IDENTIFICATION AND PURIFICATION OF FACTOR A-GHRH FROM HYPOTHALAMI WHICH RELEASES GROWTH HORMONE

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SUMMARY

Two separable hormone entities have been found by exploratory purifications and assay for release of growth hormone (GH) by radioimmunoassay. In recognition of frequent multiple activities of peptide hormones, these two hormonal entities are provisionally designated factors A-GHRH and B-GHRH until they are chemically characterized and their dominant functionality clarified. The A- and B-GHRH designations merely define the assay guiding isolation. Factor A-GHRH was found by filtration on Bio-Gel P-2, and purified over Sephadex G-25 in two partition chromatographic systems, and by Sephadex LH-20. The two partitions and stage LH-20 also differentiated the two active entities. Fractions of A-GHRH were active at 100-200 μg. Factor A-GHRH is inhibited by somatostatin.

INTRODUCTION

In the accompanying report by Johansson et al.[1] on factor B-GHRH from hypothalami, citation of relevant studies by other groups of investigators on the release of growth hormone (GH) by initial extracts of hypothalami have been made. These citations need not be concurrently repeated herein.

Unexpectedly, our exploratory purifications and assay of chromatographic fractions subsequent to the initial steps of extracting hypothalamic tissue led to the identification of two regions of chromatographic fractions each of which released in vitro GH by a radioimmunoassay. It became increasingly clear that we were fractionating two chemical entities which released growth hormone either on a primary or some nature of a secondary basis. There was no doubt about the chromatographic steps which separated these two biologically active entities once they had been found and identified. Designations for both entities were needed so one entity was designated as factor A-GHRH and the other as factor

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B-GHRH until further research permits their chemical and biological characterization in such a manner that their primary hormonal functions are clear. The abbreviation GHRH has been linked to the letters A and B merely to indicate the releasing activity which is presently guiding the isolation of the substance. It is no longer novel to recognize that hypothalamic peptide hormones can have more than one biological activity, particularly since it was observed that pGlu-His-Pro-NH_2 releases both thyrotropin and prolactin and that pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 releases both the luteinizing and follicle stimulating hormones. There are abundant older examples of physiologically important peptides which have multiple activities.

Since factors A-GHRH and B-GHRH were found on a related basis, Johansson et al.[1] reported on factor B-GHRH, and we describe herein the identification and purification of factor A-GHRH and describe some of the chromatographic properties that differentiate factors A-GHRH and B-GHRH.

METHODS

Purification of Factor A-GHRH from Porcine Hypothalami. - Lyophilized hypothalamic fragments in batches of 5,000-10,000 were homogenized in a mixture of methanol and acetic acid. After filtration of the homogenate, the filter cake was resuspended in fresh methanol-acetic acid, and with effective stirring. The mixture was filtered, and the residue was resuspended in the solvent mixture, and this operation was repeated for a total of five times. The filtrates were combined and evaporated to dryness. The extractives were then defatted and lyophilized to provide material at Stage SP. The extractives at Stage SP on 1 M acetic acid were passed over a column of Bio-Gel P-2 (exclusion limit 1800 Daltons) to give material at Stage P-2. Partition chromatography was then carried out on Sephadex G-25 in two systems. Solvent system A consisted of 0.1% acetic acid-butanol-pyridine (11:5:3) which was Stage PC-A. System B was butanol-acetic acid-water (4:1:5) which was designated Stage PC-B. These partition systems were used after gel filtration. Sephadex LH-20 was also used with water-butanol as the mobile phase. Bioassays were conducted on the extractives after pooling the appropriate fractions at each stage. The aliquots for assay were lyophilized and the residues were dissolved in 0.05 M sodium phosphate buffer, pH 7.4; 50 µl aliquots were added to the incubation medium at the beginning of the third hour (I_3) for the in vitro assay.

Assay Procedure. - The in vitro assay procedure, which was based on the radioimmuno method of Parlow, was carried out as described in the companion report on factor B-GHRH by Johansson et al.[1]

RESULTS AND DISCUSSION

The data on the stages of fractionation of factor A-GHRH from porcine hypothalami and corresponding assay results are in Table 1.

