DATA ON STRUCTURE OF BOVINE LRH BY INACTIVATION

by

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SUMMARY - Data on the structure of the bovine leuteinizing hormone releasing hormone (LRH) of the hypothalamus have been obtained from specific chemical and enzymatic inactivations of concentrates of the hormone as determined by in vivo assays. Bovine LRH apparently has an N-terminal pGlu moiety since it was inactivated by a pGlu-peptidase from P. fluorescens. It was inactivated by the Pauly reagent, but was not inactivated by ninhydrin; therefore, bovine LRH appears to have a His or Tyr or other Pauly-reactive amino acid(s) in one or more moieties, and no free amino group.

Several enzymatic inactivation studies have been reported on purified LRH preparations. McCann and Ramirez (1) and Fawcett et al. (2) have found that bovine LRH was inactivated by pepsin and trypsin. Guillemin (3) and Gregory et al. (4) have showed that ovine LRH was inactivated by pepsin, trypsin and chymotrypsin. Basova and Ginodman (5) recently reported that porcine LRH was inactivated by pepsin, trypsin, and chymotrypsin. In contrast to these results, Schally et al. (6,7) have reported that porcine LRH was not inactivated by pepsin and that it was inactivated by chymotrypsin; porcine LRH was also inactivated by trypsin, but only at a very high ratio of enzyme to substrate. Ovine LRH was also chemically inactivated by performic acid and DNFB (4).

Amoss et al. (8) reported that purified ovine LRH was Pauly positive in four TLC systems. Since they stated that their material "was not proved to be homogeneous", it is uncertain whether the Pauly spot is derived from LRH or impurities. Since their preparation was inactivated by a pGlu-peptidase from B. subtilis, a pGlu (9) moiety appeared to be present. They also reported that ovine LRH was ninhydrin-negative. Schally et al. (10) reported that purified porcine LRH was ninhydrin-positive.

*Hypothalamic Hormones XVIII.

Frozen hypothalami of bovine origin were lyophilized, defatted, pulverized, and extracted with absolute methanol. Then, gel filtration on Sephadex G-25 and two filtrations on Bio-Gel P-2 yielded concentrates of LRH which were used to obtain information about the structure of LRH as revealed by chemical and enzymatic inactivations. Samples of the bovine LRH and TRH concentrates corresponding to 2-10 hypothalamic fragments were used in the chemical and enzymic inactivations. Such reactions at different stages of advancing purification can be more meaningful than at just one stage of purification, and interpretation of the results on the basis of hypothalamic fragments can be more enlightening than on a weight basis. The data on LRH are in Table I and that on TRH as a control are in Table II.

Pauly reagent was prepared by dissolving diazotized sulfanilic acid (11) (100 mg) in a 10% solution (20.0 ml) of sodium carbonate. Aliquots of this solution were used immediately for reaction with the LRH concentrate. After incubation with the Pauly reagent at room temperature for 10 minutes, the solution was neutralized with 5N HCl, lyophilized, and the residue was dissolved in distilled water. The results of the bioassays are recorded in Table I and

TABLE I. BOVINE LRH CONCENTRATE INACTIVATION

	Fragment Equiv./Dose	LRH[a] Concentrate		Reaction[b] Material		Reagent[c] Control	
		mμg LH/ml serum					
		before	after	before	after	before	after
Exposure to Pauly Reagent	3.25	4.6	186	4.0	4.0	4.0	5.0
		4.0	>286	4.0	4.0	4.0	4.0
	1.0	4.0	25.0	4.0	4.0	4.6	6.8
		6.8	20.1	4.0	<4.0	4.0	5.4
Exposure to Ninhydrin Reagent	2.6	<4.0	85.0	5.6	285.6	4.0	5.0
		4.0	90.0	4.0	111.0	4.0	4.0
	1.0	<4.0	12.6	<4.0	20.8	<4.0	<4.0
		4.0	18.2	<4.0	17.4	<4.0	<4.0
Exposure to pGlu-Peptidase	2.45			<4.0	<4.0		
		4.0	78.0	<4.0	<4.0		
		3.0	82.0	<4.0	<4.0		
				22.0	21.0		
	0.56			4.2	4.4		
				<4.0	<4.0		
	0.56	4.0	44.0	4.0	4.0		
		<4.0	160.0	<4.0	<4.0		
	0.48			6.4	7.4		
				6.8	7.4		

a)LRH preparation and the reagents (e.g. buffer, etc.), except reactive species.
b)LRH preparation and all the reagents necessary for reaction.
c)All the reagents except the LRH concentrate.

