Isolation and Properties of Porcine Thyrotropin-releasing Hormone*

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SUMMARY

Isolation on a preparative scale of thyrotropin-releasing hormone from porcine hypothalami is described. The yields from two batches of 100,000 and 165,000 hypothalami were 2.8 mg and 4.4 mg, respectively. The material with thyrotropin-releasing hormone activity, calculated to be purified about 570,000 times, was active in mice at doses of 1 ng in vivo, and stimulated the secretion of thyroid-stimulating hormone from rat pituitaries in vitro at doses of 10 pg. Thin layer chromatography, electrophoresis, and paper chromatography indicated that the material was homogeneous. Histidine, glutamic acid, and proline, which were present in an equimolar ratio, accounted for about 32% of the dry weight of the active material. However, eight synthetic peptides containing histidine, proline, and glutamic acid or glutamine had no thyrotropin-releasing hormone activity at doses as large as 10 pg. Biological activity of thyrotropin-releasing hormone was abolished by diazotized sulfanilic acid, N-ethylmaleimide, or acid hydrolysis, but was not affected by periodate or by incubation with proteolytic enzymes.

There is now convincing evidence that the hypothalamus controls the secretion of thyrotropic hormone from the anterior pituitary gland by a neurohormone designated thyrotropin-releasing hormone (TRH) (1-4). The case for the existence of control of thyroid-stimulating hormone release by a hypothalamic substance (TRH) was built upon the results of physiological experiments reviewed recently by Reichlin (3) and Schally (4) as well as recent biochemical studies (1, 5-7). The strongest support for the concept of a TRH is offered by the fact that very potent TRH preparations were obtained from hypothalami of sheep (5, 6, 8), cattle (1, 9), and pigs (1, 10). Recently, our laboratories obtained conclusive evidence that porcine TRH stimulates TSH release in man (16).

This paper is concerned both with the isolation of TRH from porcine hypothalami and with some of its chemical properties. Isolation of TRH from two different batches of hypothalami was accomplished by essentially the same procedure on two separate occasions. A brief description of some of our results with the first batch of TRH has been reported already (1, 10). However, in the interest of completeness, the isolation process is described in detail here, as well as modifications introduced during preparation of the second batch of TRH. The description of the methodology used is important in view of the difficulties and small yields reported for the isolation of ovine TRH (6, 8, 11).

EXPERIMENTAL PROCEDURE

Dissection and Extraction—Fragments of the ventral hypothalami of pigs, consisting mainly of the pituitary stalk and median eminence, were dissected 20 to 30 min after death, frozen on Dry Ice, and lyophilized. This operation was carried out in its entirety by the staff of Oscar Mayer and Company, Madison, Wisconsin. Lyophilized hypothalami (dry weight, 11 to 34 mg each) were canned under nitrogen and shipped to our laboratory on Dry Ice, where the hypothalami in batches of 20,000 to 40,000 were pulverized on Dry Ice. The powder was defatted by stirring for 5 hours with 5 to 6 liters of acetone. It was then collected by filtration through the Whatman No. 541 filter paper and washed with 4 to 5 liters of acetone and 4 to 5 liters of petroleum ether (40-60°). The defatted powder was extracted four to five times with 2 N acetic acid at 8°C, with 1.2 to 1.5 liters of solvents for each extract. The extracts were centrifuged at 15,000 x g; the supernatants were heated to boiling and then chilled. Occasionally a recentrifugation at 20,000 to 50,000 x g was used to remove a fine suspension which sometimes resulted after repeated grinding. The supernatants were then lyophilized and concentrated by reextraction with 3 to 4 liters of glacial acetic acid divided into four to six portions (17). This concentrate was diluted with glass-distilled water and lyophilized again. The details of this extraction procedure have been described previously (17).

Gel Filtration on Sephadex—This was performed as described previously (17, 18) except that large columns were used.
For processing the concentrate from Batch I, a column 170 cm was used and a larger column (15.5 x 180 cm) with a hold-up volume of 11.8 l was utilized during purification of Batch II. This column was constructed by Pharmacia and had only glass and plastic parts in contact with the eluant, the gel, and the effluent. The columns were operated by gravity and the hydrostatic head required was only 1.5 m.

**Phenol Extraction**—This step was carried out essentially as described previously (8). The fractions with TRH activity from Sephadex were dissolved in distilled water saturated with hydrogen sulfide, and extracted three to five times with redistilled phenol, rather than twice (9). The TRH active materials were recovered from phenol by re-extraction into the aqueous phase, after the addition of several volumes of redistilled diethyl ether.

**Chromatography, Countercurrent Distribution, and Electrophoresis**—The procedure for ion exchange chromatography on carboxymethyl cellulose has been described previously (9).

Countercurrent distribution was carried out in automatic all glass apparatus (H. O. Post Scientific Company). Both a model B-3, 200 cells with 10 ml in each phase and a model C-2, 100 cells with 50 ml capacity in each phase, were utilized. The system consisted of 0.1% acetic acid-1-butanol-pyridine (11:5:3) as recommended by Craig (19) and utilized previously by Lo, Dixon, and Li (20). 1-Butanol was used for countercurrent distribution and partition chromatography (see below) was treated with zinc powder and redistilled. Similarly, pyridine was treated with sodium hydroxide pellets and redistilled. The water used in all of the experiments was triple distilled in glass.

After the countercurrent distribution, the materials were recovered from the upper phase by adding 2 to 3 volumes of redistilled benzene in order to displace all of the materials into lower phase, which was then flash-evaporated to a small volume and lyophilized.

