Thyrotropin-Releasing Hormone: Biosynthesis by Rat Hypothalamic Fragments in vitro

Abstract. *Biosynthesis of thyrotropin-releasing hormone (L-pyroglutamyl-L-histidyl-L-proline amide) in vitro was studied. Rat hypothalamic fragments were incubated in Krebs-Ringer bicarbonate buffer that contained either ^{14}C-labeled proline, histidine, or glutamic acid (the three probable precursor amino acids) and for control purposes each of 16 other naturally occurring amino acids. A number of labeled peptides were synthesized. With the use of synthetic thyrotropin-releasing hormone, detected by the Pauly reagent or with ^{125}I-labeled thyrotropin-releasing hormone as a marker, thin-layer chromatograms, paper electrophoresis, and carboxymethyl cellulose ion exchange chromatography revealed that only proline, histidine, and glutamic acid were consistently incorporated into peptides associated with the thyrotropin-releasing hormone region. This synthesizing activity was found in stalk median eminence, ventral hypothalamus, and dorsal hypothalamus but not in neural lobe or cerebral cortex. Because the biosynthetic peptide has identical properties with L-pyroglutamyl-L-histidyl-L-proline amide, it is probable that rat thyrotropin-releasing hormone is similar or identical to both bovine and porcine thyrotropin-releasing hormone and that the native material is present in the pyroglutamyl form in tissues.*

The chemical identity of sheep and pig thyrotropin-releasing hormone (TRH) has been reported to be L-pyroglutamyl-L-histidyl-L-proline amide (*1–5*). We now demonstrate that rat hypothalamic fragments incubated in vitro incorporate glutamic acid, histidine, and proline into a polypeptide that has chromatographic characteristics identical with those of synthetic TRH.

Hypothalamic tissues were removed from decapitated male rats (body weight 250 to 400 g) in three sections: stalk median eminence, ventral hypothalamus (including arcuate and ventromedial nuclei), and dorsal hypothalamus (including tissue from the anterior commissure anteriorly, the anterior margin of the mammillary nuclei posteriorly, and the paraventricular nuclei superiorly). The tissue fragments, in 1.0 ml of Krebs-Ringer bicarbonate buffer solution that contained 10 or 20 μc of labeled amino acid, were incubated (95 percent oxygen and 5 percent carbon dioxide) with shaking for 2 hours at 37°C. The tissues were then homogenized in 90 percent methanol, a solvent chosen on the basis of the solubility of TRH in various organic solvents (*6*). The supernatant was removed after centrifugation and subjected to thin-layer chromatography (TLC) on silica gel G plates, paper electrophoresis on Whatman paper No. 1 in barbital buffer (0.1*N* at *p*H 8.6), Sephadex gel filtration (G-25 and G-10) in 1.0*N* acetic acid, and carboxymethyl cellulose (CM-cellulose) ion exchange chromatography with a discontinuous system of 0.002*M* and 1.0*M* ammonium acetate buffer modified from the method of Schally (*2*). The position of carrier TRH on TLC was identified by the Pauly reagent (*7*); TRH position on Sephadex or CM-cellulose fractionation was identified by the location of ^{125}I-labeled TRH. Synthetic TRH was prepared from the methyl ester of L-pyroglutamyl-L-histidyl-L-proline acetate (*8*) followed by ammonolysis. This preparation was identical to one from Abbott Laboratories in its behavior in TLC systems. The TRH was iodinated with $Na^{125}I$ (Isoserve) by the Hunter and Greenwood (*9*) method for iodination of proteins modified slightly (*10*). A fraction of the iodinated material separated by Sephadex chromatography on G-10 had identical mobility on TLC with unlabeled TRH. Protein in the column fractions was determined by the Folin-Lowry method (*11*).

Radioactivity of ^{14}C and ^{3}H in column fractions was determined by counting: 200 μl portions were placed in glass vials containing 1 ml of NCS solubilizer and 10 ml of toluene-based counting solution. The radioactivity was expressed as disintegrations per minute per 200 μl.

Attempts to separate [^{14}C]proline–labeled peptides from unreacted amino acids on Sephadex G-10 or G-25 were unsuccessful because of overlap between incorporated and unreacted proline. For this reason, TLC was used for separation of labeled amino acids from polypetides. Initial study showed that methanol extracts of rat hypothalami could be chromatographed directly on thin-layer sheets. Hypothalamic, neural lobe, and cerebral cortex fragments were incubated with [^{14}C]proline (20 μc/ml); the methanol extracts to which was added 25 μg of synthetic TRH as a marker, were subjected to two-dimensional TLC, with the use of the following solvent systems: chloroform, methanol, acetic acid (60 : 40 : 20); and chloroform, methanol, concentrated ammonia (60 : 45 : 20) (*4*). Radioautographs were prepared by exposure to Kodak medical x-ray film BB-54 for 2 weeks, and the positions of radioactive areas were compared with the Pauly reagent reactive spot. A radioactive area that corresponded to TRH was noted in extracts of incubated stalk median eminence, ventral hypothalamus, and dorsal hypothalamus. No coincident Pauly-positive radioactive area was observed in extracts of incubated neural lobe or cerebral cortex.

