^{3}H-LABELLING OF A SYNTHETIC DECAPEPTIDE HAVING LH AND FSH RELEASING ACTIVITY (LH-RH/FSH-RH)

P. MARCHE, J.L. MORGAT and P. FROMAGEOT
Service de Biochimie, CEN Saclay, B.P. no. 2, 91-Gif-sur-Yvette, France

and

B. KERDELHUE and M. JUTISZ
Laboratoire des Hormones Polypeptidiques, CNRS, 91-Gif-sur-Yvette, France

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1. Introduction

A decapeptide capable to stimulate the release of LH and FSH from pituitary has been recently isolated from hypothalami of porcine origin and its amino acid sequence has been established as (Pyro)Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly-NH_2 [1, 2]. An identical decapeptide has been found in sheep hypothalami [3, 4].

Several laboratories described syntheses of LH-RH/FSH-RH either by the solid phase technique [5, 8] or by the classical approach [9, 10].

Previous studies in our laboratory have permitted the tritiation of several peptidic hormones with subsequent retention of their biological activities. The method used, described by Morgat et al. [11, 12], consists in a catalytic hydrogenolysis of iodinated tyrosyl residue(s). Recently, by a similar procedure, Pradelles et al. [13] and Menez et al. [14] have obtained [^{3}H]TRH and *Naja nigricollis* [^{3}H]α-neurotoxin, respectively, in which the histidyl side chain carried the label.

The present report describes the tritium labelling of LH-RH/FSH-RH. The product had a specific radioactivity of 75 Ci/mmole, it was biologically active on the release of both LH and FSH, and not distinct in this respect from the unlabelled decapeptide.

2. Experimental

2.1. *Materials*

LH-RH/FSH-RH synthesized via the classical approach of peptide synthesis [10] was generously supplied by Drs. R.O. Studer and D. Gillessen (Hoffman-La Roche, Basel). The final material was purified by partition chromatography on Sephadex G-25 using 1-butanol–ethanol–pyridine–0.2 N acetic acid (4:1:1:7) as solvent system [10]. ICl used for the halogenation was from Rhône Poulenc (Paris) and ^{125}ICl used as tracer, from New-England Nuclear Corporation. The catalyst Pd/Al_2O_3 (10%) was supplied by Engelhardt (Roma), the ion exchange resins AG 11 A8 and Bio-gel P2 by BioRad laboratories. Tritium gas was made by the Commissariat à l'Energie Atomique (France). Tritium determinations were made with a Nuclear-Chicago liquid scintillation counter and ^{125}I was counted with SAIP-Gammatic solid scintillation device. [^{3}H]LH-RH/FSH-RH was stored in liquid N_2.

2.2. *Iodination of LH-RH/FSH-RH*

Iodination was accomplished at 4°; 4.3 μmoles ICl (100 μl of anhydrous methanolic solution in the presence of ^{125}ICl as tracer) were slowly added to 1.1 μmoles (1.3 mg) LH-RH/FSH-RH dissolved in 1 ml

0.1 M phosphate buffer pH 7.4. The reaction was stopped by addition of 50 μl 0.1 M Na thiosulfate solution. By paper electrophoresis of a reaction aliquot (pyridine–acetic acid 0.075 M, pH 6.5, 12 V/cm), 2.5 iodine atoms were found incorporated per mole LH-RH/FSH-RH. The iodinated mixture was purified by passage through an AG 11 A8 column which removed the salt. Its UV spectrum showed i) no alteration of the tryptophan residue and ii) the presence of a di-iodotyrosyl residue (maximum at 312 nm at pH 11). With respect to the amount of iodine atoms bound to the peptide, it appeared that the histidyl residue was also partially iodinated.

2.3. *Tritiation*

After lyophilization, iodo-LH-RH/FSH-RH redissolved in 1 ml glacial AcOH, was transferred to the tritiation flask and then frozen. 10 mg of catalyst Pd/Al_2O_3 were added and the tritiation flask was connected to the vacuum. At a pressure of 10^{-4} Torr, 10 Ci of pure tritium gas were introduced (500 Torr). The mixture was thawed and stirred for 0.5 hr. Millipore filtration was employed for removal of the catalyst followed by successive flash evaporation for exchange of labile hydrogens.

