Purification of Hypothalamic Releasing Hormones[1] of Human Origin

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ABSTRACT. Acetic acid extracts of 339 defatted human hypothalami were subjected to a precipitation procedure and then to separation by several successive steps in order to purify and characterize human releasing hormones.[1] Growth hormone-releasing hormone (GH-RH) and FSH-releasing hormone (FSH-RH) were measured by stimulation of release of GH and FSH, respectively, from rat pituitaries *in vitro*. LH-releasing hormone (LH-RH) and thyrotropin-releasing hormone (TRH) were followed by *in vivo* assays. The TSH, GH, FSH and LH released were measured by bioassays. LH and FSH were also measured by radioimmunoassays. Gel filtration on Sephadex G-25 separated GH-RH from TRH, LH-RH and FSH-RH. GH-RH was further purified by free-flow electrophoresis and chromatography on carboxymethylcellulose (CMC). TRH, LH-RH and FSH-RH were concentrated by phenol extraction and then chromatographed on CMC, which separated TRH from LH-RH and FSH-RH. In the systems described, as well as others, the behavior of human hypothalamic releasing hormones was similar to or identical with that of hypothalamic releasing hormones from pigs and cattle. LH-RH and FSH-RH of human origin were also tested clinically in humans. It is concluded that hypothalamic releasing hormones are present in man and that their chemical characteristics (molecular weights, isoelectric points, etc.) are very similar to those of domestic animals. (*J Clin Endocr* **31**: 291, 1970)

STUDIES performed during the past few years have provided important information concerning the physiological and biochemical properties of some hypothalamic hormones[1] regulating anterior pituitary function (1–4). Recently we determined the structure of porcine thyrotropin-releasing hormone (TRH) to be (pyro)glu.his.pro.amide and carried out the synthesis of this peptide (5–7). The molecular structure of ovine TRH was shown to be identical with that of pig (8).

However, all these studies were carried out using extracts and materials purified from hypothalami of sheep, cattle and pigs. There is little information on the properties of releasing hormones obtained from hypothalami of man, except for the demonstration that crude hypothalamic extracts of human origin can release ACTH, TSH, LH, FSH and GH when tested in assay systems employing rats and mice (9). Although LH-releasing hormone (LH-RH) and TRH of porcine or ovine origin stimulate LH release (10, 11) and TSH release (12), respectively, in human subjects, tests in monkeys (13) and men (Kastin, Schally, Glick, Bowers and Gaul, unpublished) utilizing GH-releasing hormone (GH-RH) purified from porcine hypothalami have so far given only negative results. It would seem of interest to compare the effectiveness in man of GH-RH and other releasing hormones purified from hypothalami of humans with similar materials obtained from hypothalami of domestic animals. Thus, in conjunction with investigation of the biochemical and physiological characteristics of human hypothalamic releasing hormones, our studies were designed to provide small quantities of these purified materials for tests in human subjects.

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[1] These substances were formerly designated releasing factors. However, the nomenclature proposed by Schally *et al.* (1) will be used in this report.

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Materials and Methods

Human hypothalamic extracts. The ventral part of the hypothalamus between the optic chiasm and the mammillary bodies, including portions of the pituitary stalk, was removed from the brain during routine autopsies. These fragments weighed 400–1000 mg and were obtained from unselected males and females, most of whom had been 50–80 yr of age. There was a time lapse of 2–48 hr between death and excision of tissues, which after excision were kept frozen until lyophilized.[4] All 339 stalk-median eminence (SME) fragments were carefully lyophilized on a tray drying chamber (Model 10-MR-SA, Virtis & Co.). The mean dry weight after lyophilization was 140 mg/SME. These fragments were then defatted and extracted according to the following procedure, which was similar to that developed for porcine hypothalami (14, 15). The 339 lyophilized fragments, weighing 47.5 g, were carefully ground into a fine powder on dry ice using a mortar and pestle. A few crystals of EDTA were added to the tissues during the grinding. The powder was desiccated *in vacuo* and then stirred for 2 hr with 1 liter of acetone, filtered and washed on the filter with 1 liter of acetone and 1 liter petroleum ether (40–60 C) (14, 15). Traces of the solvents were removed *in vacuo*. The dry material was extracted with 2N acetic acid at 8 C using 5 portions of about 400 ml each (14, 15). Each extract was centrifuged at 15,000 $\times g$ for 60 min at 4 C, quickly heated to 95 C over 30 min, and rapidly chilled. The extracts were recentrifuged at 15,000 $\times g$ at 4 C for 1 hr. The combined extracts were lyophilized and concentrated by re-extraction with glacial acetic acid (14, 15). This was performed 5 times with portions of 250–400 ml acid. The combined glacial acetic acid extracts were diluted 4 times with glass distilled water and lyophilized.

Gel filtration on Sephadex G-25 (fine beads) was performed as described previously (15, 16) using a column 7.5 $\times$170 cm and 1M acetic acid as an eluant. R_f values for migration rates on Sephadex were calculated as suggested by Porath and Schally (16). They were based on the comparison of the migration rate of the hypothalamic material *vs.* the migration rate of a substance which is completely excluded from the column (16). The use of substances such as albumin, Blue Dextran 2000 or hemoglobin permits a convenient determination of the void volume (Vo) of Sephadex G-25 columns.

