DISCOVERY OF MODIFICATION OF THE SYNTHETIC TRIPEPTIDE-SEQUENCE OF THE THYROTROPIN RELEASING HORMONE HAVING ACTIVITY.

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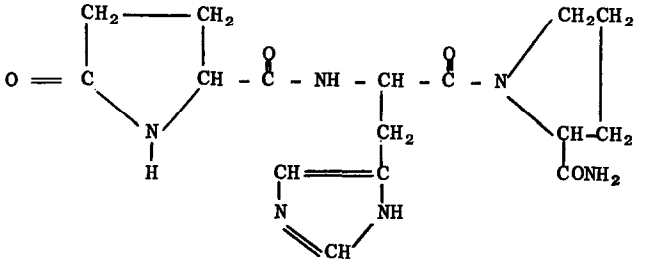
The isolation of the thyrotropin releasing factor or hormone (TRF or TRH) in a state of "near-purity" from porcine hypothalami was achieved by Schally et al., in 1966. Since only 2.8 mg. of TRH was obtained from the hypothalami of 100,000 pigs, the investment of this amount in further work to "absolute-purity" or in structure determination became one of stepwise decision. Most notably, this TRH was subjected to acid hydrolysis and three amino acids were obtained; they were histidine, glutamic acid and proline. These three amino acids were present in essentially equimolar amounts and accounted for about 30% of the particular sample which was hydrolyzed. This evidence indicated that His, Glu and Pro are moieties in TRH. It was advantageous that TRH showed only one Pauly-positive spot (the histidine moiety) on chromatograms.

Extension of the isolation process to 165,000 hypothalami has recently been described in detail, and the TRH appeared to be homogeneous by TLC, electrophoresis and paper chromatography (Schally et al., 1969). Such isolated TRH released thyrotropin (TSH) from the pituitary glands of mice in doses of about one nanogram. In vitro, about ten picograms of such TRH stimulated the secretion of TSH. Again, only three amino acids, histidine, glutamic acid and proline, were obtained in equimolar ratio by hydrolysis. The structural sequence of the three amino acids was determined by the Edman procedure to be Glu-His-Pro.

The knowledge of the presence of the three amino acids was the first basis for organic syntheses on this problem. Dr. Frederick W. Holly of the Merck, Sharp and Dohme Research Laboratories had kindly provided in 1966 gift-samples of the following sequences of the three amino acids and Gln modifications of Glu: Pro-Glu-His; Glu-Pro-His; His-Glu-Pro; Glu-His-Pro;

His-Pro-Glu; Pro-His-Glu; His-Pro-Gln; Pro-His-Gln. Although these samples included Glu-His-Pro, none of them showed the hormonal activity of TRH, either in vitro or in vivo at relatively high dose levels (Schally et al., 1968).

The lack of activity of the unsubstituted tripeptide, Glu-His-Pro, is not particularly surprising since TRH does not appear to have a free amino or carboxyl group; TRH appears to be a substituted or modified form of Glu-His-Pro. Taking into account the known structural characteristics of TRH, including the knowledge of the sequence, synthetic experiments were carried out on Glu-His-Pro to modify both the amino and carboxyl groups. This tripeptide was treated with anhydrous methanol containing hydrogen chloride to form a dimethyl ester. The dimethyl ester was dissolved in anhydrous methanol saturated with ammonia at -5° and allowed to stand for 24 hours at room temperature to yield preparation A. The conditions used to give preparation A may be expected (Coleman, 1951; Beecham, 1954, Shiba et al., 1958) to give predominately, but not exclusively, (pyro)Glu-His-Pro(NH_2) (I). Preparation A was not specially purified partly because the tripeptide used as starting material was estimated to be not over 80% pure, and the reactions were carried out on a milligram-basis. Preparation A was ninhydrin-negative and Pauly-positive.



I.

Samples of preparation A were subjected directly to biological tests for hormonal activity by the T_3-TRH method in mice (Bowers et al., 1965, '67, '69). The response is determined by the increase of I^{125} in the blood as Δ cpm two hours after the iv. injection of TRH and the synthetic preparations. The increase is proportional to the amount of TSH released from the pituitary. Since the doses of the synthetic preparations represent the weights of the starting material, they are the "relative" rather than the actual amounts of the products given; although products were visualized chromatographically, the yields are unknown. Nevertheless, levels of "6 - 54" nanograms of preparation A in the mouse increased I^{125} in the range of Δ cpm 670 - 8000 and in comparison with Δcpm 140 - 170 for acid-saline controls. Levels

of 2 - 6 - 18 nanograms of porcine TRH increased I^{125} in the range of Δ cpm 2000 - 6000. It is significant that preparation A is extremely active in comparison with porcine TRH although a precise comparison of activities is not yet known. Graded responses were obtained when the doses of preparation A was increased, and two of the important biological characteristics of natural TRH were observed: (a) the degree of the response depended upon the amount of T_3 injected; (b) incubation for fifteen minutes at 37°C in normal human plasma inactivated the synthetic preparation.

The synthetic preparation was also active in vitro by the method of Bowers et al. (1965). The amount of TSH released from the pituitary into the medium was estimated by the release of I^{125} from the thyroid gland of mice. Activity is measured as I^{125} in Δ cpm and is proportional to the amount of TSH present in the medium. More TSH (Δ cpm, 2300 - 3100) was released when "50" nanograms of the preparation A was added to the medium than in the control (Δ cpm 275). It appeared that T_3 added, in vitro, or given, in vivo, partially or completely inhibited the activity.

A comparison was made of the changes in blood levels of I^{125} in mice at various time-intervals after the i.v. injection of acid-saline, the synthetic preparations, porcine and bovine TSH. The time-response curves of the active compounds are essentially the same. There was a definite rise at 60 minutes; the levels were higher at 90 minutes and remained elevated at 120 and 180 minutes.

Preparation A elevated the plasma levels of TSH in rats (Bowers et al., 1965), and produced an increase within two minutes in plasma levels of TSH , after its i.v. injection in mice (Bowers et al., 1967, 1969). The plasma levels of TSH were highest at 10 and 15 minutes, and remained elevated for 60 minutes, and started to fall at 120 minutes. Preparation A was active in mice when given i.v., i.p., but not i.m. or orally.

Similar synthetic reactions were carried out upon Glu-Pro-His; Pro-His-Gln; Pro-His-Glu, but none of the resulting preparations showed the hormonal activity of TRH under comparable conditions. To this limited extent, the sequence of Glu-His-Pro shows organic structural specificity for hormonal activity.

While these structural and synthetic studies have been on TRH isolated from porcine hypothalami, Guillemin and his group have similarly investigated TRH which they isolated from ovine hypothalami. Burgus et al., 1969, found TRH-activity at microgram levels for acetylation products of Glu-His-Pro and observed that TRH is active at 1×10^{-3} of the level of these particular synthetic preparations.

These mutually independent synthetic studies on porcine and ovine TRH show at this stage of structural elucidation that the hormone from the two mammalian species may be chemically identical.

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