Table 1. DATA ON THE RELEASE OF GROWTH HORMONE BY FACTOR A-GHRH

Stage	Description	µg Dose	ng GH/ml P_2	ng GH/ml I_3
Saline Control	-	-	740	625
Saline Control	-	-	600	225
Saline Control	-	-	700	285
P-2	76-100[a]	300	1680	>2560
	101-125	11,500	1260	>2560
P-2	76-100	700	980	2120
	101-125	11,400	415	470
P-2	76-100	3600	615	780
	101-125	9600	770	760
P-2	76-101	700	1135	2410
	102-128	5700	1160	1800
P-2	76-100	600	775	1025
	101-125	2600	220	485
P-2	76-100	700	990	1105
	101-125	3200	170	1035
Saline Control	-	-	1880	1520
Saline Control	-	-	1290	1160
B-PC	11-20[b]	90	360	870
B-PC	21-30	150	755	>2560
B-PC	31-40	110	550	1480
B-PC	41-50	140	1150	1515
B-PC	6-15	40	560	1780
B-PC	16-25	100	430	1990
B-PC	26-35	90	485	1600
B-PC	36-45	110	645	2200
B-PC	46-55	190	747	2116
B-PC	56-65	320	1000	2260
B-PC	66-75	470	500	1670
B-PC	76-85	310	590	1770
B-PC	86-95	280	250	955
Saline Control	-	-	1005	620
Saline Control	-	-	650	330
Saline Control	-	-	485	410
A-PC	11-15[c]	200	545	2090
A-PC	16-20	200	675	1830
A-PC	21-25	200	860	1620
A-PC	26-30	200	695	1590
A-PC	31-35	200	485	1325
A-PC	36-38	200	431	704
Saline Control	-	-	550	545
Saline Control	-	-	1185	770
LH-20	5-6[d]	200	845	1630
LH-20	7-8	200	1280	1660
LH-20	9-10	200	725	880

a. Each fraction was 14.5 ml, column dimensions 5.0 x 140 cm.
b. Each fraction was 10 ml, column dimensions 2.0 x 90 cm.
c. Each fraction was 12 ml, column dimensions 2.5 x 90 cm.
d. Each fraction was 10 ml, column dimensions 1.5 x 90 cm.

Gel filtration on Bio-Gel P-2 turned out to be surprisingly beneficial and permitted our finding of two well separated groups of fractions which possessed active entities that released growth hormone as measured by the in vitro assay. Fractions from Bio-Gel P-2 in the range 76-125 contained the active entity which has been designated factor A-GHRH. The active entity in the range of fractions 201-600 and higher contained the active entity designated factor B-GHRH. The subsequent behavior of these two sets of fractions in several chromatographic systems including partition chromatography on Sephadex G-25 in solvent systems A and B, and by Sephadex LH-20 clearly showed that the original apparent separation of factors A-GHRH and B-GHRH on Bio-Gel P-2 did constitute the separation of two chemical entities. The results on the six columns at Stage P-2 in Table 1 show reasonable consistency for separation of factor A-GHRH. Its R_f value was about 0.6-0.7. Although there was some variation in the fractionation and assay data on factor A-GHRH, its presence and activity were generally found in the fractions 76-100.

The behavior of factor A-GHRH on Bio-Gel P-2 was similar to that of the decapeptide, LHRH.

Significant additional purification of factor A-GHRH was obtained by partition chromatography over Sephadex G-25 in solvent system B according to the data for Stage B-PC in Table 1. These data demonstrate the consistent purification of A-GHRH with concomitant reduction of weight so that Stage B-PC is a good purification step.

Table 2. IN VITRO EFFECT OF PARTIALLY PURIFIED PORCINE FACTOR A-GHRH ON RELEASE OF GH FROM PITUITARIES OF 20 DAY OLD FEMALE RATS AND INHIBITION OF FACTOR A-GHRH BY SOMATOSTATIN

Additions to Incubation Medium	ng GH/ml medium					
	P_2	I_3	Δ	I_4	Δ	p
Saline Control	550	545	- 5	130	-420	
	1185	770	-415	320	-865	
	Ave Δ = -426 ± 175					-
Factor A-GHRH	585	705	+120	900	+315	
	840	1165	+325	1430	+590	
	Ave Δ = 338 ± 96					<0.01[a]
Factor A-GHRH + Somatostatin, 1 μg	1120	895	-225	1200	+ 80	
	910	915	+ 5	960	+ 50	
	Ave Δ = -23 ± 69					<0.02[b]

a) Factor A-GHRH vs. Saline.

b) Factor A-GHRH vs. Factor A-GHRH + Somatostatin.

Further purification of factor A-GHRH by partition chromatography with solvent system A resulted in fractions that consistently gave a release of GH at a dosage of 100-200 μg. In partition chromatographic system A, factor A-GHRH had an R_f of about 0.38 and factor B-GHRH had an R_f of about 0.17. Although there was relatively less loss of weight in purification on Stage A-PC, this purification step does clearly differentiate factors A-GHRH and B-GHRH.

Further purification of factor A-GHRH on Sephadex LH-20 (Table 1) demonstrated again the difference between the two hormones. On Sephadex LH-20, factor A-GHRH had an R_f of about 0.71 and factor B-GHRH had an R_f of 0.31-0.04 when using a water-butanol system.

The data in Table 2 demonstrate that the release of GH by factor A-GHRH is inhibited by somatostatin[2,3] at a dose of 1 μg.

Further purification of factors A-GHRH and B-GHRH is in progress.

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