[4] Some hypothalamic fragments were shipped on dry ice from Cleveland to New Orleans, where lyophilization, extraction and purification took place.

Phenol extraction. This step was carried out essentially as described previously (15, 17). Fractions from Sephadex with TRH, LH-RH and FSH releasing hormone (FSH-RH) activity were lyophilized, dissolved in 200 ml distilled water saturated with hydrogen sulfide, and extracted 3 times with 100 ml redistilled phenol. The active materials were recovered from phenol by re-extraction into the aqueous phase, after the addition of 1500 ml of redistilled diethyl ether.

Chromatography on carboxymethylcellulose (CMC) was performed as described previously (15, 17, 18). Free-flow electrophoresis was performed in an Elphor FF continuous electrophoretic separator (Brinkman) as described previously (16, 18).

Thin layer chromatography (TLC) was carried out on plates of cellulose MN 300 HR (Brinkman) using a layer of 250 μ. TRH spots were revealed by spraying with diazotized sulfanilic acid (Pauly's reagent) (15).

Bioassays. The assays for pressor activity were carried out in the rat treated with Dibenzyline and urethane as recommended by Dekanski (19). Melanoycte-stimulating hormone (MSH) activity was determined *in vivo* in hypophysectomized frogs as described by Kastin and Ross (20). Tests for ACTH activity were performed in hypophysectomized rats using plasma corticosterone levels as an index of adrenocorticotropic activity (21).

TRH activity was measured *in vivo* by the release of ^{125}I from the thyroids of mice placed on a low iodine diet for 7-14 days and then given radioactive iodide and 0.08 μg triiodothyronine (22).

LH-RH activity was determined *in vivo* by stimulation of release of LH in ovariectomized rats pretreated with estrogen and progesterone (23). The details of this method, as carried out in our laboratories, were described previously (24). Plasma LH activity was measured by the OAAD assay (25) and/or by radioimmunoassay for rat LH (26).

FSH-RH activity was detected *in vitro* by measuring the stimulation of FSH release from the pituitaries of intact male rats (27). Ten pituitary halves were incubated for 6 hr in 10 ml Krebs-Ringer bicarbonate medium containing 200 mg/100 ml glucose (KRBG) (28). Released FSH in incubation medium was determined by the Steelman-Pohley assay (29) and/or by radioimmunoassay for rat FSH (30).

GH-RH activity was determined *in vitro* by stimulation of the release of GH from isolated rat pituitaries (18, 31). Ten pituitary halves were incubated in 5 ml KRBG for 7 hr after the

incubation. The medium was diluted to 10 ml with saline; 1.2 ml of this solution was injected per assay animal during 4 days. GH activity in incubation medium was measured by the "tibia test" (32); 6–7 hypophysectomized rats were used per group.

Attempts to locate corticotropin-releasing hormone (CRH) activity were made utilizing the *in vivo* assay described by Arimura *et al.* (33).

Results

After 339 lyophilized human hypothalami, weighing in all 47.5 g, were ground and defatted, the weight was reduced to 39 g. Extraction of this material with 2N acetic acid followed by lyophilization produced 24.1 g extract or 71 mg/SME. Concentration of the lyophilized extract by re-extraction with glacial acetic acid reduced the weight to 12.5 g. Previous work (9) made it possible to omit assays for releasing hormones at this stage of purification. However, the pressor activity of the glacial acetic acid extract was determined to be 2.6–3 mU/mg and MSH activity was 4.3 MSH U/mg; these values were 3–5 times higher than those obtained previously with human materials (8). The residue from this re-extraction weighed 11.6 g and assayed 0.06–0.25 pressor mU/mg and about 1.0 MSH U/mg. The exact values for the residue were difficult to obtain owing to the presence in them of materials which may have interfered with some of the bioassays, but it was estimated that most of the biological activities were in the glacial acetic acid extract of human hypothalami, as was the case with porcine and bovine hypothalami (1, 14, 15, 17, 18, 24).

Gel filtration on Sephadex. Lyophilized glacial acetic acid extracts (12.5 g) were stirred with 100 ml 1M acetic acid for two hours and then centrifuged at 30,000 $\times g$. The material which did not dissolve was re-extracted three more times with 50 ml 1M acetic acid. The dry weight of the residue was 2 g and its pressor activity was only 0.04 mU/mg. The combined washings were subjected to gel filtration on a column of Sephadex G-25. The pattern of this separation, as followed by OD reading at 278 mμ and bioassays of aliquots, can be seen in Fig. 1. There were two areas with MSH activity, the first probably corresponding to ACTH and the second, with an R_f (16) of 0.6, corresponding to human β-MSH. Arginine vasopressin behaved as determined previously, giving an $R_f = 0.38$ (14, 16, 17, 24). When GH-releasing activity was assayed *in vitro* with 250 and 500 μl aliquots of each tube, fractions 160–179 induced a slight increase in GH release in both assays, while the preceding and following fractions caused a strong inhibition of GH release. Since large amounts of still relatively crude material from Sephadex could exert a possible toxic influence and account for the inhibition of GH release and the blunting of the response to GH-RH *in vitro*, the dose of active fractions was reduced to 100 μl in the third assay. The results of this assay, shown in Table 1, indicate that fractions 160–179 contained GH-RH activity (mean $R_f = 0.56$) since they induced a considerable stimulation of GH release. Other fractions were not assayed at smaller doses. The GH-RH activity was clearly separated from other known releasing hormones and from vasopressin.

