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Preparation of Biologically Active ^{125}I LH-RH Suitable for Membrane-Binding Studies (38806)

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Since the availability of the synthetic decapeptide, LH-RH, several groups of investigators have produced iodine-labeled hormone for immunological studies. Both the chloramin-T (1–3) and the lactoperoxidase iodination methods have been used (3). However, few data are available on the biological activity of the iodinated product, or on the contamination of ^{125}I LH-RH with unlabeled hormone. In 1973, a group (3) reported that the ^{125}I LH-RH possessed 13% of the bioactivity of the native hormone, but did not comment on possible contamination with unlabeled hormone. Again in 1973, another group (4) produced bioactive ^{125}I LH-RH using a lactoperoxidase technique, and were able to separate labeled from unlabeled hormone by means of polyacrylamide gel electrophoresis.

The aim of the present study was to prepare biologically active ^{125}I LH-RH free of carrier hormone which would be suitable for both immunological and pituitary cell membrane-binding studies.

Materials and Methods. Preparation of ^{125}I LH-RH. The iodination system was based on the lactoperoxidase method using a hydrogen peroxide generating system described by Miyachi *et al.* (4). Reagents were added to the iodination vial in the following order: 15 μl 0.01 *M* PBS (0.01 *M* phosphate buffer, 0.15 *M* saline, pH 7.5); 5 μg synthetic LH-RH (Abbott) in 5 μl of 0.01 *M* PBS; 1.5 mCi of ^{125}I (Industrial Nuclear); Lactoperoxidase (Sigma) 50 ng in 10 μl of 0.1 *M* sodium acetate (pH 5.6); 5 μl glucose oxidase (Miles); 25 μl of 0.1% glucose. All reagents were at room temperature (21°C) when added except for the glucose oxidase (4°C). The reaction was allowed to continue for 1½ min at room temperature, mixing being achieved by finger-flicking. Then, 0.5 ml of 0.01 M phosphate (pH 7.5) was added, and the iodination mixture transferred to 200 mg of Dowex I X 10 (200–400 mesh) in 0.5 ml of 0.01 *M* phosphate to remove excess unreacted iodine. This was then mixed for 10 sec and centrifuged. The supernatant, containing the iodinated LH-RH, was removed and transferred to a 20 × 0.9-cm column of carboxymethyl cellulose (Sigma) equilibrated in 0.002 *M* ammonium acetate (pH 4.5). The column was eluted with 20 ml of the same buffer, before changing the eluant to 0.06 *M* ammonium acetate (pH 4.5) in a single step. Three hundred milliliters of the eluant were passed through the column at 45–50 ml/hr, 5-ml fractions being collected.

Elution of unlabeled LH from the column. To determine the elution profile of unlabeled LH-RH, 5 μg in 1.0 ml 0.01 *M* phosphate (pH 7.5) was added to an identical CMC column and eluted as above. Each fraction was stored at 4°C prior to measurement of the LH-RH concentration by immunoassay.

Immunological studies and calculation of the specific activity of ^{125}I LH-RH. The antisera (R3) used was prepared in rabbits against an LH-RH bovine thyroglobulin conjugate, the materials being linked through the thyroxine residue of LH-RH by a diazo reaction. All binding studies and immunoassays were performed using 100 μl of diluted antiserum, 100 μl diluted label, 100 μl of 0.1 *M* EDTA, and 1% normal rabbit serum in PBS to a total volume of 1 ml. A second antibody system was used to separate bound from free hormone.

The small amount of protein emerging from the CMC column in the ^{125}I peak could not be measured by the micro-Folin–Lowry method. Thus, in order to assess the specific activity of the ^{125}I LH-RH, the LH-RH concentration was measured by immunoassay.