2.4. *Purification*

The [^{3}H]LH-RH/FSH-RH mixture was filtered on a Bio-gel P2 column (50–100 mesh, eluted by 10^{-2} M AcOH). Elution profiles were monitored by absorbance at 280 nm and by ^{3}H and ^{125}I counting. These analyses and amino acid measurements with the Technicon auto-analyzer indicated recovery of 180 μg [^{3}H]LH-RH/FSH-RH. This product was iodine-free and its specific radioactivity was 75 Ci/mmole.

The chemical purity was verified by thin-layer chromatography (Cellulose MN 300, nBuOH/AcOH/H_2O 75:10:25, v/v/v) and scanning; the UV spectrum of the [^{3}H]LH-RH/FSH-RH was found to be exactly the same as that of the original material.

2.5. *Biological assays*

Biological assays for measuring the hypothalamic releasing hormone activity were performed using indirect methods involving a 2-step system: i) action on the *in vitro* release of gonadotropins from the pituitary, ii) the radioimmunoassay of gonadotropins.

Hemipituitaries (4 per flask) from ovariectomized, steroid primed rats [15] were rinsed twice with 2 ml of Krebs-Ringer bicarbonate–glucose buffer (KRB) and "pre-incubated" for a period of 30 min in 2 ml of the same medium saturated with 93% O_2/7% CO_2. After changing the medium, incubation proceeded for 4 hr period in 2 ml of KRB containing none or various doses of unlabelled or tritiated LH-RH/FSH-RH [16].

Following incubation, groups of hemipituitaries were weighted within 0.05 mg. Rat LH and FSH were measured in incubation medium using radioimmunoassays previously described [17, 18].

Table 1 shows the release of LH and FSH from rat pituitaries under the action of both unlabelled and labelled LH-RH/FSH-RH assayed 10 days and 60 days after tritiation. No significant difference was found between tritiated and non tritiated products.

3. Discussion

The analytical, spectral and biological characteristics indicate that the labelled peptide is indistinguishable from the unlabelled one. The tritium radioactivity is in agreement with the incorporation of 2.5 iodine atoms per mole LH-RH/FSH-RH and their quantitative replacement by 2.5 tritium atoms. This confirms the fact that ICl has reacted not only with the tyrosyl but also with the histidyl residue. It is worth pointing out that the [^{3}H]LH-RH/FSH-RH tryptophyl residue has not been oxidized during the halogenation step. In parallel experiments, free tryptophan and tryptophan + glutathione mixtures, have been treated with ICl under similar conditions. The indole moiety was immediately altered as indicated by a pink coloration which appeared and remained after thiosulfate addition. The resistance of the tryptophan side chain of LH-RH/FSH-RH to the oxidizing action of ICl may correspond either to the much faster reaction rate of ICl with the tyrosine and histidine side chains or to the inaccessibility of the tryptophan side chain to the reagent. The latter possibility appears more likely as an excess of ICl was used, and suggests in the peptide a specific environment for the tryptophan, an essential residue for the biological activity of the molecule [19].

Thus, as in the case of the *Naja nigricollis* α neurotoxin [14], tryptophan containing peptides

Table 1
Effect of unlabelled and labelled LH-RH/FSH-RH on the *in vitro* release of LH and FSH from rat pituitary glands[1].

LH-RH/FSH-RH (ng/ml)	μg LH released[2]/mg of tissue		μg FSH released[3]/mg of tissue	
	Unlabelled LH-RH/FSH-RH	[^{3}H]LH-RH/FSH-RH	Unlabelled LH-RH/FSH-RH	[^{3}H]LH-RH/FSH-RH
	Assayed 10 days after tritiation[4]			
Control	0.58 ± 0.13		0.06 ± 0.020	
1	1.24 ± 0.13	1.06 ± 0.07	0.11 ± 0.01	0.13 ± 0.010
10	1.35 ± 0.12	1.50 ± 0.15	0.17 ± 0.015	0.15 ± 0.013
100	2.09 ± 0.20	1.97 ± 0.20	0.31 ± 0.03	0.24 ± 0.030
	Assayed 60 days after tritiation[4]			
Control	0.40 ± 0.1		0.03 ± 0.007	
1	0.73 ± 0.08	0.77 ± 0.07	0.07 ± 0.007	0.06 ± 0.005
10	1.60 ± 0.16	1.88 ± 0.19	0.11 ± 0.01	0.12 ± 0.009
100	1.80 ± 0.20	1.83 ± 0.18	0.11 ± 0.01	0.12 ± 0.006