FSH-RH activity was located in fractions 190–229 (the greatest activity being in 200–209) and LH-RH activity in tubes 190–239 (Table 2). The R_f of human LH-RF was 0.45, similar to that of pig or beef LH-RH. TRH activity was eluted in tubes 190–219 with an average $R_f = 0.47$, similar to what we reported previously for bovine and porcine TRH (14, 15, 17).

Since fractions 190–229, containing TRH, LH-RH and FSH-RH activities, were to be desalted and chromatographed on CMC, they were combined The lyophilized material weighing 3.57 g was extracted with phenol. After recovery of the phenol soluble substances and lyophilization, 334 mg material was obtained. Since this procedure results in the recovery of virtually all the LH-RH and TRH (1, 17, 24), a purification factor of more than 10-fold was prob-

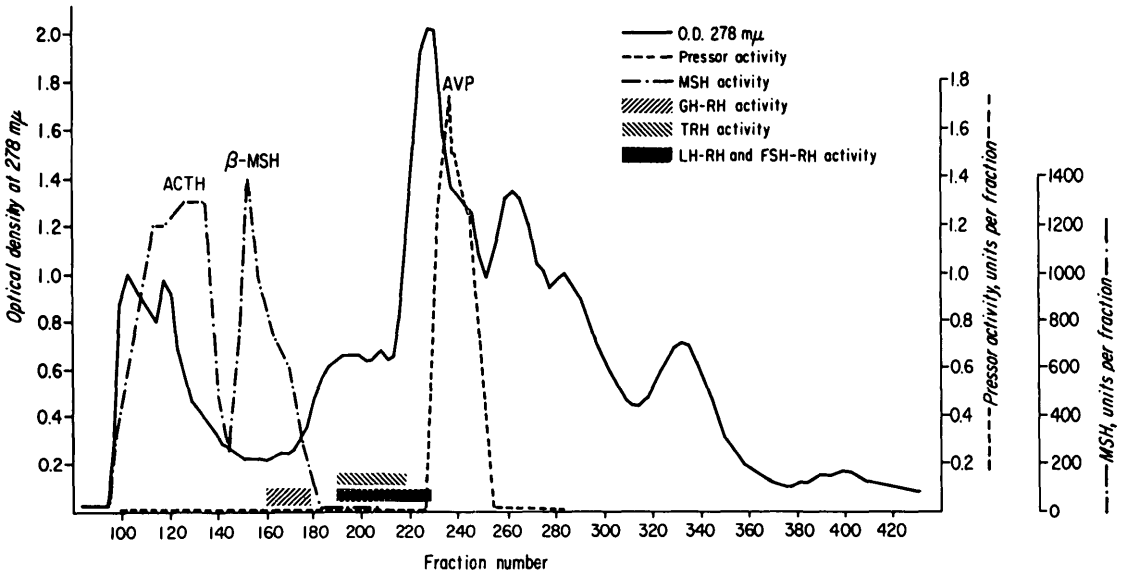

FIG. 1. Gel filtration of 10.5 g of human hypothalamic extracts on Sephadex G-25, fine grade. The column (7.5×170 cm) had a hold-up volume (H-U.V.) of 2300 ml. The solvent was 1M acetic acid. Fraction size was 25 ml. Flow rate = 200 ml/hr. Aliquots of effluents evaporated to dryness and assayed for biological activity were as follows: 100, 250 and 500 μl/beaker for GH-RH (3 assays); 500 μl/beaker for FSH-RH; 30 and 40 μl/rat for LH-RH (2 assays); 10 and 30 μl/mouse for TRH (2 assays).

ably achieved in this step. At the same time the conductivity fell from 9000 to 175 μMHOS (for the same volume of solution), indicating that a very effective desalting had also taken place.

Chromatography on CMC. The phenol extract containing TRH, LH-RH and FSH-RH activities was subjected to ion-exchange chromatography on CMC in order to effect a further separation and purification of biological activities. Fig. 2 shows the location of pressor, TRH, LH-RH and FSH-RH activities. Small aliquots of each tube were evaporated and tested for biological activities. TRH was eluted in fractions 81–90 (Table 3), with an average conductivity of 900 μMHOS, similar to that found in the case of porcine and bovine TRH. Lyophilization of fractions 81–90

TABLE 1. GH-RH activity in fractions from Sephadex G-25 column measured by stimulation of GH-release *in vitro*

Fraction no.	Dose† beaker	GH activity of medium			p value *vs.* control (Duncan's MRT test)
		Tibia cartilage width (μ) ±SE	Total GH* (μg)	Increment (μg GH)	
Control	—	202 ±4.6	190	—	—
160–169	100 μl	226 ±2.4	361	171	0.01
170–179	100 μl	225 ±2.9	352	162	0.01
Standard GH (NIH-GH-S9)					
0		158 ±1.6			
20 μg		197 ±4.5			
80 μg		249 ±6.6			

Ten pituitary halves were incubated in 5 ml KRBG for 7 hr. Medium was diluted to 10 ml with saline after incubation. 1.2 ml of this solution was injected per assay animal during 4 days, 6–7 hypophysectomized rats/group.

