PREPARATION OF HIGHLY LABELLED ^{3}H-THYREOTROPIN RELEASING HORMONE (PGA–HIS– PRO(NH_2)) BY CATALYTIC HYDROGENOLYSIS

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Received 22 February 1972

1. Introduction

The synthetic tripeptide pyroglutamyl–histidyl–prolineamide (TRH) has been shown to possess the biological potencies of the natural thyreotropin releasing factor (TRF) [1, 2]. This achievement encouraged a large variety of physiological investigations and efforts towards the preparation of labelled hormone. The first attempts reported concerned tritium labelling of extractive TRF [3]. The specific radioactivity obtained, of the order of 8 mCi/mg was not sufficient for precise cellular localisation under physiological conditions. The simplicity of TRH structure suggested a labelling of the molecule by total synthesis, using labelled amino acids. This aim has been reached by Flouret [4] and by Monahan and Young [5] using, respectively, ^{14}C-histidine or ^{3}H-proline. The specific radioactivities obtained were those of the precursor amino acids, 250 mCi/mmole and 50 Ci/mmole, respectively.

Previous work had shown that a variety of hormonal peptides could be tritium labelled by catalytic replacement of iodine atoms previously bound on tyrosyl residues(s) [6, 7]. The specific radioactivities were those expected from the number of iodine atoms exchanged. The possibilities to separate the iodinated peptide either from the non-reacted or from the tritiated peptide allowed the preparation of biologically active labelled material with nearly the theoretical specific radioactivities.

It was gratifying to establish that our technique could be used for introducing a tritium atom on a histidyl residue belonging to a large peptide, the toxin of *Naja nigricollis* [8], which contains two histidines and a buried tyrosine. This result prompted us to investigate the tritium labelling of a short peptide; the present report indicates that two tritium atoms could be bound to the histidine side chain of TRH with no loss of the biological activity tested.

2. Experimental

2.1. *Materials*

TRH was kindly provided by Dr. R.O. Studer (Hoffman-La Roche, Basel). Halogenation was carried out by ICI from Rhone-Poulenc. ^{125}ICI used as tracer was from New England Nuclear Corporation. The

Table 1
Release of TSH in rats following injection of identical amounts of tritium labelled and standard TRH.

Amount injected (ng)	TSH μU/ml	
	Standard TRH	^{3}H-TRH
50	9.5 ± 1.7	8.2 ± 1.6
100	17.4 ± 3.3	16.0 ± 3.3
250	25.3 ± 5.4	22.5 ± 4.5
500	39 ± 7.9	40 ± 8.1
1000	54.1 ± 11.7	63.8 ± 11.8

palladium/Al_2O_3 catalyst was from Engelhardt (Rome). The ion exchange resin AG II A8 came from Bio Rad Laboratories. The tritium gas was made by the Commissariat à l'Energie Atomique (France). TRH weight determinations were carried out by amino acid measurement with the Technicon Autoanalyzer. Tritium determination was made with SL 30 Intertechnique liquid scintillation counter and ^{125}iodine was counted with SAIP-Gammatic solid scintillation device.

2.2. *Preparation of iodo-TRH*

20 μmoles ^{125}ICI, dissolved in 50 μl dry methanol are slowly (10 sec) added to 2.7 μmoles TRH dissolved in 1 ml 0.2 M Na acetate solution pH 6.5, chilled to 4°; after 45 sec excess Na thiosulfate is added.

2.3. *Purification of iodo-TRH*

The mixture is passed through an AG II A8 column (41 × 1.3 cm) to removed the salts. Elution with distilled water yielded two peptide peaks. Peak 1 (5–10%) had the same amino acid composition as TRH, and contained no iodine. Peak II (85–90%) corresponded to halogenated material. Thin-layer chromatography on cellulose (acetone:water, 80:40) of peak II indicated a trace of a non-halogenated compound beside iodo-TRH.

2.4. *Tritiation*

Peak II was flash evaporated and redissolved into 500 μl 0.1 M phosphate buffer pH 7.3 . 10 mg Pd/Al_2O_3 catalyst were added to the cup of the tritiation flask containing the iodo-TRH. The latter was frozen and connected to the vacuum line. After 10^{-4} Torr were reached, 10 Ci 99% pure tritium gas were introduced (pressure 200 Torr) flushing the Pd catalyst which was afterwards dropped into the frozen solution. Gradual melting was allowed. After 20 min the catalyst was removed by Millipore filtration and the labile hydrogens exchanged by successive flash evaporation.