Free flow electrophoresis was carried out in an Elphor FF continuous electrophoretic separator (Brinkmann) as described previously (21). Pyridine acetate buffer, pH 6.3 (0.37 M pyridine, 0.035 M acetic acid), was used in the separation chamber, while the buffer for washing the electrodes consisted of 0.93 M pyridine and 0.083 M acetic acid. The dosing speed was about 2.7 to 3 ml per hour and the buffer flow was approximately 50 ml per hour. Other conditions are given in the legend to Fig. 5.

Partition chromatography was carried out on columns of Sephadex G-25 (fine beads) as described by Yamashiro (22). Columns initially packed in 0.2 n acetic acid were first equilibrated with the lower phase of a system of 0.1% acetic acid-1-butanol-pyridine (11:5:3, v/v) or 1-butanol-acetic acid-water (4:1:5) and then with the upper phase of the same system before application of material. The material was dissolved in the upper phase for application on the column. An attempt was made to control the temperature within a 1° range. A specially designed dephlegmator was used to remove the bottom phase, which splits out of the upper phase after equilibration and separation. After 15 to 20 hold-up volumes were passed through the column, the uneluted material was displaced with 0.2 n acetic acid-pyridine (100:70, v/v) (22). The RF values were calculated as suggested by Yamashiro (22).

Adsorption on charcoal was carried out essentially by the method of Guillenin **et al.** (8). Norit, Grade A, alkaline charcoal, acid-washed (Pfanstiehl Laboratories, Waukegan, Illinois), was washed with 2 n acetic acid, water, and redistilled absolute ethanol and dried under reduced pressure before use. Micro-columns of charcoal were packed in 5 ml volumetric pipettes, Normax-K A No. 6588 (0.5-cm inside diameter), cut at the 3-mI mark and provided with a glass wool plug at the tip. These were connected through Nalgan tubing (P73-223) with a glass reservoir and a pressure-regulating device (1-13-A-Azet, Victor Equipment Company, San Francisco, California). Nalgan tubing was soaked in 0.1 M acetic acid and 99.5% ethanol for 1 week before use. However, a few months after this treatment, some cracks formed in the wall of tubing and additional amounts of soluble material could be washed out with 50% ethanol. The columns were operated under a pressure of 5 to 18 psi to maintain a flow rate of 4 to 10 ml per hour. The material was applied in 0.01 M acetic acid and the elution of TRH was carried out in stepwise fashion with 30%, 50%, and 99.5% ethanol, rather than by the use of gradient (8). In some cases when the use of too much charcoal resulted in poor recovery of TRH, the adsorbed TRH was displaced by a mixture of phenol-pyridine-ethanol (20:20:3, v/v). The separation pattern in stages prior to the second partition chromatography was followed by the Folin-Lowry reaction (23) or by optical density readings at 278 mμ.

**Homogeneity and Composition Tests**—Thin layer chromatography and electrophoresis were carried out on plates of cellulose MN 300 HR (brinkmann), with De-Saga Brinkmann thin layer chromatography and thin layer electrophoresis equipment for the separations. The spots were visualized by spraying with diazotized sulfanilic acid (Pauly’s reagent) or with chlorine-o-tolidine reagent (24). At times, the spots were visualized by exposing the paper or thin layer chromatographic plates to iodine vapor (25). Paper chromatography was carried out on washed Whatman No. 52 or No. 54 filter paper.

Attempts to find an NH₂-terminal residue were performed by the use of Dansyl chloride (1-dimethylaminonaphthalene-5-sulfonylchloride) (26, 27). An attempt to detect the COOH-terminal residue was carried out by the method of Netkov and Genov (28). Amino acid analyses were performed in an automatic Spinco-Beckman model 120-B analyzer provided with microcuvettes and a 1-mv range card, after hydrolysis of the sample in 6 n hydrochloric acid for 22 hours at 110° under reduced pressure.

**Inactivation Experiments**—The reaction with Pauly’s reagent was carried out as follows (29). Diazotized sulfanilic acid solution (5 μl of 1% sulfanilic acid in n HCl plus 5 μl of 0.7% aqueous sodium nitrite) was added to a solution of 50 ng of TRH in 0.01 ml of 2% acetic acid and allowed to stand for 30 min at which time 0.01 ml of 5% Na₂CO₃ solution was added. The reaction mixture was brought to final volume so as to give ε final concentration of both Na₂CO₃ and diazo reagent of less than 0.0003 M. This solution was subsequently injected into assay mice.

The reaction with N-bromosuccinimido was performed as follows (30): 0.05 M N-bromosuccinimide (recrystallized before use) was made up in 0.01 M phosphate buffer, pH 7.4, and 0.2 mI of TRH (24) and 0.4 mI of 0.01 M of 2% acetic acid was added to 80 ng of TRH in 0.01 ml of 2% acetic acid. The solution was allowed to react for 1 hour at 56°, after which

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3 We are grateful to Dr. R. Burgus, Department of Physiology, Baylor University College of Medicine, Houston, Texas, for advice on the use of this method.
time the solution was diluted to 1.5 ml with 0.9% NaCl solution for assay in mice.

TRH was also reacted with resorcinol reagent (1% resorcinol in 8 M HCl) (31). HCl, 0.05 ml concentrated, and 0.005 ml of resorcinol reagent were added to 80 ng of TRH in 0.01 ml of 2% acetic acid. This solution was then heated at 56° for 2 hours, dried over NaOH under reduced pressure, and taken up in 0.9% NaCl solution and neutralized for the assay.