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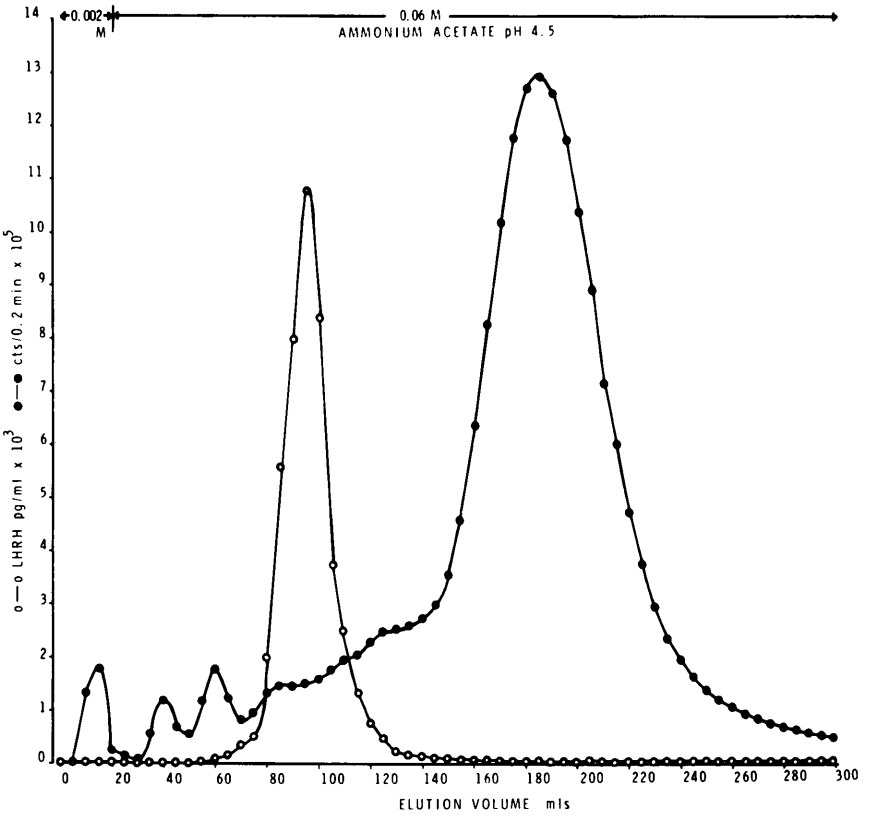

FIG. 1. Separation of unlabeled LH-RH from iodinated LH-RH on a carboxymethyl cellulose column. ●——● radioactivity, O- - - - -O LH-RH measured by immunoassay. Glassware and column saturated with albumin prior to use.