2.5. *Purification*

The ^{3}H-TRH soltuion was filtered on an AG II A8 column, and the peptide eluted with distilled water. Two peaks were found. The first represented ^{3}H-TRH as shown by thin-layer co-chromatography with control substance on silica gel ($CHCl_3$–CH_3OH– conc. NH_4OH, 60/45/20). The UV spectrum of the ^{3}H-TRH was not different from the control. After acid hydrolysis of an aliquot of the labelled compound (6 N HCl, 110°, 16 hr) the three constitutive amino acids were found. Their quantitative estimation indicated a total amount of 120 μg ^{3}H-TRH. On that basis the specific radioactivity was found to be 60 Ci/mmole.

2.6. *Biological tests*

To check that the labelled compound (^{3}H-TRH) obtained was biologically active two assays were per-

Table 2
Action of cold and tritiated TRH on the prolactin released in the media by GH_3 cells, after 48 hr of contact (ng of prolactin per ml).

Nature of the TRH	TRH doses (ng/ml)			Control
	0.1	1	10	
TRH	67.3 ± 4.6*	60.6 ± 2.4*	97.3 ± 4.6*	53.7 ± 3.5**
^{3}H-TRH	62.0 ± 5.0*	82.6 ± 4.8*	92.6 ± 4.0*	57.3 ± 1.2**

*Average of 3 wells by dose. **Average of 9 control wells.

formed: the release of TSH and the release of prolactin.

2.6.1. *Release of TSH in the rat*

Increasing amounts of ^{3}H-labelled and control TRH were dissolved into 0.5 ml of isotonic NaCl solution, injected intraperitoneally into 200–250 g Wistar rats anesthetized by Nembutal (4 mg/100 g). Eight animals were used for each dose. 0.5 ml of blood were withdrawn after 15 and 30 min and the TSH concentration estimated by radioimmunoassay [9–11]. The TSH base line for control animals was the mean value of the levels found at 15 and 30 min. The figure found for Nembutal anesthetized rats was 3 ± 0.8 μU/ml, with extreme at 1 and 10.5 μU/ml. As shown in table 1 equal doses of ^{3}H-TRH and control TRH released the same mean amounts of TSH.

2.6.2. *Release of prolactin*

The response to TRH of GH_3 clonal strain (rat prolactin cells [12]) has been previously established [13, 14]. Cells from this clone were plated in 6 "disposo trays" (Linbro FB6-TC) at the density of 2.5×10^4 cells/well, in 2 ml medium (medium HAM F 10 + 15% horse serum + 2.5% foetal calf serum). After 48 hr the medium was changed and the TRH introduced at 3 different doses: 0.1, 1 and 10 ng/ml. Each tray contained 3 treated wells and 3 control wells. For each dose of cold or ^{3}H-TRH, we used a separate tray. A new dose of TRF was added after 24 hr. The prolactin concentration in the medium after 24 hr and 48 hr of contact with the TRH was measured with a radioimmunoassay using the NIAMD kit for rat prolactin [15] (minimum detectable amount: 10 picogram). The stimulation of the prolactin production was evident after 48 hr of contact and reached the same level with cold as well as with labelled TRF (table 2). In addition to their biological responsiveness to the tritiated TRF, the GH_3 cells possess the ability to selectively bind this molecule (manuscript in preparation).

3. Discussion

The analytical characteristics as well as the biological potencies of the ^{3}H-TRH indicated that the labelled peptide was indistinguishable from the original material. Thus the labelling technique used seems to be valuable for the rapid preparation of ^{3}H-TRH. The specific radioactivity reached, 60 Ci/mmole, corresponded to the replacement of 2 H by 2 tritium atoms per mole in positions 2 and 4 of the imidazole side chain.

As well as those obtained with the ^{3}H-proline labelled peptide made by Monahan and Young (5), it is indicated that even in a short peptide devoid of a large variety of side chains, substitution of 2 H by 2 tritium atoms on two essential rings, did not modify the ability of the peptide to bind and to trigger the biological responses investigated. It is thus demonstrated that tritium labelling of substance acting by virtue of their structure apparently leads to compounds as close as possible to their non-labelled counterpart.

Acknowledgements

We express our gratitude to Dr. R.O. Studer (Hoffman-La Roche, Basel) who provided TRH and made this investigation possible. We are indebted to Dr. Kerdelhué and Dr. M. Jutisz, Collège de France, for performing the prolactin radioimmunoassays.

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