TRH was subjected to periodate oxidation under the following conditions (32). Sodium metaperiodate (Baker) (0.05 ml of 0.01 m solution) was added to 100 ng of TRH in 0.02 ml 2% acetic acid and allowed to stand for 2 hours, the final pH being 4.5. After this time a 5-fold excess of glucose was added in 2 μl of water to stop the reaction. After 15 min the solution was diluted to 2 ml and assayed for TRH. High concentrations of periodate seemed to interfere with the TRH assay, but 0.001 m periodate did not influence the response appreciably. The attempts to acetylate TRH were carried out at 37° by mixing equal volumes (50 to 200 μl) of redistilled pyridine and acetic anhydride with TRH. After 2 hours, fresh portions of both reagents were added and the reaction was allowed to proceed overnight. After the reaction, the mixture was taken down to dryness, and then redissolved in water and re-evaporated several times.

Digestion with neuraminidase (Clostridium perfringens, EC 3.2.1.19, splits off N-acetyl neuraminic acid and a variety of glycoproteins) was performed as follows (33). Neuraminidase (General Biochemicals), 1 μl (500 milliunits), was added to 100 ng of TRH, followed by 0.10 ml of pH 5.8 acetate buffer and 3 or 19 hours. In all three cases the enzyme to substrate ratio was 1:10 and the incubation temperature was 37°.

Inactivation of TRH by plasma was carried out by adding TRH to rat plasma and incubating at 37° for 15 to 30 min (34).

Nuclear magnetic resonance spectroscopy was carried out on 1.0 mg of TRH Preparation I and 7.1 mg of TRH Preparation II in pyridine or D2O (Bio-Rad, 99.9% pure, redistilled), with a Varian HA-100 instrument. Mass spectra were determined in a XB 9000 spectrometer.

RESULTS AND COMMENTS

Two preparations of TRH from porcine hypothalami were processed. The steps in the extraction and purification procedure (1, 17) of these SME fragments as well as the yields and activities after each step are summarized in Table I. The reason for the higher relative yield of 2 N acetic acid extract (6.5 mg per SME) in Preparation II with respect to Preparation I (5.2 mg per SME) is that the extraction of Preparation I was done in smaller batches (10,000 to 25,000) of hypothalami. This permitted a better centrifugation of the extracts (see "Experimental Procedure"). The second TRH preparation was extracted in batches of 25,000 to 40,000 SME and the residue remaining after the fourth extraction with 2 N acetic acid was also ascertained by elevation of plasma TSH levels in thyroidectomized rats treated with T3 (36) or depletion of pituitary TSH content in mice (37). TRH activity in vitro was measured by stimulation of TSH release from isolated rat pituitaries (1, 14). TSH released in vitro also was measured by the McKenzie assay (36).

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FIG. 1. Gel filtration of pig TRH concentrate (45 g) on Sephadex G-25 column, 15.5 X 180 cm. Solvent, 1 M acetic acid. Fraction size, 25 ml. Bioassays were carried out on 200-μl aliquots. Vertical bars indicate ±S.E.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Change in blood TSH levels at 2 hr</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl solution</td>
<td>+23 ± 22</td>
<td>0.01</td>
</tr>
<tr>
<td>Extract 1</td>
<td>+3298 ± 770</td>
<td>0.01</td>
</tr>
<tr>
<td>Extract 2</td>
<td>+473 ± 78</td>
<td>0.01</td>
</tr>
<tr>
<td>Extract 3</td>
<td>+2776 ± 507</td>
<td>0.01</td>
</tr>
<tr>
<td>Residue 1</td>
<td>+457 ± 228</td>
<td>0.01</td>
</tr>
<tr>
<td>Residue 1</td>
<td>+708 ± 354</td>
<td>0.05</td>
</tr>
<tr>
<td>Residue 2</td>
<td>+540 ± 315</td>
<td>NS*</td>
</tr>
<tr>
<td>Residue 2</td>
<td>+160 ± 262</td>
<td>NS</td>
</tr>
<tr>
<td>Residue 3</td>
<td>-200 ± 140</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Not significant.

The pattern of separation which occurred on the column 15.5 X 180 cm can be seen in Fig. 1. The effluents from 12 identical columns were combined into 13 areas and lyophilized. The main TRH activity emerged in Fractions 913 to 1082 (average Rf = 0.48; Fig. 1), but there was also some TRH activity in Fractions 833 to 912. Fractions 913 to 992 contained some growth hormone-releasing hormone activity; consequently the 90 g of material corresponding to Fractions 673 to 992 were subjected to rechromatography on the same large column of Sephadex in two batches of 45 g each. After rechromatography, TRH activity began to emerge in Fraction 920 (Table II, Experiment 2), and was completely separated from growth hormone-releasing hormone, which was eluted in Fractions 750 to 900 (40). Fractions 911 to 1260 from rechromatography on Sephadex were lyophilized, yielding 29 g of material. This material was combined with the other batches for phenol extraction.

Phenol Extraction—TRH fractions from the Sephadex contained luteinizing hormone-releasing hormone activity located in Fractions 913 to 1200 and follicle-stimulating hormone-releasing hormone activity in Fractions 993 to 1260. Since these fractions had to be desalted and chromatographed on CM-cellulose under identical conditions, they were combined for phenol extraction. Thus 380 g of material from Sephadex (Preparation II) were subjected to phenol extraction in three batches, each batch being extracted with about 800 ml of phenol divided into five portions. After recovery of the phenol soluble materials, 83 g of extract were obtained. The TRH activity of the phenol extract is shown in Table II. The residual of Batch I showed some TRH activity at doses 20 to 100 times greater than Extract I, but the residues of Batch II and III were inactive at doses 20 to 33 times greater than those for Extrac I or III. It was estimated that 281 g of residue contained less than 1% of total TRH activity.