In none of the tissues did the radioactive area correspond exclusively with TRH, thus indicating the presence of other biosynthesized polypeptides. We assumed that the failure to demonstrate an amino acid in association with the TRH marker could be used to exclude that amino acid from consideration as a precursor of TRH.

A systematic examination was then made of the pattern of polypeptide incorporation into hypothalamic fragments of 19 naturally occurring amino acids with the use of the two-dimensional system described above. The various amino acids were separately incubated with dissected stalk median eminence, ventral hypothalamus, and dorsal hypothalamus. In each of these control experiments tissue from two rats was used, and each amino acid was tested in three separate incubations. Proline, histidine, and glutamic acid (the three probable precursors of TRH) and four other amino acids (alanine, glycine, serine, and threonine) were incorporated into peptides that had R_F values similar to or identical with TRH (although not exclusively so). Not one of the other amino acids tested was found in the TRH region and they could, therefore, be disregarded from consideration as being precursors of TRH. Each of the three parts of the hypothalamus showed the same pattern of incorporated amino acids.

Paper electrophoresis of methanol extracts of dorsal hypothalami incubated separately in each of 19 naturally occurring amino acids was carried out. Radioactivity was localized by scan-

ning, and TRH standards were localized by the Pauly reaction and also by the distribution of [^{125}I]TRH. Proline, histidine, glutamic acid, and glycine alone were incorporated into peptides which coincided with TRH. All the others were separate from TRH. Double-labeled extracts similarly separated are illustrated in Fig. 1.

Further proof of the precursor specificity of proline, histidine, and glutamic acid to TRH was sought by CM-cellulose ion exchange chromatography. Hypothalami were incubated in the seven amino acids previously shown by TLC to be associated with the TRH region. Methanol extracts were subjected to CM-cellulose chromatography on a column (2.5 by 45 cm) equilibrated with 0.002*M*, *p*H 4.6, and eluted with a gradient to 0.1*M*, *p*H 7.0, ammonium acetate buffer. The fractions that contained TRH were shown by distribution of ^{125}I-labeled TRH to be located in tubes 42 to 50. Peptides containing each of the seven listed amino acids appeared in several peaks after chromatographic separation, but a common peak of proline, glutamic acid, and histidine was found only in tubes 42 to 50.

To study further the anatomical location of TRH-synthesizing regions of the hypothalamus, extracts of stalk median eminence, ventral hypothalamus, and dorsal hypothalamus were chromatographed on CM-cellulose. Each of the regions showed incorporation of proline, glutamic acid, and histidine into the tubes that corresponded to the distribution of [^{125}I]TRH.

Thus rat hypothalamic fragments incubated in buffer solutions synthesize a peptide from glutamic acid, proline, and histidine; this peptide has characteristics identical with synthetic TRH in three chromatographic separation systems. Our findings support the proposition of Schally, Guillemin, and their collaborators (*1–5*) that TRH is a biologically significant product synthesized in the hypothalamus and suggest, moreover, that rat TRH is similar to both porcine and bovine TRH. Because the biosynthetic material was extracted by methanol under mild conditions, it is probable that the pyroglutamic acid group is present as such in native TRH. These observations imply that hypothalamic tissues contain a glutamic acid cyclase that alters the glutamic acid either before or after its incorporation into TRH. This conclusion is reinforced by the finding that labeled L-pyroglutamic acid is not incorporated into TRH (*10*). The TRH-synthesizing system is most probably enzymatic in that we have found activity in a soluble fraction extracted from hypothalamic tissue (*10*). The material is synthesized by the three parts of the hypothalamus tested: stalk median eminence, ventral hypothalamus, and dorsal hypothalamus, but not in the neurohypophysis or in the cerebral cortex. The relatively wide distribution of TRH-synthesizing tissue corresponds to the hypophysiotropic area of Halasz *et al.* (*12*) and provides further corroborative evidence of Guillemin's statement that TRH is distributed throughout the hypothalamus without special distribution to the stalk median eminence (*1*). It is important to recognize that regions of the hypothalamus electrically excitable for TSH release are also widely distributed throughout the same region, bounded by the preoptic nucleus anteriorly, the paraventricular nucleus superiorly, the anterior margin of the mammilary nuclei posteriorly, and the median eminence inferiorly (*13*).

