PREPARATION OF OXYTOCIN SPECIFICALLY ^{14}C-LABELED IN THE TYROSINE RESIDUE

P. L. HOFFMAN and Roderich WALTER

Department of Physiology and Biophysics, University of Illinois at the Medical Center, Chicago, Illinois 60612 USA

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1. Introduction

Oxytocin and vasopressin containing [^{14}C]glycine in the C-terminus [1] have significantly aided research on new metabolic pathways of these hormones. Such studies demonstrated the presence in various tissues of enzymes which catalyze the hydrolysis of peptide bonds located in acyclic portion of the hormones [2,3] and have led to additional investigations of these kinds of enzymatic activities in other biological systems [4–9]. Enzymes which release glycinamide or the C-terminal dipeptides of oxytocin and vasopressin have now been partially purified [10,11]. Additional enzymatic mechanisms capable of neurohypophyseal hormone inactivation are known, including disulfide reductase in liver [12] as well as enzymes which can degrade the hormones from the N-terminus [13–15].

In order to investigate in greater detail the mechanisms of neurohypophyseal hormone inactivation from the N-terminus, we have synthesized and report here oxytocin labeled with [^{14}C]tyrosine. [2-^{14}C(U)Tyrosine]oxytocin was synthesized by the general solid-phase procedure [16].

Nomenclature is in accordance with IUPAC–IUB Rules on Biochemical Nomenclature, Biochem. J. 126, 773 (1972); and J. Biol. Chem. 242, 555 (1967). All optically active amino acids are of the L configuration. Other abbreviations: ONp, nitrophenyl ester; HOAc, acetic acid; DMF, *N,N'*-dimethylformamide; DCCI, dicyclohexylcarbodiimide; t.l.c., thin layer chromatography. All t.l.c. were run on silica gel plates from Quantum Industries; unlabeled compounds were visualized by the method of H. Zahn and E. Rexroth (1955) Z. Analyt. Chem. 148, 181–186. On high voltage electrophoresis, unlabeled compound was detected with ninhydrin. The biological activity of the hormone was measured against the U.S.P. Posterior Pituitary Reference Standard; the four-point design was used for the assay.

2. Results and discussion

Boc-glycine was esterified to chloromethylated copolystyrene–2% divinylbenzene [17] (1.06 mmol Cl/g) at a substitution of 0.36 mmol/g resin as determined by hydrolysis in 6 N HCl/propionic acid (1:1) [18] followed by amino acid analysis [19] (Beckman 120C amino acid analyzer). An individual cycle for incorporation of each additional amino acid involved washing with HOAc followed by CH_2Cl_2, removal of the N-protecting group with 25% TFA in CH_2Cl_2, followed by neutralization with 10% diisopropylethylamine, and then washing with CH_2Cl_2 and acylation with the appropriate N-protected amino acid. The final washes consisted of DMF/CH_2Cl_2, 1:1, v/v and $CHCl_3$/MeOH, 2:1, v/v. Boc-Leu, Boc-Pro, Boc-Cys(Bzl), Boc-Ile and Boc-^{14}C(U)Tyr were added in two-fold molar excess along with a two-fold molar excess of DCCI and the reaction was carried out in CH_2Cl_2 for 2 h. Extent of acylation was monitored by the ninhydrin reaction [20] and in most cases a repeat of the coupling reaction (in DMF/CH_2Cl_2, 1:1, v/v) was required. Boc-Gln, Boc-Asn, and the N-terminal Z-Cys(Bzl) were incorporated as their nitrophenyl esters (5 meq) in the presence of 1,2,4 triazole (1.3 meq) [21] in DMF during an overnight reaction. For incorporation of ^{14}C(U)tyrosine, 30 mCi of uniformly labeled [^{14}C] tyrosine (486 Ci/mol, ~ 0.06 mmol; Amersham-