Similar results were obtained with Preparation I from Sephadex, 20 g of extracts being obtained, which released TSH at doses of 50 μg. The residue (80 g) of Preparation I was inactive at doses of 100 μg (1, 10).
CM-cellulose Chromatography—The phenol extract of preparation II was subjected to ion exchange chromatography on CM-cellulose columns (2.8 × 60 cm or 2.8 × 80 cm) in batches of 5.7 to 8.0 g. Thirteen chromatographic runs were necessary to process the 83 g of the phenol extract. The location of TRH activity in aliquots of effluent from the shorter CM-cellulose column (2.8 × 60 cm) is shown in Fig. 2. It can be seen that chromatography on CM-cellulose completely separated TRH from luteinizing hormone-releasing hormone, follicle-stimulating hormone-releasing hormone, and lysine vasopressin. TRH activity was eluted in Fractions 54 to 87, with a conductivity of 800 to 1700 µmhos. On the longer CM-cellulose column, TRH activity was correspondingly shifted to the right, which is to be expected, owing to prolongation of the ion exchange processes. The fractions with TRH activity from all of the columns were combined, lyophilized, and reasayed for TRH activity, this time on the basis of an accurate dry weight. The results of these tests indicated that Fractions 53 to 75 (5.0 g) were active at doses of 5 μg and Fractions 76 to 92 (11.6 g) were active at doses of 1 μg. It was estimated that Fractions 9 to 40 (52.1 g) and 41 to 52 (32.6 g) contained about 1% and Fractions 3 to 115 (37.3 g) about 5% of the total TRH activity.

The material from TRH Preparation I was similarly processed a batch of about 2.5 g each (1, 10). About 2.1 g of material were obtained which was active in TRH bioassays at doses of 0.3 μg (1, 10).

Countercurrent Distribution—Fractions 53 to 75 and 76 to 2 from CM-cellulose (total 16.6 g) of Preparation II were subjected to 200 transfers by countercurrent distribution in a system of 0.1% acetic acid-1-butanol-pyridine (11:5:3 v/v). The number of transfers was 200. Peptide analyses were carried out on 50-μl aliquots lower phase. The bioassay data shown are based on responses to 1 μg of material.

The profile of separation, as followed by the Folin-Lowry reaction, and the location of TRH activity, are shown in Fig. 3.

TRH activity appeared to be concentrated in Fractions 44 to 63. After recovery of TRH with benzene (displacement of TRH activity into the aqueous layer by the addition of benzene, followed by lyophilization), 4.98 g of solids were obtained from Fractions 43 to 63 (mean partition coefficient K = 0.36). This material was now active in vivo in doses of 0.33 μg dry weight. TRH Preparation 1, 2.1 g, from CM-cellulose was similarly subjected to countercurrent distribution in the same system but with the model B-3 (10 ml of each phase). In both of these countercurrent distribution experiments about 1 g of material was subjected to 400 transfers. The results are seen in Fig. 4. TRH activity was located in Fractions 100 to 130 with K = 0.42. The yield of material obtained from both these countercurrent distribution runs was 800 mg. This material was active in vivo in doses of 0.2 to 1 μg (1, 10). It is not clear why a slightly lower partition coefficient (K = 0.36) for TRH activity...
resulted during the countercurrent distribution with the model C-2 apparatus as compared with values obtained in smaller scale countercurrent distribution experiments in the model B-3 for porcine TRH ($K = 0.42$ to 0.44) or ovine TRH ($K = 0.43$) (8, 11). In addition to the possibility that ammonium acetate or another material was incompletely removed by lyophilization of TRH Preparation II, which might have caused a change in the $K$ value, incomplete draining of both phases during the decantation and transfer stages has been noticed during operation of model C-2. This incomplete draining could not be eliminated since the timing of steps of decantation and transfer on each cycle is controlled by the robot mechanisms and the times for these steps could not be increased. The time allowed for draining on decantation and transfer on model A and B (8 to 10 ml each phase) is more than adequate.

**Free Flow Electrophoresis**—Of the TRH concentrate obtained by countercurrent distribution of Preparation II, 4.98 g were dissolved in 75 ml of pyridine-acetate buffer, pH 6.3, and subjected to free flow electrophoresis for 30 hours. The pattern of separation is seen in Fig. 5. The results of bioassays indicated that TRH activity migrated slightly toward the cathode and was found in tubes 27 to 37. After lyophilization of Fractions 27 to 38, 538 mg were obtained which had significant TRH activity in doses of 30 ng. By increasing the doses, higher responses were obtained. The TRH was concentrated in this step about 9.3 times.

A higher degree of purification was obtained when free flow electrophoresis was carried out on a smaller scale. When 800 mg of TRH Preparation I from countercurrent distribution were subjected to FFE under similar conditions as those shown in Fig. 5, 18 mg of TRH were obtained which were active in doses 5 to 30 ng (1, 10). Thus the purification factor for a small scale free flow electrophoresis was 45-fold. A poorer separation when free flow electrophoresis is performed on a large scale is due to incomplete separation of the TRH area from a large peak (tubes 18 to 26, Fig. 5), which contained most of the inactive material (over 4.3 g). In both experiments TRH activity migrated slightly toward the cathode, which would indicate a positive charge, in agreement with its behavior to Cm-cellulose.