* NIH-GH-S9.

† 100 μl of each tube = equiv 0.5 SME = approx 250 μg dry weight per beaker for active fractions.

TABLE 2. LH-RH activity in fractions from Sephadex G-25 measured by elevation of plasma LH in ovariectomized rats pretreated with estrogen and progesterone

Sample	Dose μl/rat	Plasma LH activity OAA change μg/100 mg ±SE	p value	RIA of plasma LH ng/ml†
Saline control	—	−10.0±2.0	—	10.6
170–179	40*	−15.0±2.7	NS	13.8
180–189	40	−20.5±3.9	0.05	11.6
190–199	40	−17.0±3.9	NS	75.0
200–209	40	−24.7±4.6	0.05	>100.0
210–219	40	−22.5±2.5	0.05	>100.0
220–229	40	−20.8±1.5	0.01	66.3
230–239	40	−17.8±2.5	0.05	60.0
240–249	40	− 9.0±2.2	NS	10.0
250–259	40	− 5.3±2.9	NS	8.3
260–269	40	− 4.5±1.6	NS	9.5
Saline control	—	−10.5±1.3	—	8.0
200–209	30	−23.8±2.1	<0.1	>100
210–219	30	−22.4±1.5	<.001	>100
219–229	30	−17.6±2.0	< .05	72.5

* 40 μl of each tube = equiv 0.2 SME ±approx 2 mg dry wt per rat for active fractions.
† As NIH-LH-S-14 (mean of 2 determinations), read directly on the standard curve constructed with NIH-LH-S-14.

yielded 61.6 mg TRH active material. When 100 and 300 μg aliquots of this purified human TRH were subjected to TLC in 1-butanol:ethyl acetate:acetic acid and water = 1:1:1:1, a spot with an R_f identical with natural porcine TRH and synthetic (pyro) glu.his.pro.amide was revealed by Pauly's reagent. The spot intensity of 300 μg of purified human TRH was equivalent to about 1 μg of synthetic (pyro)glu.his.pro. amide. However, further purification in other solvent systems followed by bioassays indicated that the actual amount of human TRH in the sample purified by CMC was much smaller than one part in 300. These studies and the provisional identification of human TRH as (pyro)glu.his.pro.amide will be reported in detail elsewhere (Bowers and Schally, in preparation).

The area with LH-RH and FSH-RH activity (average conductivity 7000 μMHOS) was clearly separated from that with TRH activity. The peak of pressor activity overlapped the LH-RH and FSH-RH active area, but it was devoid of these activities when tested at the doses indicated. The LH-RH and FSH-RH activities are shown in Tables 4 and 5, respectively. Most LH-RH activity was in fractions 171–180. However, fractions 181–190, when tested *in vitro*, appeared to have essentially the same FSH-RH activity as the preceding fractions 171–180 (Table 5). High FSH-RH activity of fractions 191–200, shown in the case of radioimmunoassay only, could be due to an error. Lyophilization of fractions 171–180 yielded 3.3 mg and of fractions 181–190, 3.0 mg material. The pressor activity of these fractions was 155 and 160 mU/mg,

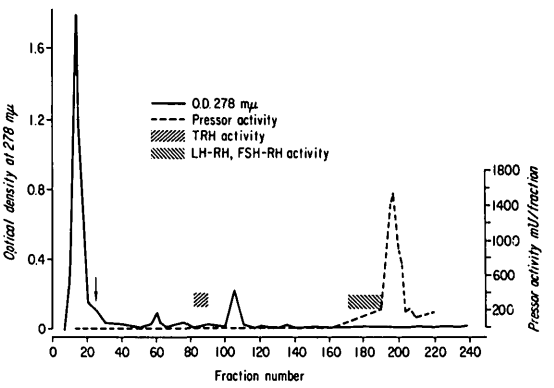

FIG. 2. Ion exchange chromatography of 334 mg of phenol concentrate on CMC column 2.8×60 cm, equilibrated with 0.002M, pH 4.6, ammonium acetate buffer. Gradient to 0.1M pH 7 buffer through 2000 ml mixing flask applied at tube 25. Flow rate 250 ml/hr. Fraction size 25 ml. Aliquots of each tube used for TRH tests were 18 μl/mouse, for LH-RH bioassays 30 μl/rat, and for FSH-RH 500 μl/beaker.