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Searle) was examined by t.l.c. (95% EtOH/H_2O, 7.3, v/v; PrOH/H_2O 7:3, v/v) and found to be 98% radiochemically pure. The labeled amino acid was diluted to a specific radioactivity of 93.7 Ci/mol (0.32 mmol) by addition of unlabeled tyrosine, and was treated with Boc-azide according to the method of Schnabel [22]. The reaction was followed by t.l.c. ($CHCl_3$/MeOH/HOAc, 85:10:5, v/v/v; S_1) and after 3 days the reaction mixture was extracted with Et_2O, the aqueous phase acidified with citric acid, and again extracted with ethyl acetate and ether. This organic layer was dried to an oil which revealed two radioactive spots on t.l.c. (S_1), corresponding to Boc-tyrosine and di-Boc-tyrosine. Based on radioactivity, the yield of this reaction was 85%. The oil was added to 0.45 g of free heptapeptide-resin (0.16 mmol heptapeptide). The resultant octapeptide was elongated to the nonapeptide as described, the protected nonapeptide was cleaved from the resin by ammonolysis, precipitated from DFM/H_2O, and repeatedly washed with water by centrifugation. This material contained two radioactive components upon t.l.c. (S_1 and $CHCl_3$/MeOH, 8:2). The nonapeptide was deprotected with Na in liquid ammonia [23], oxidized to the disulfide with ferricyanide [24], and subjected to partition chromatography [25] on a column of Sephadex G-25 (200–400 mesh, 108 × 0.9 cm) which had been equilibrated with the lower phase of the solvent system 1-BuOH/benzene/pyridine/HOAc/H_2O (600:200:100:1:900). The hormone was eluted with the upper phase of the solvent system and detected by scintillation counting (Beckman LS-355 Liquid Scintillation Spectrometer; scintillation cocktail 3A70 from Research Products Intl.) at an R_f value of 0.22 (R_f of 0.24 reported for oxytocin [25]).

Fractions containing the radioactive peak were pooled, the organic phase was removed under reduced pressure, and the aqueous phase was adjusted to 0.2% acetic acid. An aliquot of this material was examined by t.l.c. in two solvent systems and by high voltage paper electrophoresis (see fig.1). The material exhibited an avian vasodepressor activity [26,27] of 463 ± 11 U/mg*, a value comparable to that reported for purified oxytocin [28,29]. After bioassay, 0.5 ml aliquots of the hormone solution, at a concentration of 0.1 mg/ml (2.06×10^7 dpm/ml) in 0.2% acetic acid, were sealed in ampules which were sterilized in a steam bath for 10 min and stored at 4°C.

The specific radioactivity of this hormone is 93.7 Ci/mol, which is significantly higher than that previously reported for ^{14}C-labeled neurohypophyseal hormones [1], and should overcome some of the problems encountered in the past in using ^{14}C-labeled neurohypophyseal hormones for in vivo studies.

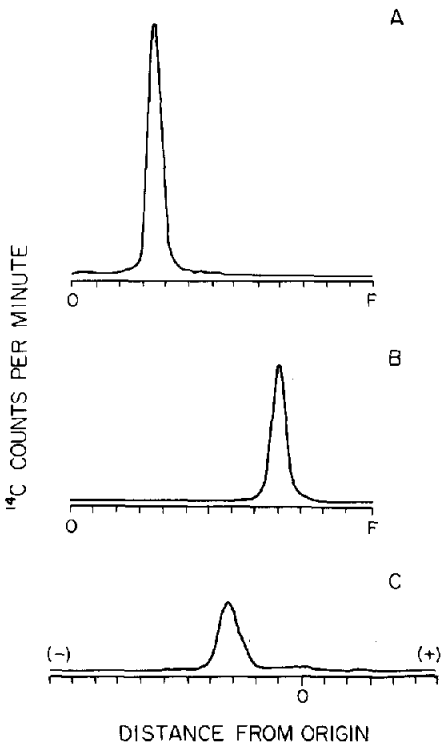

Fig.1. Thin-layer chromatography and high voltage electrophoresis of [2-^{14}C(U)Tyr]oxytocin. In each case, 125 000 cpm of radioactivity (spec. act. of compound, 93.7 Ci/mol) was applied. Peaks were detected using a Packard Model 7201 chromatogram scanner; unlabeled oxytocin was used as standard. (A) Thin-layer chromatography on silica gel in the solvent system 1-BuOH/HOAc/H_2O, 4:1:1, v/v/v. (B) Thin-layer chromatography in the solvent system 1-BuOH/pyridine/H_2O, 20:10:11, v/v/v. (C) High voltage electrophoresis in 10% pyridine acetate buffer, pH 3.5. Electrophoresis was carried out at 3000 V for 1 h. O = origin, with distances given in cm. F = solvent front.

*Concentration of the hormone was determined on the basis of specific radioactivity of the hormone and cpm/ml of solution.

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