**Partition Chromatography**—TRH Preparation II from free flow electrophoresis was subjected to partition chromatography on Sephadex G-25 in a system of 1-butanol-acetic acid-water (4:1:5). This was accomplished as follows. Material from free flow electrophoresis 538 mg was divided into two batches and each batch was applied on a partition column in 5-ml upper phase. The column was developed with the same phase. The pattern of separation and the location of biological activity can be seen in Fig. 6. TRH activity, as determined by injecting 0.5-ml aliquots of every tube, was located in Fractions 42 to 63. This area had an $R_p = 0.06$ to 0.1 and contained very little peptide as ascertained by the Folin-Lowry reaction. By combining the TRH active area from both columns, 46.9 mg of

*Calculated according to Yamashiro (22).
material were obtained. This material was active in doses of 10 ng.

The above material, 46.9 mg, was now subjected to partition chromatography on an analytical scale with another solvent system, consisting of 0.1% acetic acid-1-butanol-pyridine (11:5:3). Since the peptide content of this material was very low, peptide analyses were not carried out. Similarly the ultraviolet pattern could not be followed because of the pyridine. The distribution of biological activity in the fractions is shown in Fig. 7. After concentration by flash evaporation and lyophilization of tubes 48 to 67, 44.9 mg of material were obtained which was active in TRH assays in a dose of 12 pg. The \( R_f \) of TRH was approximately 0.2, in agreement with the observations of Burgus and Guillemin (41) on ovine TRH. This lack of significant purification on the second partition system may be due to the fact that the same solvent system was used for counter-current distribution.

When 18 mg of TRH Preparation I from free flow electrophoresis was purified by partition chromatography on an analytical column of Sephadex G-25 in a system of 1-butanol-acetic acid-water (4:1:5), the amount of TRH-active material obtained was 2.8 mg (1, 10). This material with an \( R_f \) of about 0.1 was essentially homogeneous by thin layer chromatography and electrophoresis (see below) and was active in vivo in 1-ng amounts (1, 10) and in vitro in doses of 10 pg (1, 14).

Some typical responses in vivo are shown in Table III.

### Charcoal Adsorption

- Of this highly purified, apparently homogeneous TRH Preparation I, 0.825 mg was subjected to charcoal adsorption on a charcoal column, 0.5 x 2.5 cm. The material was applied in 0.01 M acetic acid and the column was developed first with the same solvent. After 15 ml of 0.01 M acetic acid had passed, TRH was eluted stepwise with 30% alcohol. In the first 8-ml effluent, 500 pg of TRH were recovered active in 0.6- to 1-ng amounts (Table III). Elution with 50% alcohol led to recovery of an additional 300 pg of material with some TRH activity. Since no significant increase in potency resulted (Table III), it is not known whether adsorption on charcoal of Batch I of TRH led to any significant purification.

Since the large scale Preparation II of TRH after the second partition step was still not pure, it was decided to repurify it by charcoal adsorption. TRH, 44.9 mg, was divided into two portions of about 24 mg and each portion was applied on a column, 0.5 x 4.8 cm. The columns were developed first with 0.01 M acetic acid (tubes 1 to 6), then 30% ethanol (tubes 7 to 21), 50% ethanol (tubes 22 to 40), 99.5% ethanol (tubes 41 to 59), and finally pyridine-phenol-ethanol (25:20:5). The TRH activity in the effluents was followed by assay in vivo in mice. As can be seen in Fig. 8, TRH activity emerged in both the 30% alcohol and the 50% alcohol fractions. This dual location of TRH was probably due to increased length of charcoal columns as compared with small scale experiment, since it was observed previously that in the case of bovine TRH, when several grams

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**Table III**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Dose (dry weight)</th>
<th>Change in blood ( ^{32} )I or ( ^{131} )I levels of mice treated with 0.005 ( \mu g ) of L-T4</th>
<th>( p ) versus control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
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<td>0.0</td>
<td>0.005</td>
</tr>
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<td>TRH Ia</td>
<td>1.0</td>
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<td>+16 ± 9.0</td>
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<td>0.01</td>
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<tr>
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<td>+17 ± 18.0</td>
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<td>0.01</td>
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<tr>
<td>TRH Ia</td>
<td>3.0</td>
<td>+290 ± 249.0</td>
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| a NaCl (0.9%) solution. |
| b Mice treated with codeine and 1.0 \( \mu g \) of thyroxine. |
| c From Schally et al. (1) (courtesy of Academic Press). |
| d Before charcoal rechromatography. |
| e After charcoal rechromatography. |
| f Not significant. |
| g After exposure to D20. |

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**Fig. 7.** Partition chromatography of 46.9 mg of porcine TRH on a column of Sephadex G-25, 0.9 x 76 cm. The solvent system consisted of upper phase of 1-butanol-0.1% acetic acid-pyridine = 11:5:3. Fraction size, 1.6 ml. H.U. volume, 20 ml. Aliquots, 0.16 ml, of every other tube were assayed for TRH.
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**Fig. 8.** Adsorption chromatography of 24 mg of TRH on charcoal column, 0.5 × 4.8 cm. At points indicated with arrows applied increasing concentrations of aqueous ethanol. Flow rate, 8 ml per hour. Fractions, 2 ml, were collected. Of original solution 0.2 ml was assayed for TRH activity in mice.

**Fig. 9.** Adsorption chromatography of 28.65 mg of TRH on charcoal column, 0.5 × 2.1 cm. At points indicated with arrows applied increasing concentrations of aqueous ethanol. Flow rate, 10 ml per hour. Fractions, 2 ml, were collected. Of original solution 0.2 ml was assayed for TRH activity in mice.