TABLE 3. TRH activity in fractions from CMC measured by release of ^{125}I from thyroid glands of mice

Fraction	Dose* μl/mouse†	Change in blood ^{125}I levels at 2 hr Δ cpm ±SE	p value
Saline	—	− 29 ±12	—
40–50	18 μl	− 10 ±3	NS
51–60	18 μl	− 283 ±100	NS
61–70	18 μl	− 63 ±25	NS
71–80	18 μl	− 44 ±30	NS
81–90	18 μl	+2887 ±230	.001
91–100	18 μl	− 106 ±55	NS
101–110	18 μl	− 74 ±35	NS
111–120	18 μl	+ 7 ±8	NS
Pig TRH	10 ng	+9801 ±102	.001

* 18 μl of each tube/mouse = equiv 0.2 SME /mouse = approx 40 μg dry wt/mouse for active fractions.
† 5–6 mice per fraction.

respectively. These materials were tested clinically in man (34) after toxicity studies.

Human GH-RH concentrate from Sephadex was repurified by free-flow electrophoresis (FFE), followed by chromatography on CMC. The 191 mg material representing the GH-RH active area from Sephadex (fractions 155–185, Fig. 1) was dissolved in 10.0 ml pH 6.3 pyridine acetate buffer and subjected to FFE for eight hours. The results of this separation were monitored by the Folin-Lowry reaction for peptide linkages (35) and are shown in Fig. 3. Aliquots (50 μl) of each tube were evaporated to dryness *in vacuo* and assayed for GH-RH activity. The GH-RH activity was found mainly in fractions 9–14 and 15–20 (Table 6). The electrophoretic mobility of human GH-RH was identical with that of porcine GH-RH (18). Although fractions 21–28 induced a small stimulation of GH release, which was marginally significant, it was assumed that this activity would be present only in tubes 21 and 22 overlapping the major GH-RH active area (No. 9–20). Consequently, fractions 9–22 were combined and lyophilized, yielding 57.8 mg material. Most of this human GH-RH, purified and desalted (18) by FFE, was then subjected to chromatography on CMC. Thus, 54.5 mg was dissolved in 10 ml 0.0002M, pH 4.6 ammonium acetate (the resulting conductivity was 640 μMHOS) and applied on an analytical column of CMC. The pattern of separation is seen in Fig. 4 and the results of bioassays of 50 aliquots of each fraction in Table 7. Human GH-RH activity appeared principally in fractions 66–91 with a conductivity of 900–4000 μMHOS, but there was also some GH-RH activity in fractions 52–65. Such a pattern of distribution of activity would have been expected also from porcine and bovine GH-RH. After lyophilization, fractions 52–65 yielded 2.6 mg and 66–91, 16.2 mg. These materials were used for clinical studies in humans.

Fractions 151–190 and 205–235 from

TABLE 4. LH-RH activity in fractions from CMC measured by elevation of plasma LH in ovariectomized rats pretreated with estrogen and progesterone

Fraction no.	Dose* μl/rat	RIA of LH ng LH/ml plasma† (mean of 3 rats) +SE		p value (Student's *t* test)
		before iv injection	15 min after iv injection	
Control saline	—	5.5 ±0.3	3.9 ±0.2	
140–150	30	4.3 ±0.3	3.5 ±0.5	NS
151–160	30	5.5 ±0.7	4.3 ±1.1	NS
161–170	30	6.2 ±0.7	5.1 ±0.2	NS
171–180	30	5.5 ±0.3	107.5 ±9.0	0.001
181–190	30	7.4 ±1.5	16.5 ±1.7	0.01
191–200	30	6.6 ±0.5	5.2 ±0.3	NS
201–210	30	6.9 ±0.3	4.7 ±0.7	NS
210–220	30	5.7 ±0.3	4.1 ±0.3	NS

* 30 μl of each tube/rat = equiv 0.4 SME/rat or approx 4 μg dry wt/rat for active fractions.
† As NIH-LH-S-14, read directly on the standard curve.

CMC (Fig. 2) and 23–48 from FFE (Fig. 3) were also tested for corticotropin-releasing hormone (CRH) activity at doses of 20 μg material. However, these tests gave negative results.

Discussion

The results reported here confirm and extend previous observations that human hypothalamic extracts contain activities capable of inducing the release of LH, FSH, GH and TSH in laboratory animals (9). The extracts obtained in this work were several times more potent than those reported previously (9), indicating possible improvements in the storage and extraction procedure. The bioassays selected for following the respective biological activities of releasing hormones were thought to be the most specific now available. Thus, for GH-RH and FSH-RH, *in vitro* bioassays were used, based on the stimulation of release of GH (18, 31) and FSH (27, 28), respectively, from isolated rat pituitaries. Inhibition of GH release, which was obtained *in vitro* with many of the hypothalamic fractions preceding and following GH-RH, does not necessarily indicate the presence of a specific GH-release inhibiting factor (GIF) (1, 18).