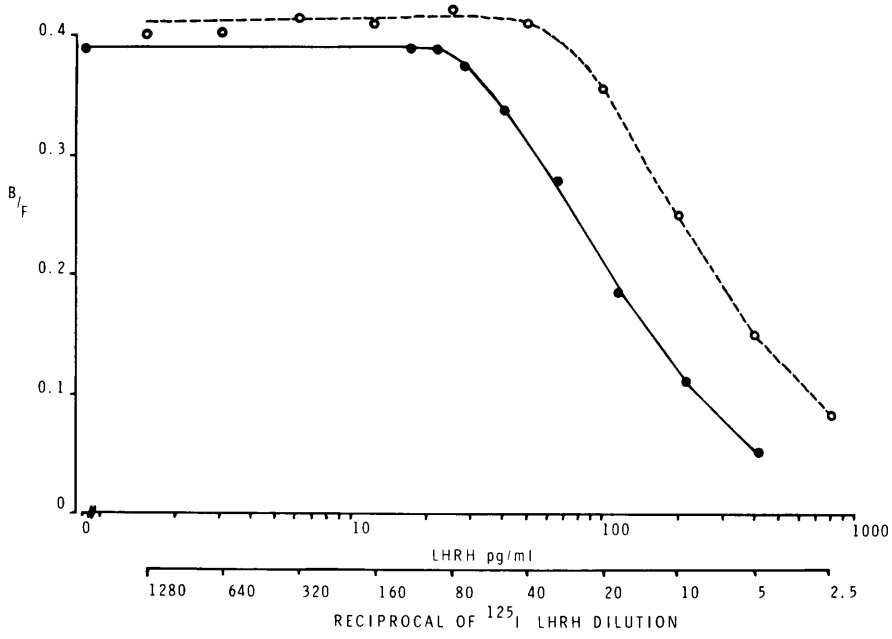

FIG. 2. Estimation of the specific activity of ^{125}I LH-RH by immunoassay. ●——● LH-RH + 15 pg ^{125}LH-RH used as tracer, O- - - - -O ^{125}I LH-RH.

TABLE I. BIOLOGICAL ACTIVITY OF ^{125}I LH-RH.[a]

	Dose of LH-RH (nM)	LH (ng/ml)	FSH (ng/ml)
Controls			
Medium alone	0	<3	
Medium + ^{125}I LH-RH + fetal calf serum	0	<3	
Cells + 2.6 n*M* NH_4 acetate	0	25.8 ± 1.06	
Cells + medium	0	25.1 ± 2.53	
LH-RH	0.1	27.7 ± 8.9	
	0.3	69.1 ± 8.2	
	1.0	101.7 ± 20.8	
	3.0	231.7 ± 28.4	
^{125}I LH-RH	0.29	62.0 ± 4.4	
	0.97	81.2 ± 15.7	
	2.91	197.0 ± 19.7	

[a] Values for rat LH and FSH are means (± SD) of three culture dishes for each dose level assayed at two dilutions.

Unlabeled LH-RH was used to produce a standard curve and serial doubling dilutions of ^{125}I LH-RH were added to identical tubes containing antiserum (R3) at a final dilution of 1:40,000. The standard curve (unlabeled LH-RH + ^{125}I LH-RH tracer) was constructed as a B/F ratio, and the B/F ratios of the tubes containing ^{125}I LH-RH alone were read off the standard curve to give the LH-RH content at each dilution.

Bioassay. The ^{125}I LH-RH was kindly bioassayed by Dr. W. Vale using the cultured enzyme dispersed rat anterior pituitary cell method (5). Three dilutions of ^{125}I LH-RH were assayed in triplicate, and synthetic LH-RH was used as a standard. Dishes containing no cells, but medium alone and medium + ^{125}I LH-RH + fetal calf serum, and dishes with cells + 2.6 m*M* ammonium acetate were set up as controls. The media were removed at the end of the incubation period and stored at −20°C prior to assay. Rat LH and FSH were immunoassayed using NIAMD reagents, and FSH RP1 and LH RP1 as standards.

Membrane binding. Pituitary glands were excised from male rats after decapitation and the anterior pituitary homogenized in ice-cold 0.3 *M* sucrose containing 0.001 *M* magnesium chloride (pH 7.7). After centrifugation, at 600*g* for 5 min (4°C), the supernatant was again centrifuged at 10,800*g* for 10 min. The subsequent pellet was resuspended in cold 0.01 M Tris–HCl (containing 0.001 *M* $MgCl_2$ and 0.001 *M* dithiothreitol, pH 7.7) to give approximately 700 μg protein/ml. Four hundred microliters of this pituitary membrane preparation, 100 μl of concentrations of LH-RH, and 100 μl of ^{125}I LH-RH with Tris–HCl buffer, were added to tubes prewashed with 2% BSA to a final volume of 800 μl. After incubation at 4°C for 40 min, the membrane-bound LH-RH was separated by centrifugation at 15,000*g* and the ppt counted.

Results. *Iodination and purification.* Reliable and reproducible iodination was achieved using a 1½-min exposure time at 21°C. Paper electrophoresis showed that some 55% of ^{125}I was incorporated into peptide.