If charcoal were used, TRH could not be displaced even with 99.5% alcohol, but only with pyridine-phenol-ethanol (20:25:5). Fractions 9 to 39 from both experiments on TRH Preparation II were combined, flash-evaporated, and lyophilized, yielding 35 mg. The material looked oily and, since Fractions 40 to 58 yielded 236 mg, it was suspected that the plasticiser, butyl phthalate, was washed out from the Nalgene tubing, in spite of its prior treatment (see “Experimental Procedure”). The 35 mg of TRH active material were extracted with ether and then subjected to a vacuum of 10 μ, whereby the weight was reduced to 28 mg. This material had TRH activity in doses of 5 ng, whereas the ether washings were inactive in amounts of 40 to 2000 ng. In view of the relatively low specific activity of TRH, it was decided that further repurification was necessary. To 28 mg of TRH Preparation II from charcoal was added 0.65 mg of TRH Preparation I (partition chromatography step). A shorter column of charcoal, 0.5 × 2.1 cm was used, and this time most of the TRH activity was eluted in the 30% alcohol fraction in tubes 8 to 20 (Fig. 9) as ascertained by assaying 0.2-μl aliquots.

After lyophilization of Fractions 8 to 20, 15.1 mg of material

**Fig. 10.** Analytical gel filtration of 15.1 mg of TRH on Sephadex G-25, 0.9 × 147 cm. H.U. volume, 31 ml. Solvent 0.1 M pyridine acetate, pH 5.9. Applied 15.1 mg of TRH from the second charcoal adsorption. Flow rate, 5 ml per hour. Fraction size, 1.5 ml. Aliquots, 0.16 μl, were tested for TRH activity. The p values for TRH activity in Fractions 36 to 42 were 0.01.

**Fig. 11.** 1, thin layer chromatography of TRH Preparation I in 1-butanol-acetic acid-water, 4:1:5. Layer: MN cellulose 200 H.R., 0.25 mm. Rf = 0.52. 2, thin layer chromatography of TRH Preparation I in ethyl acetate-1-butanol-acetic acid-water, 1:1:1. Layer same as in 1. Rf = 0.74. 3, paper chromatography (descending) of TRH Preparation II in ethyl acetate-1-butanol-acetic acid-water, 1:1:1:1, on Whatman No. 54 filter paper. Rf = 0.52. 4, paper chromatography (ascending) of TRH Preparation II in acetone-water, 70:30, on Whatman No. 54 filter paper a 4°. Rf = 0.45. All of the spots were visualized with Pauly's reagent.
were obtained which was active in TRH assay in a dose of 3 ng.
Fractions 21 to 37 (1.3 mg) were inactive in 10-ng amounts, but
active in a relatively high dose of 50 ng. Other fractions were
inactive in doses as high as 100 ng.

Final Purification of TRH Preparation II—The main TRH
area from the second charcoal column (Fractions 8 to 20, 15.1
mg) was precipitated by analytical scale gel filtration on Sephadex
G-25. The distribution of activity in the eluents is shown in
Fig. 10. TRH activity emerged sharply in Fractions 36 to 42
(mean \( R_F = 0.51 \)). Lysophosphatidylcholine of this fraction yielded
7.1 mg of material.

Final purification of TRH Preparation II was carried out by
desalting paper chromatography on washed Whatman No. 54
filter paper in a system of acetone-water (70:30) at 4°C (see Fig.
11, 4). Marker spots were revealed by spraying with Paul's
reagent (24), and the TRH area (mean \( R_F = 0.75 \)), which paralleled the Pauly-positive spots, was eluted with 2 N acetic
acid. After lyophilization, 5.05 mg of TRH were obtained,
which was repeatedly active in mice at doses of 1 ng (Table III).

Under conditions in vitro, TRH Preparation I released TSH
from isolated rat pituitaries in doses of 10 pg (Table IV). Thus
it's activity was comparable to TRH Preparation I (Table V).
In addition, this TRH released \(^{131}I\) from thyroid glands of rats
(1), prepared as described by Yamazaki, Sakiz, and Guillemin
(42), depleted pituitary TSH content in mice (1, 39) and in-
creased incorporation of \(^{14}C\) amino acids into the rat pituitary
tissue (1). This material was purified about 570,000 times, like
TRH Preparation I (Table V). The biological activities of both
TRH preparations (Tables III, IV, and V) as well as their
chemical properties (see below) appeared to be identical.

Homogeneity Tests and Amino Acid Analyses—Thin layer
chromatography of porcine TRH (Preparations I and II) in four
solvent systems—\( 1, \text{butanol-acetic acid-water, } 4:1:5 \) (v/v) \( (R_F =
0.52) \); \( 2, 1\)-propanol-\( N\) acetic acid, \( 3:1 \) (v/v) \( (R_F =
0.48) \); \( 3, \text{ethyl acetate-butanol-acetic acid-water, } 1:1:1:1 \) (v/v)
\( (R_F = 0.71) \); \( 4, \text{acetone-water, } 2:1 \) (v/v) \( (R_F =
0.48) \)—revealed only one spot after exposure to iodine vapor or
spraying with Paul's reagent. After treatment with acetic anhydride in pyridine and paper chromatography
only one spot was revealed by iodine vapor or Paul's reagent.

After acid hydrolysis TRH was shown to contain histidine,
glutamic acid, and proline, which were present in an equimolar ratio and which accounted for about 32% of the dry
weight of TRH (Table V). Thin layer chromatography of TRH
in three solvent systems and thin layer electrophoresis did not
lead to any detectable changes in the amino acid content of
TRH. In each case TRH activity was associated with
Pauly-positive spots, yielding histidine, glutamic acid, and proline
on hydrolysis.