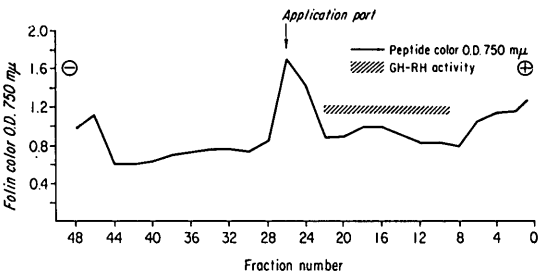

FIG. 3. Free-flow electrophoresis of human GH-RH. Voltage = 1900 volts, current 160 mA, T = 5 C, buffer flow about 50 ml/hr. 191 mg material from Sephadex was infused over a 5-hr period, followed by washings for 2 hr and then an elution with the current for 1 hr. Dosing speed 2.0 ml/hr. Buffer for the separation chamber was pH 6.3, pyridine acetate (0.37M pyridine, 0.035M acetic acid). The buffer for rinsing the electrodes (0.93M pyridine, 0.083M acetic acid, pH 6.3). 100 μl aliquots were taken for Folin-Lowry analyses (34).

For LH the *in vivo* test proposed by Ramirez and McCann (23) was utilized. The LH released was measured by both bioassay (25) and radioimmunoassay (RIA) (26). The combination of the two bioassay methods (23, 25) gives specific and reliable results in the measurement of LH-RH (1, 24). Recently we have shown that the LH-RIA can be successfully substituted for OAAD assay in this system (36). TRH was measured by a specific *in vivo* method in mice (1, 15, 22).

TABLE 5. FSH-RH activity in fractions from CMC tested by stimulation of FSH release *in vitro* from pituitaries of male rats

Fraction no.	Dose* μl/beaker	FSH activity of medium			RIA of rat FSH‡ ng/ml
		Ovarian wt mean ± SE	p *vs.* control	μg FSH† /total	
Control	—	64 ± 4.4	—	265	1120
140–150	500	57 ± 5.9	NS	220	920
151–160	500	57 ± 1.9	NS	220	880
161–170	500	75 ± 11.0	NS	335	1500
171–180	500	119 ± 12.9	<0.05	610	2580
181–190	500	102 ± 15.3	<.05	525	1480
191–200	500	56 ± 5.4	NS	215	2880
201–210	500	65 ± 6.6	NS	275	1080
211–220	500	66 ± 4.5	NS	280	1150
NIH-FSH-S4	100 μg	115 ± 16.0	—	—	—
NIH-FSH-S4	200 μg	180 ± 29.7	—	—	—

* 500 μl of each tube/beaker = equiv 3 SME = approx 60 μg dry wt/beaker for active fractions.
† As NIH-FSH-S4.
‡ As NIAMD-Rat-FSH-RP-1.

TABLE 6. GH-RH activity in fractions from FFE followed by stimulation of GH release *in vitro*

Group no.	Fraction no.	Dose* μl/beaker	GH activity of medium: Tibia cartilage width (μ) ±SE†	GH activity of medium: Total GH content of medium (μg)‡	GH activity of medium: Increment μg GH‡ *vs.* 1	p *vs.* 1 or 8 Duncan's MRT test
1	Control	—	198 ±4.0	166	—	—
2	1–8	50	206 ±3.5	203	37	NS
3	9–14	50	216 ±2.8	260	94	0.05
4	15–20	50	221 ±2.8	294	128	0.05
5	21–28	50	209 ±6.8	218	52	0.05
6	29–37	50	205 ±5.7	198	32	NS
7	38–48	50	207 ±2.4	208	42	NS
8	Control	—	200 ±2.5	174	8	—
Standard GH (NIH-GH-S8)						
0			151 ±1.9			
20 μg			198 ±1.6			
80 μg			254 ±5.3			

* 50 μl of each tube = 2 SME equiv = approx 120 μg dry wt in case of active fractions.
† 6–7 rats per group for the tibia test.
‡ As NIH-GH-S8.

Since the extracts were subjected to several purification steps, in addition to boiling in 2N acetic acid, they were free of the corresponding tropic hormones. Moreover, the design of the assays excluded the possibility that contamination with these hormones could have influenced our results.

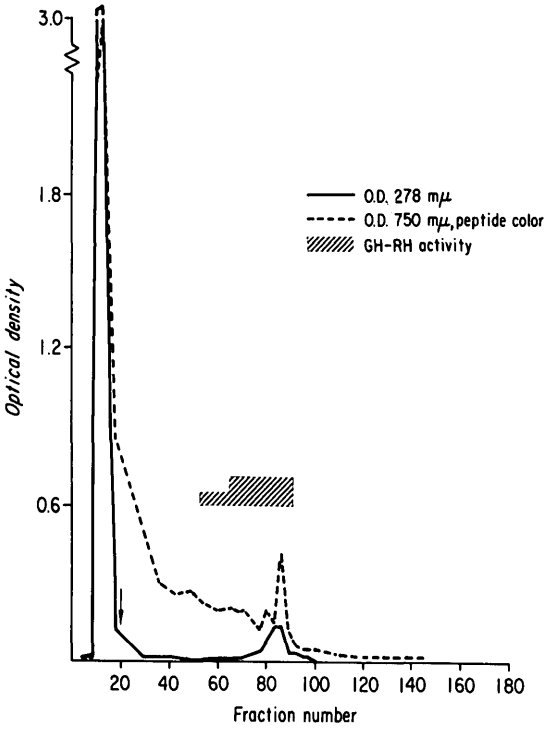

FIG. 4. Chromatography of 54.5 mg of human GH-RH from FFE on CMC. Column 1.0×60 cm, equilibrated with 0.002M, pH 4.6, ammonium acetate buffer. Gradient to 0.1M, pH 7, buffer started at tube 20 through a 250 ml mixing flask. Fraction size 5 ml. The aliquots for Folin-Lowry analyses were 200 μl. The flow rate was 40 ml/hr.

