedlund, M. T., & Rippel, R. H. (1973) *J. Med. Chem. 16*, 359.

Flouret, G., Terada, S., Yang, F., Nakagawa, S. H., Nakahara, T., & Hechter, O. (1977) *Biochemistry 16*, 2119.

Flouret, G., Terada, S., Kato, T., Gualtieri, R., & Lipkowski, A. (1979) *Int. J. Pept. Protein Res. 13*, 137.

Greenwood, F. C., & Hunter, W. M. (1963) *Biochem. J. 89*, 114.

Hartdegen, F. J., & Rupley, J. A. (1967) *J. Am. Chem. Soc. 89*, 1743.

IUPAC-IUB Commission on Biochemical Nomenclature (1972) *Biochem. J. 126*, 773.

Kaiser, E., Colescot, R. L., Bossinger, C. D., & Cook, P. I. (1970) *Anal. Biochem. 34*, 595.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Marshall, J. C., & Odell, W. D. (1975) *Proc. Soc. Exp. Biol. Med. 149*, 351.

Merrifield, R. B. (1963) *J. Am. Chem. Soc. 85*, 2149.

Miyachi, Y., Chrambach, A., Mecklenburg, R. S., & Lipsett, M. B. (1973a) *Endocrinology 92*, 1725.

Miyachi, Y., Mecklenburg, R. S., Hansen, J. W., & Lipsett, M. B. (1973b) *J. Clin. Endocrinol. Metab. 37*, 63.

Porath, J., & Flodin, P. (1959) *Nature (London) 183*, 310.

Raub, W. F. (1974) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33*, 2390.

Sakakibara, S., & Shimonishi, Y. (1965) *Bull. Chem. Soc. Jpn. 38*, 141.

Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190.

Terada, S., Nakagawa, S. H., Yang, D. C., Lipkowski, A., & Flouret, G. (1979) *Peptides 1978* (Siemion, I. Z., & Kupryszewski, G., Eds.) p 515, Wroclaw University Press, Wroclaw, Poland.

White, W. F., Hedlund, M. T., Rippel, R. H., Arnold, W., & Flouret, G. (1973) *Endocrinology 93*, 96.

Yamashiro, D. (1964) *Nature (London) 201*, 76.