No free NH2-terminal or phenolic-OH groups could be de-
tected by the Dansyl method (26, 27). Also, no free COOH-
terminal residues could be shown by the Neldov-Genov method
(28) of hydrazinolysis. The reaction with Paul's reagent indicated the presence of the imidazole ring, which is probably
unsubstituted. A weak reaction of TRH with the chlorine-o-
tolidine reagent suggests the presence of some peptide bonds.
Nuclear magnetic resonance analysis of 7 mg of TRH in D2O
was carried out by Drs. G. Albers-Schonberg and B. Arison at
the Merck Institute for Therapeutic Research. The nuclear
magnetic resonance spectra, aside from residual acetic acid (2
moles), showed no obvious impurities.

Comparison of the signal areas for the imidazole protons of
TRH and of a histidine solution of known concentration indi-
cates a molecular weight of about 1000 per histidine moiety.
Of considerable interest is an ethyl group, causing a sharp
methyl triplet at 8.7 \( r_s \), which integrates, within the error of the
measurement, for 3 hydrogens relative to each of the low field

<table>
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<td><strong>Some properties of porcine thyrotropin-releasing hormone</strong></td>
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<td>Minimal active dose ( \text{in vivo} )</td>
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<td>Total amino acid content (% dry weight)</td>
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* After deducting 0.65 mg of material from TRH Preparation I.
histidine-CH. Because of the sharpness of the CH₃ triplet, only one such group and consequently only one histidine moiety are likely in TRH. The hormone would then have a molecular weight of about 1000 and not a multiple of this. However, there is still some doubt about the authenticity of the ethyl group as part of TRH. The CH₂ absorption, identified by spin decoupling at 6.4 ppm, indicates an N-ethyl group which is unlikely for biogenetic reasons. On the other hand, the unresolved character of the CH₂ signal must be due to a crowded or unsymmetrical molecular environment as one may find in material of biological origin.

The bulk of the absorption is interpretable by amino acid-like structures. The proline, glutamic acid, and histidine moieties can be clearly identified. Other signals could originate from N-alkylated amino acids which are anhydride-negative and therefore not necessarily detectable by Spino analysis.

**Effect of Various Treatments on Biological Activity of TRH**

- **Digestion of samples of TRH with pepsin, subtilisin, and Nagarse was found not to decrease the TRH activity, as assayed in mice.**
- We have previously shown that incubation of TRH with trypsin, chymotrypsin, pepsin, carboxypeptidase B, and aminopeptidase did not affect biological activity (1), in agreement with findings of Burgus (43). However, incubation of TRH with rat, porcine, bovine, and human serum at pI 7.4 abolished biological activity in 15 to 30 min (34), indicating the presence of an enzyme in plasma which inactivates TRH. Incubation with neuraminidase seemed to inactivate about one-half of TRH activity in 3 to 19 hours. Spraying with diazotized sulfanilic acid, and consistently caused about 80% to 95% loss of activity. Similarly, additional dialysis of sulfanilic acid or of A-bromosucinimide to TRH solutions caused complete inactivation. This indicates that an intact imidazole ring or a closely related structure is necessary for the full biological activity of TRH. Overnight hydrolysis with 6 N hydrochloric acid at 105° also caused complete inactivation of TRH. Periodate oxidation did not affect TRH activity; an observation indicating that two adjacent hydroxyls or a hydroxyl and an amino on adjacent carbons were absent (44). After treatment with resorcinol the TRH samples were inactive. However, since heating with hydrochloric acid is involved in this reaction, the inactivation may not be due to resorcinol itself. Overnight treatment with acetic anhydride in pyridine did not affect TRH activity.