The content of prolactin release-inhibiting hormone (PRIH) (1) was not studied. The behavior of human CRH could not be determined, possibly because of inactivation during the purification and/or before the dissection and storage of hypothalami.

It is apparent from the results reported here that the behavior of human hypothalamic releasing hormones during separation procedures on Sephadex, CMC and free-flow electrophoresis (and, in the case of TRH, also on TLC) is similar to or identical with the behavior of bovine and porcine hypothalamic hormones. This might have been expected of TRH and LH-RH since these hormones, prepared from the hypothalami of pigs (or sheep), induce release of TSH (12) and LH (10, 11), respectively, in man.

It was also interesting to observe that the behavior of human GH-RH on Sephadex, free-flow electrophoresis and CMC closely paralleled that of porcine GH-RH (18). This indicates that human GH-RH is most likely an acidic polypeptide and that its molecular size and isoelectric point are very similar to the porcine hormone. The porcine GH-RH was reported to be a pentadeca-

TABLE 7. GH-RH activity in fractions from CMC measured by stimulation of GH-release *in vitro*

Group no.	Fraction no.	Dose*/ beaker	GH activity of medium			p *vs.* 1 or 8 Duncan's MRT test
			Tibia cartilage width (μ) ±SE	Total GH content of medium (μg)†	Increment μg GH† *vs.* 1	
1	Control	—	185 ±2.9	165	—	—
2	8–31	25 μl	181 ±4.4	150	−15	NS
3	32–51	25 μl	191 ±2.1	190	25	NS
4	52–65	25 μl	202 ±2.9	245	80	0.01
5	66–91	25 μl	214 ±3.9	326	161	0.01
6	92–180	25 μl	189 ±3.9	182	17	NS
7	Pure porcine GH-RH	20 pg	211 ±3.3	303	138	0.01
8	Control	—	186 ±1.3	169	4	—
Standard (NIH-GH-S9)						
	20 μg		185 ±5.5			
	80 μg		244 ±4.2			

* 25 μl of each tube = 1.5 SME equiv = approx 13 and 80 μg dry weight, respectively, of fractions 52–65 and 66–91.
† As NIH-GH-S-9.

peptide (18), but the latest structural work (Schally, Baba, Nair and Barrett, in preparation) indicates that the molecule is probably a hendecapeptide.

The behavior of human LH-RH on Sephadex and CMC was identical with that of LH-RH of porcine and bovine origin (1, 24). Unfortunately, the amount of material available did not permit the comparison of human and porcine LH-RH in other physicochemical systems. LH-RH fractions were associated with considerable FSH-RH activity. It is not known whether this FSH-RH releasing activity is intrinsic to human LH-RH or is due to contamination with FSH-RH. Similar observations were reported for porcine LH-RH (10, 28). Preliminary studies indicate that LH-RH of human origin, as expected, stimulated LH release in men (34).

The characteristics of human TRH were identical with those of bovine, porcine and ovine TRH (1, 15, 17). In addition to a similar mobility in Sephadex, human TRH displayed the same behavior on chromatography or CMC and the same R_f on TLC in ethyl acetate:butanol: acetic acid:water =1:1:1:1. Further studies, which will be reported in detail elsewhere (37), utilizing glass paper chromatography in eight different solvent systems, indicate that the structure of human TRH is probably (pyro)glu.his.pro.amide, the same as that reported for porcine (5–7) or ovine TRH (8, 38).

The findings reported here establish that hypothalamic releasing hormones are present in man. These findings combined with previous observations on the effectiveness of hypothalamic hormones of animal origin in humans (9–12) support the concept that in man the release of pituitary hormones is controlled by the respective hypothalamic releasing neurohumors through neurohumoral pathways suggested previously (1–4). The methods described may facilitate the study of the level of hypothalamic hormones in certain pathological conditions involving the pituitary and hypothalamus. An extension of the methods described here and/or their repetition on a somewhat larger scale may make possible the preparation of adequate amounts of human hypothalamic hormones for a comparison of their effectiveness with synthetic materials or natural materials of animal origin.