[1] In each case 4 pituitary halves (per flask) of ovariectomized female rats treated with estradiol benzoate and progesterone (EBP) were incubated for 4 hr.
[2] In terms of a rat LH laboratory preparation (1 × NIH-LH S3).
[3] In terms of a rat FSH (FSH-S-1-1, 100 × NIH-FSH-S1) supplied by the NIAMD.
[4] The product was stored in liquid nitrogen.

may survive the halogenation step required for the present tritiation method.

Lastly, it may be mentioned that after two months of storage in liquid N_2, [^{3}H]LH-RH/FSH-RH retained both its chemical and biological properties.

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References

[1] A.V. Schally, R.M.G. Nair, T.W. Redding and A. Arimura, J. Biol. Chem. 246 (1971) 7230.
[2] Y. Baba, H. Matsuo and A.V. Schally, Biochem. Biophys. Res. Commun. 44 (1971) 459.
[3] M. Amoss, R. Burgus, R. Blackwell, W. Vale, R. Fellows and R. Guillemin, Biochem. Biophys. Res. Commun. 44 (1971) 205.
[4] R. Burgus, M. Butcher, M. Amoss, N. Ling, M. Monahan, J. Rivier, R. Fellows, R. Blackwell, W. Vale and R. Guillemin, Proc. Natl. Acad. Sci. U.S. 69 (1972) 278.
[5] H Matsuo, A. Arimura, R.M.G. Nair and A.V. Schally, Biochem. Biophys. Res. Commun. 45 (1971) 822.
[6] M. Monahan, J. Rivier, R. Burgus, M. Amoss, R. Blackwell, W. Vale and R. Guillemin, Compt. Rend. 273 (D) (1971) 508.
[7] H. Stevertsson, J.K. Chang, C. Bogentoft, B.L. Currie K. Folkers and C.Y. Bowers, Biochem. Biophys. Res. Commun. 44 (1971) 1566.
[8] P. Rivaille, A. Robinson, M. Kamen and G. Milhaud, Helv. Chim. Acta 54 (1971) 2772.
[9] R. Geiger, W. König, H. Nissman, K. Geisen and F. Enzman, Biochem. Biophys. Res. Commun. 45 (1971) 767.
[10] B. Kerdelhué, M. Jutisz, D. Gillesen and R.O. Studer, Biochim. Biophys. Acta, submitted for publication.
[11] J.L. Morgat, Lam Thanh Hung and P. Fromageot, Biochem. Biophys. Acta 207 (1970) 374.
[12] J.L. Morgat, Lam Thanh Hung, R. Cardinaud, P. Fromageot, J. Bockaert, M. Imbert and F. Morel, J. Label. Compounds 6 (1970) 276.
[13] P. Pradelles, J.L. Morgat, P. Fromageot, C. Oliver, P. Jacquet, D. Gourdji and A. Tixier-Vidal, FEBS Letters 22 (1972) 19.
[14] A. Menez, J.L. Morgat, P. Fromageot, A.M. Ronseray, P. Boquet and J.P. Changeux, FEBS Letters 17 (1971) 333.
[15] V.D. Ramirez and S.M. McCann, Endocrinology 73 (1963) 193.

[16] M. Jutisz, B. Kerdelhué and A. Berault, in: The Human Testis, eds. E. Rosemberg and C.A. Paulsen (Plenum Press, New York, 1970) p. 221.
[17] B. Kerdelhué, A. Bérault, C. Courte and M. Jutisz, Compt. Rend. 269 (D) (1969) 2413.
[18] B. Kerdelhué, G. Kann and M. Jutisz, in: Colloquium on the Pituitary Glycoprotein Hormones, ed. Marian Jutisz (INSERM Publisher, Paris, 1972) p. 177.
[19] Y. Baba, A. Arimura and A.V. Schally, Biochem. Biophys. Res. Commun. 45 (1971) 483.