**DISCUSSION**

The procedure reported briefly for the isolation of TRH from 100,000 mg hypothalamus (1, 10) is described in detail in this report. In addition, certain modifications in the processing of TRH from a second batch of 165,000 mg hypothalamus are reported in detail. Since this preparation involved a larger batch of hypothalamus, additional purification on partition columns, adsorption on charcoal, rechromatography on Sephadex, and paper chromatography were necessary before the material achieved a degree of biological potency and chemical purity observed for the first batch of TRH. In both cases, a relatively high yield of TRH was achieved. This yield was estimated to be about 75 times higher (7) than that reported for TRH from ovine hypothalamus (6, 8, 11). As the material was purified about 570,000 times, the specific biological activity increased proportionately. Because of contamination of starting materials with TSH and melanocyte-stimulating hormone it is difficult to compute the exact percentage of recovery. However, we determined previously that the glacial acetic acid extract of the 2 N acetic acid extract of hypothalamic tissue contains about 85% to 90% of the pressor, oxytocic, and MSH activities, as well as the bulk of the activity of hypothalamic releasing hormones (1, 7, 9, 17, 40). The purification methods used lead to no detectable inactivation and over 90% of the total TRH activity applied is recovered after each step. When bovine TRH was purified it was found to behave like porcine TRH during this isolation procedure (1). The porcine TRH consistently released TSH in mice at doses of 1 mg or less. It also stimulated secretion of TSH in doses of 10 mg systems based on short term incubation (1, 14) or in tissue cultures (15) of rat anterior pituitaries. In the latter work the “multiplier ratio” (TSH produced/TRH added) observed was 70,000 (15), as compared to a ratio of 200 to 2,000 times reported for studies (1, 14) utilizing short term incubation. Since the same material appeared to increase incorporation of labeled amino acids into pituitary tissue (1) and induced release as well as synthesis of TSH in tissue cultures (15), the same hormone (one substance) is probably responsible for the release as well as synthesis of TSH. The same preparation of porcine TRH also stimulated release of TSH from isolated slices of goat and sheep pituitaries. These studies in vitro clearly showed that TRH acts directly on anterior pituitary tissue. In addition to being active in vivo in mice (1, 12, 13, 35, 39), TRH stimulated secretion of TSH after intravenous administration in the albino rat (1, 38), in the nutria (Myocastor coypus Molina) (45) and in man (16). The criteria of TSH stimulation in man after administration of TRH were based both on radioimmunoassays as well as bioassays (16). Thus it appears that TRH is not species-specific in mammals. The biological effects described are presumably due to a substance with a molecular weight of about 1000 to 1200, which contains three amino acid-α part of the molecule. However, the estimation of molecular weight is only tentative, being based on behavior on Sephadex, content of histidine, glutamic acid, and proline, and measurement of absorption intensity of XMR spectra. Studies on the structure of TRH now being carried out utilizing ultraviolet, infrared, mass spectra, and nuclear magnetic resonance techniques have not yet revealed all the features of the structure of TRH. Mass spectra studies on undervolatilized TRH indicated formation of the histidyl-prolyl diketopiperazine as the only volatile pyrolysis product. This suggests a His-Pro or Pro-His linkage. Derivatives designed to mask polar groups in order to achieve higher volatility gave fragments of mass greater than 1000, but the pyrolysis was not substantially reduced. Our efforts therefore are now concentrated on identification of the products of partial acid hydrolysis and products of partial, base-catalyzed degradation. The structure of the moiety which forms about 68% of the structure of TRH is still unknown. Guillemain et al. (8) indicated that their preparation of ovine TRH had a low amino acid content (6.8%) and suggested that ovine TRH was not a polypeptide or at any rate not a simple polypeptide. This ovine TRH exhibited the same K on countercurrent distribution in 0.1% acetic acid-1-butanol-pyridine (11:5:3) and the same Rp on partition chromatography in the system above as well as in 1-butanol-acetic acid-water (4:1:5) as did porcine TRH (41).

1 T. W. Redding, A. Arimura, and A. V. Schally, unpublished data.
support of this view that TRH is not a simple polypeptide they showed that certain proteolytic enzymes did not inactivate TRH (43). Our own experiments with papain, subtilisin, and Nagerse substantiate this view. However, since both of our preparations of porcine TRH showed an amino acid content of about 32% and paper chromatography, electrophoresis, or charcoal adsorption did not cause detectable changes in this amino acid content, it is probable that histidine, proline, and glutamic acid are part of the molecule. In their latest report Lüscher and Guillaumin (41) revised their initial view and reported that ovine TRH contains 62 to 70% amino acid material with proline, glutamic acid, and histidine occurring in a molar ratio of 1:1:1 (41). A direct comparison of the potency of ovine TRH preparations and our porcine TRH was not carried out, but, since similar assays were utilized for assaying ovine and porcine TRH preparation, it appears that our preparation of porcine TRH may be 10 to 15 times more potent (7) than the ovine TRH of Guillaumin et al. (8, 41). It may be of interest to report that the following eight tripeptides synthesized by Merek Sharp and Dohme were inactive in vivo in 10-µg doses (i.e. at levels 10,000 times greater than those used for TRH): L-His-L-Glu-L-Pro; L-Pro-L-His-L-His; L-Glu-L-Pro-L-His; L-His-L-Pro-L-Glu; L-Pro-L-His-L-Glu; L-His-L-Pro-L-Gln; and L-Pro-L-His-L-Gln. This indicates that TRH is not a simple unsubstituted tripeptide of histidine, glutamic acid, and proline, and that some other moiety (or moieties) may be essential for biological activity. Although TRH is negative to ninhydrin and does not form a readily identifiable derivative with dansyl-chloride, the behavior on Cn-cellulose or electro- phoresis indicated that at pH 4.4 to 6.3 the TRH molecule has a slight positive charge. Lack of effect of propranolol may indicate that TRH lacks two hydroxyls or an amino and a hydroxyl group on adjacent carbon atoms (44). The conclusion that histidine is present as part of the molecule is reinforced by the inactivation of TRH by diazotized sulfanilic acid and N-bromosuccinimide (46). Since tryptophan and tyrosine are absent, both procedures would presumably attack only the imidazole ring of histidine.

In conclusion, a large body of evidence (1-16, 34, 35, 37-43) supports the existence of a hypothalamic neurohumor (TRH) which regulates TSH secretion from the anterior pituitary gland. From the results reported in this paper, it would appear that the isolation of porcine TRH has been achieved.

**Addendum**—The amino acid sequence of the peptide portion of TRH was established to be Glu-His-Pro. This was derived by the method of Gray and Hartley (27), after an initial cleavage with N-bromosuccinimide (30).

**Acknowledgments**—We are deeply grateful to Drs. F. Olson and R. Maas, Oscar Mayer and Company, Madison, Wisconsin, for generous gifts of porcine hypothalamus. We wish to acknowledge the technical assistance of Miss Edda Mareconi in the bioassays. The mass spectrophotometric and nuclear magnetic resonance studies were carried out by Dr. G. Albers-Schoenberg and Dr. B. Arison at Merek Institute for Therapeutic Research, Rahway, New Jersey. We thank Dr. F. W. Holly, Merek Sharp and Dohme, for supplying the eight synthetic tripeptides containing histidine, proline, and glutamic acid or glutamine. We are grateful to Dr. William Locke, Dr. Clyde Huggins, and Dr. William Cohen for editorial advice.

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