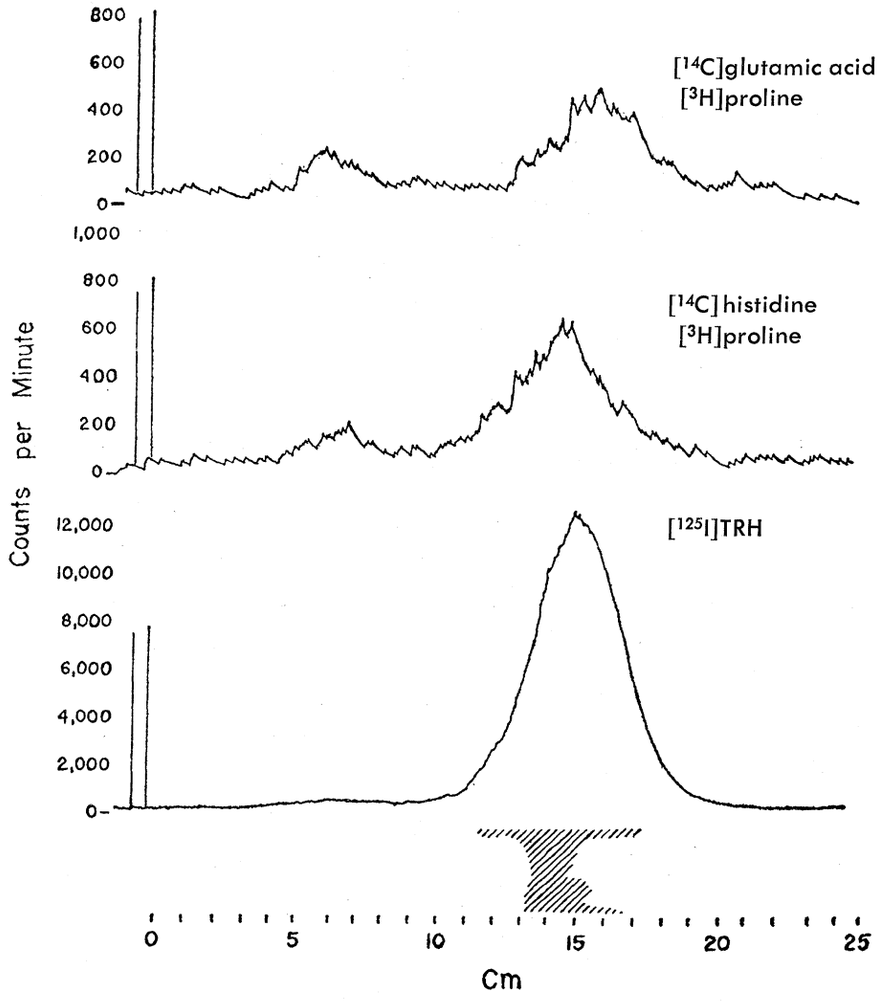

Fig. 1. Paper electrophoresis of synthetic TRH (shown at the bottom as a hatched area corresponding to Pauly-positive material), of ^{125}I-labeled TRH, and of methanol extracts of hypothalamic tissue that had been incubated for 2 hours in buffer solution containing [^{14}C]glutamic acid and [^{3}H]proline or [^{14}C]histidine and [^{3}H]proline. The Pauly-positive material coincides with the ^{125}I-labeled TRH and with the principal peak of the labeled amino acids. Unreacted amino acids migrate to distinctly different regions of the electrophoretogram (not illustrated).

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Diisopropylphosphorofluoridate and Tabun: Enzymatic Hydrolysis and Nerve Function

Abstract. *Squid nerve contains an enzyme that hydrolyzes the nerve gas Tabun at about one-tenth the rate it hydrolyzes diisopropylphosphorofluoridate (DFP), and at about one-third to one-fourth the rate it hydrolyzes Sarin and Soman. Tabun is a more potent inhibitor of acetylcholinesterase than is DFP, is both lipid- and water-soluble, and penetrates readily into the squid giant axon in its inhibitory form. The failure of Tabun to block or markedly decrease the conducted action potential in the squid axon makes it likely that the blocking of conduction caused by DFP is probably not due to inhibition of acetylcholinesterase. Substrate specificity with regard to organophosphate metabolism by squid enzyme has possible implications for the disposal and detoxication of nerve gases in the ocean.*

