ISOLATION OF THYROTROPIN RELEASING FACTOR (TRF) FROM PORCINE HYPOTHALAMUS

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Hypothalamic control of the secretion of pituitary thyrotropic hormone (TSH) is exercised through the hypophysial portal blood and mediated by a neurohumor designated thyrotropin-releasing factor (TRF). The work of various investigators clearly established the existence in hypothalamic extracts of several animal species of TRF capable of stimulating the release of TSH (Guillemin et al., 1962; 1963; Reichlin, 1964; Schreiber et al. 1961; Schally et al. 1965; 1966 a; Bowers et al. 1965; Sinha and Meites, 1965). The purification of ovine TRF was reported by Guillemin et al. 1962; 1965, while our laboratory described the purification of porcine and bovine materials (Schally et al. 1965; 1966 a; 1966 b; 1966 c). Recently Guillemin et al. (1966) described a new sequence of purification of ovine TRF and suggested that their preparation may not be a polypeptide as it has been considered to be so far.

This report describes the isolation of TRF from porcine hypothalamic extracts. Purified material is active at one nanogram (millimicrogram) in vivo and appears to be not a simple polypeptide since amino acids account for only 30% of its composition. The biological activity of TRF was followed in vivo by the release of I^{131} in mice treated with codeine and 1 μg thyroxine (Redding et al. 1966), elevation of plasma level of TSH in thyroidectomized rats treated with triiodothyronine (Bowers et al. 1965) and depletion of

pituitary TSH in mice. That TRF acts directly on the anterior pituitary tissue to stimulate the release of TSH was clearly shown by experiments *in vitro*. Incubation of rat anterior pituitary tissue (Bowers *et al*. 1965; Saffran and Schally 1955) with 0.1 nanograms of TRF resulted in significant stimulation of release of TSH.

The isolation of TRF from 100,000 hypothalami was accomplished essentially in 9 steps. Fragments of pig hypothalamic tissue containing essentially the pituitary stalk and median eminence (SME) (kindly supplied by Oscar Mayer Co., Madison, Wis.) were dehydrated by lyophilization. Lyophilized fragments (total weight for 100,000 SME = 1600 grams) were chilled on dry ice and ground into a fine powder. The powder was defatted with acetone and petroleum ether and extracted with 2 N acetic acid at 8°C. The lyophilized extract (yield 520 g) was reextracted with glacial acetic acid and lyophilized (yield 360 g). Glacial acetic acid extracts in 10 g batches were subjected to gel filtration on a column of Sephadex G-25 (7 x 17 cm) in 1 M acetic acid. Fractions containing TRF activity emerged between α-MSH and lysine vasopressin and had a R_f (Porath and Schally, 1962) of 0.48 identical with bovine TRF. Lyophilization of combined TRF active areas yielded 100 g material active *in vivo* at 200-500 µg. TRF activity in this material was concentrated and desalted by phenol extraction (Schally *et al*. 1966 a). TRF activity is highly soluble in phenol phase of a phenol/water system and was thus extracted yielding 20 g material active *in vivo* at 20-100 µg. This material in 2.5 g batches was subjected to ion-exchange chromatography on a column of carboxymethylcellulose (CMC)(column, 2.6 x 60 cm, equilibrated with 0.002 M, pH 4.6 ammonium acetate buffer). TRF was eluted by application of a gradient to pH 7, 0.1 M ammonium acetate (through 2000 ml mixing flask) and emerged in fractions with conductivity of 1 m MHOS before luteinizing hormone releasing factor (LRF) and lysine vasopressin. Lyophilization gave 2.1 g of material active to release TSH at 4-20 µg. This was divided into 2 batches and each batch was subjected to 400 transfers by countercurrent distribution in a system of 0.1% acetic acid: n-

butanol: pyridine = 11:5:3 (v/v). TRF active area gave a partition coefficient k=0.42, identical with that reported by Guillemin et al. (1965) for ovine TRF. TRF activity was displaced into the aqueous layer by addition of several volumes of benzene, concentrated by flash evaporation and lyophilized. 800 mg TRF were obtained, active at the dose of 1-2 µg. This material was further repurified by free flow electrophoresis in pyridine acetate buffer at 1800 V, 160 mA, 5°C in Brinkman Elphor FF continuous electrophoretic separator (Hannig 1961). The TRF activity migrated slightly toward the cathode under these conditions. 18 mg TRF were obtained and now it was active at the dose of 6-10 nanograms in vivo.

The final purification step consisted of partition chromatography on Sephadex G-25, 0.9 x 76 cm (Yamashiro, 1964). The solvent consisted of upper phase of n-butanol:acetic acid:water =4:1:5. The peak containing TRF activity was not revealed with Folin-Lowry reagent (Lowry et al, 1951) but it was detected by bioassays. After lyophilization 2.8 mg of TRF were obtained from 100,000 hypothalami. The material was active to release TSH in vivo (Redding et al, 1966) at doses smaller than 1 nanogram. Since the recovery of biological activity of each step was essentially complete, the purification was about 600,000 fold. Thin-layer chromatography (TLC) of TRF on cellulose coated plates in three solvent systems, 1. butanol:acetic acid:water = 4:1:5, 2. acetone:water = 2:1 at 6°C, 3. ethyl acetate:butanol:acetic acid:water = 1:1:1:1 v/v, revealed only one spot which was negative to ninhydrin but positive to Pauly reagent (Diazotized sulfanilic acid). Thin-layer electrophoresis (TLE) in pyridine acetate buffer at pH 6.3 also showed one Pauly positive spot with a mobility ($-6.8\ cm^2\ volt^{-1}\ sec^{-1} \times 10^{-5}$) much smaller than that of histamine ($-9.6\ cm^2\ volt^{-1}\ sec^{-1} \times 10^{-5}$). The elution of TRF spots applied parallel to the sprayed zones on TLE and on all 3 systems on TLC revealed that all the biological activity was concentrated in the area corresponding to the Pauly positive spot. The spraying with diazotized sulfanilic acid consistently caused about 80% decrease in biological activity

of TRF indicating that an intact imidazole ring is necessary for the full biological activity of TRF. The material was stable to all the commonly used proteolytic enzymes but the activity was destroyed by a 5 min incubation with human serum (Bowers et al. 1966). After acid hydrolysis TRF was shown to contain three amino acids, histidine, glutamic acid and proline, which were present in about equimolar ratio and which accounted for about 30% of the dry weight of TRF. TLC of TRF in three solvent systems and TLE did not lead to any detectable changes in the amino acid content of TRF. In each case TRF activity was associated with Pauly positive spots yielding histidine, glutamic acid and proline on hydrolysis.

The results are consistent with a hypothesis that TRF is not a simple polypeptide as has been thought previously, but nevertheless our evidence indicates that three amino acids are present in this molecule.

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