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References

1. Schally, A. V., A. Arimura, C. Y. Bowers, A. J. Kastin, S. Sawano, and T. W. Redding, *Recent Progr Hormone Res* **24:** 497, 1968.
2. Guillemin, R., *Ann Rev Physiol* **29:** 313, 1967.
3. McCann, S. M., A. P. S. Dhariwal, and J. C. Porter, *Ann Rev Physiol* **30:** 590, 1968.
4. Reichlin, S., *In* Williams, R. H. (ed.), Textbook of Endocrinology, ed. 4, section 12, W. B. Saunders Co., Philadelphia, 1968, p. 967.
5. Nair, R. M. G., J. F. Barrett, C. Y. Bowers, and A. V. Schally, *Biochemistry (Wash)* **9:** 1103, 1970.
6. Schally, A. V., R. M. G. Nair, J. F. Barrett, C. Y. Bowers, and K. Folkers, *Fed Proc* **29:** 470, 1269, 1970.
7. Bøler, J., F. Enzmann, K. Folkers, C. Y. Bowers, and A. V. Schally, *Biochem Biophys Res Commun* **37:** 705, 1969.
8. Burgus, R., T. F. Dunn, D. Desiderio, D. N. Ward, W. Vale, and R. Guillemin, *Nature (London)* **226:** 321, 1970.
9. Schally, A. V., E. E. Muller, A. Arimura, C. Y. Bowers, T. Saito, T. W. Redding, S. Sawano, and P. Pizzolato, *J Clin Endocr* **27:** 755, 1967.
10. Kastin, A. J., A. V. Schally, C. Gual, A. R. Midgley, Jr., C. Y. Bowers, and A. Diaz-Infante, Jr., *J Clin Endocr* **29:** 1046, 1969.
11. Root, A. W., G. P. Smith, A. P. S. Dhariwal, and S. M. McCann, *Nature (London)* **221:** 570, 1969.
12. Bowers, C. Y., A. V. Schally, C. Gual, W. Hawley, and A. F. Parlow, *J Clin Endocr* **28:** 978, 1968.
13. Knobil, E., J. Meyer, and A. V. Schally, *In* Muller, E. E., and A. Pecile (eds.), GH Symposium, Milan, September 1967, *Excerpta Med*, International Congress Series **158:** 226, 1968.
14. Schally, A. V., C. Y. Bowers, and T. W. Redding, *Proc Soc Exp Biol Med* **121:** 718, 1966.
15. Schally, A. V., T. W. Redding, C. Y. Bowers, and J. F. Barrett, *J Biol Chem* **244:** 4077, 1969.
16. Porath, J., and A. V. Schally, *Endocrinology* **70:** 738, 1962.
17. Schally, A. V., C. Y. Bowers, and T. W. Redding, *Endocrinology* **78:** 726, 1966.
18. Schally, A. V., S. Sawano, A. Arimura, J. F. Barrett, I. Wakabayashi, and C. Y. Bowers, *Endocrinology* **84:** 1493, 1969.
19. Dekanski, J., *Brit J Pharmacol* **7:** 567, 1952.
20. Kastin, A. J., and G. T. Ross, *Experientia* **20:** 461, 1964.
21. Guillemin, R., G. W. Clayton, J. D. Smith, and H. S. Lipscomb, *Endocrinology* **63:** 349, 1958.
22. Bowers, C. Y., A. V. Schally, G. A. Reynolds, and W. D. Hawley, *Endocrinology* **81:** 741, 1967.
23. Ramirez, V. D., and S. M. McCann, *Endocrinology* **73:** 193, 1963.
24. Schally, A. V., C. Y. Bowers, W. F. White, and A. I. Cohen, *Endocrinology* **81:** 77, 1967.
25. Parlow, A. F., *In* Albert, A. (ed.), Human Pituitary Gonadotropins, Charles C Thomas, Springfield, Ill., 1961, p. 300.
26. Niswender, G. D., A. R. Midgley, Jr., S. E. Monroe, and L. E. Reichert, Jr., *Proc Soc Exp Biol Med* **128:** 807, 1968.
27. Mittler, J. C., and J. Meites, *Endocrinology* **78:** 500, 1966.
28. Schally, A. V., J. C. Mittler, and W. F. White, *Endocrinology* **86:**903, 1970.
29. Steelman, S. L., and F. M. Pohley, *Endocrinology* **53:** 604, 1953.
30. Parlow, A. F., T. A. Daane, and A. V. Schally, Program of the 51st Meeting of The Endocrine Society, New York, 1969, p. 83 (Abstract 106).
31. Schally, A. V., E. E. Muller, and S. Sawano, *Endocrinology* **82:** 271, 1968.
32. Greenspan, F. S., C. H. Li, M. E. Simpson, and H. M. Evans, *Endocrinology* **45:** 455, 1949.
33. Arimura, A., T. Saito, and A. V. Schally, *Endocrinology* **81:** 235, 1967.
34. Kastin, A. J., A. V. Schally, C. Gual, A. R. Midgley, C. Y. Bowers, A. Cabeza, and F. Flores, *Clin Res* **18:** 371, 1970.
35. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J Biol Chem* **193:** 265, 1951.
36. Schally, A. V., A. F. Parlow, W. H. Carter, M. Saito, C. Y. Bowers, and A. Arimura, *Endocrinology* **86:** 530, 1970.
37. Bowers, C. Y., A. V. Schally, A. Weil, G. A. Reynolds, and K. Folkers, Abstract of the 6th International Thyroid Congress, Vienna, June 1970.
38. Burgus, R., T. F. Dunn, D. Desiderio, and R. Guillemin, *C R Acad Sci (Paris)* **269:** 1870, 1969.