The use of potent, relatively specific, and essentially irreversible inhibitors of acetylcholinesterase for the study of nerve function is subject to several ambiguities (*1*). For example, some inhibitors, while capable of penetrating into axons in their inhibitory form (*2*) have oil-water partition coefficients of about 0.1 (*3*). It has been suggested (*4*) that such compounds may be incapable of penetrating into all of the substructure of excitable membranes, thereby adding to the uncertainty in assigning reasons for unexpected (especially lack of) pharmacological responses. Some inhibitors are enzymatically detoxified. Thus the finding (*5*) of a high level of diisopropylphosphorofluoridate-hydrolyzing enzyme in the giant axon of the squid *Loligo pealii* has been considered as a possible explanation for the high concentration of DFP required to block conduction relative to the concentration which inhibits a solution of acetylcholinesterase. The squid head ganglion is an even richer source of the DFP-hydrolyzing enzyme than the giant axon (*6*) and is easier to obtain in quantity. During the collection of ganglia in preparation for a purification of the DFP-hydrolyzing enzyme, experiments have been performed which bear on enzyme specificity, on nerve function, and, to a more limited extent, on a matter of recent public concern, namely, the disposal of toxic compounds in the ocean.

In view of the more rapid hydrolysis of the nerve gas (so-called) ethyl *N,N*-dimethylphosphoramidocyanidate (Tabun; GA) than of DFP by various mammalian serums (*7*) and of similar data published for isopropyl methylphosphonofluoridate (Sarin; GB) and for 3,3-dimethyl-2-butyl methylphosphonofluoridate (Soman; GD), these compounds were tested as possible alternate substrates for the DFP-hydrolyzing enzyme. The DFP was obtained commercially. Tabun was synthesized according to Holmstedt (*8*); Sarin and Soman, essentially according to Reesor *et al.* (*9*). Partition of Tabun and DFP between olive oil and water was determined by measuring the inhibitor concentrations in the aqueous phase, with a standard solution of *Electrophorus* acetylcholinesterase as the test agent. Penetration of Tabun into squid giant axons was measured as described for other compounds (*2*). Enzymatic hydrolysis of the organophosphorus agents and of acetylcholine was measured titrimetrically or manometrically, the choice usually depending on considerations of sensitivity and safety. Corrections for nonenzymatic hydrolysis were made in the usual way. Dissection of axons, external recording of electrical activity, and extrusion of axoplasm were performed as previously described (*2*).

Table 1 shows that squid nerve, and especially head ganglion, hydrolyzes DFP about nine times more rapidly than it hydrolyzes Tabun, whereas there is a sixfold difference in the opposite direction for rat serum. This latter order has also been reported for rabbit plasma and a fraction of human serum (*7*). Another difference has already been noted between squid nerve DFP-hydrolyzing enzyme and that from many other sources, namely, a lack of Mn^{2+} stimulation in the former (*6*). The Michaelis constant (K_m) for DFP hydrolysis by squid head ganglion is estimated at $6.25 \times 10^{-3} M$, with a maximum velocity of approximately 1280 μmole of DFP hydrolyzed per gram of fresh tissue per hour. This should be regarded as an apparent K_m inasmuch as a crude homogenate is, so far, the enzyme source. Figure 1 shows the relative rates of hydrolysis of DFP, of Tabun, and of mixtures of the two by identical amounts of head ganglion enzyme. The enzymatic hydrolysis of the mixture in terms of CO_2 released from bicarbonate buffer is equal, within the limits of the standard deviations, to the mean for the separate hydrolyses. This suggests that squid nerve contains a single enzyme that

Table 1. Enzymatic hydrolysis of DFP and Tabun. Values obtained from squid and rat were determined manometrically in 3.0 ml of a solution of the following molar concentrations: NaCl, 0.16; KCl, 0.005; $NaHCO_3$, 0.0165. Gas phase: 95 percent N_2, 5 percent CO_2; temperature, 30°C. Nerve was homogenized in this medium to provide 10 to 18 mg of tissue per Warburg vessel or 0.1 to 0.2 ml of rat serum per vessel. Substrate concentration, 0.01*M*. Other values were determined under similar conditions. Results are expressed as micromoles of substrate hydrolyzed per hour per gram of nerve or milliliter of serum ± standard deviation.

Enzyme source	DFP	Tabun
Squid axon	80–120	< 10
Squid stellate ganglion	394 ± 4	
Squid head ganglion	750 ± 9	83 ± 18
Rat serum	12 ± 1	71 ± 6
Rabbit plasma*	80	281
Human serum, fraction IV*	80†	205‡

* See (*7*). † Arbitrary. ‡ Relative to DFP = 80.