TABLE II. BOVINE TRH AND SYNTHETIC TRH INACTIVATION

	Fragment Equiv./Dose	ng TRH/Dose	TRH[a] Concentrate	Synth.[a] TRH	Reaction[b] Material
				I^{125} Δcpm	
Exposure to Pauly Reagent	0.1	-	7844	-	697
	0.05	-	3203	-	85
	-	9	-	3140	142
Exposure to pGlu-Peptidase	0.1	-	2103	-	-13
	0.1	-	2103	-	-30
	-	25	-	6940	134
	-	25	-	6940	27

a)TRH preparation or synthetic TRH and all reagents (e.g. buffer, etc.), except the reactive species.
b)TRH preparation or synthetic TRH and all the reagents necessary for reaction.

show a loss of LRH activity. When the LRH concentrate was incubated with the reagents except the diazotized sulfanilic acid and the solution was processed in the same manner, the activity of LRH was retained.

When a bovine TRH concentrate, obtained by the same steps as the bovine LRH, and synthetic TRH (pGlu-His-Pro-NH_2) (12) were treated with the Pauly reagent under the conditions described for bovine LRH, the activity of TRH was lost according to the bioassays by the T^3-TRH method of Bowers et al. (13, 14,15). Since TRH possesses a His-moiety, these inactivations of a bovine TRH concentrate and synthetic TRH serve as control experiments for the Pauly-inactivation of bovine LRH; LRH may contain His or Tyr or another amino acid as one or more moieties which react(s) with the Pauly reagent.

Ninhydrin (20 mg) was dissolved in saturated n-butanol-water (10 ml) (16). After incubation for 5 minutes at 100° of the bovine LRH concentrate with aliquots of the ninhydrin solution, the mixture was bioassayed. The LRH activity was retained. When the LRH concentrate was incubated with only saturated n-butanol-water under the same conditions, there was also retention of activity. An amino group does not appear to be present in bovine LRH.

Doolittle et al. (17) reported that a pGlu-peptidase, which was isolated from a strain of Pseudomonas fluorescens, specifically cleaves pGlu-peptide bonds. Since TRH has a pGlu moiety and there are reports (5) on the absence of an unsubstituted N-terminus in porcine LRH, it was cogent to determine the effect of this enzyme on the bovine LRH concentrate. pGlu-peptidase was dialyzed in 0.05M phosphate buffer at pH 7.3 which contained 0.01M mercaptoethanol and 0.001M EDTA to remove the 2-pyrrolidone stabilizer which was present. The solution was then diluted to an $O.D._{280}$=1 and 100 μl aliquots were

added to samples of the LRH concentrate. For controls, a bovine TRH concentrate and synthetic TRH were similarly treated. The solutions were incubated at 30° C for 24-45 hours and then ethanol was added to inactivate the enzyme. After cooling at -5° C for 15 minutes, the solutions were centrifuged and the supernatants were lyophilized. The residue was dissolved in normal saline and the solutions were bioassayed. In all cases, there was a complete loss of activity for LRH and TRH after incubation with the pGlu-peptidase, but no loss of activity in control samples. Bovine LRH appears to have a pGlu moiety.

The bioassays for LRH activity were performed by the method of Ramirez and McCann (18). Adult Sprague-Dawley female rats were used 6 weeks to 3 months after ovariectomy. The rats were subcutaneously injected with 50 μg of estradiol benzoate and 25 mg of progesterone dissolved in sesame oil 72 hrs. before the assay. Under ether anesthesia, blood was collected from the jugular vein, and the test sample was injected into this vein; in fifteen minutes, blood was again collected. Each test sample was assayed in two rats. Serum assays for LH were performed in duplicate by the double antibody radioimmunoassay of Niswender et al. (19) using antiovine LH serum and ovine LH-^{131}I. The results are expressed in terms of mμg/ml of the LER- 1240-2-0.60 NIH-LH-SI units/mg. The results of the inactivations were determined by comparison of LH levels before and after injection of the test samples.

The bioassays for TRH activity were performed by the T^3-TRH method of Bowers et al. (12,13,14).

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