The elution profile from the CMC column is shown in Fig. 1. The first peak of radioactivity was unreacted iodine and subsequent peaks contained iodinated peptides with varying degrees of immunoreactivity. Binding of these fractions to excess R3 antiserum (final dilution, 1:1,000) was 14% (40 ml elution volume), 36% (60 ml), 47% (80 ml), 60% (110 ml), and 83% (130 ml). The main peak of radioactivity bound 94% (range 90–97% over 10 iodinations) of excess antiserum.

Chromatography of unlabeled LH-RH revealed a single, well-defined peak. Levels of unlabeled hormone at elution volumes of 170–200 ml (the peak of ^{125}I LH-RH) were 30–15 pg/ml. Thus, after dilution of label before use, and allowing for the fact that after iodination less than 5 μg unlabeled LH-RH is added to the CMC column, the contamination of ^{125}I LH-RH was <20 fg/100 μl of unlabeled LH-RH.

Specific activity and immunological studies. The results of plotting B/F ratios for serial dilutions of ^{125}I LH-RH and LH-RH are shown in Fig. 2. Fifteen picograms of ^{125}I LH-RH was added to the LH-RH curve as tracer. The ^{125}I LH-RH contained 760 pg/100 μl and the specific activity was calculated

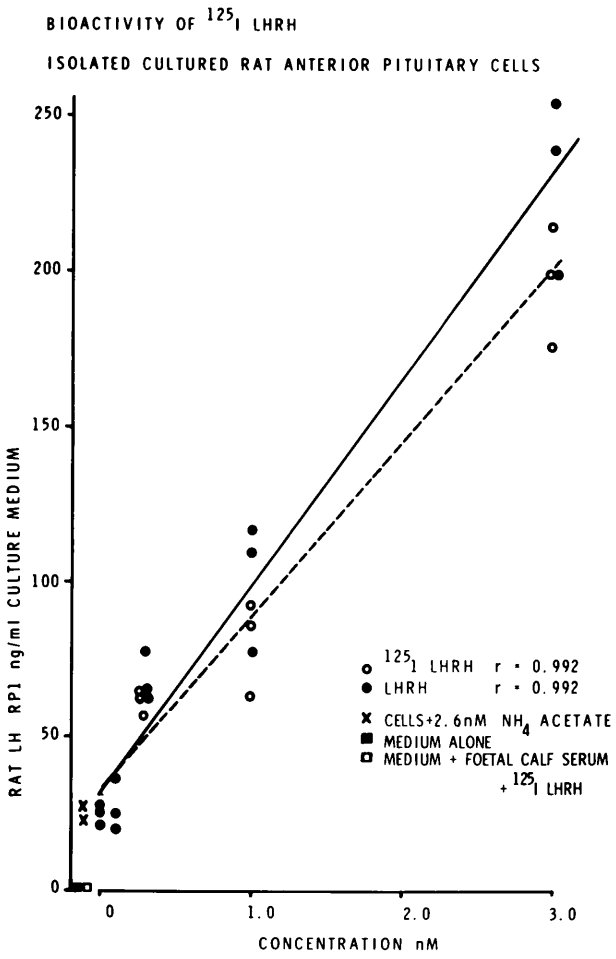

FIG. 3. Bioactivity of ^{125}I LH-RH isolated cultured rat anterior pituitary cells.

to be 1372 μCi/μg. Similar studies using other preparations of ^{125}I LH-RH revealed specific activities of 1250–1450 μCi/μg.

The ^{125}I LH-RH was stable for long periods when kept in the 0.06 *M* ammonium acetate at 4°C. Binding to excess antibody fell progressively from 96° to 90°, and nonspecific binding rose from 3.0 to 4.5% during repeated testing over a 14–wk period.

Immunoassays performed using the R3 antiserum and R42 antiserum (the latter kindly supplied by Dr. Niswender) showed that 5.0 and 0.2 pg/ml, respectively, of LH-RH could be detected in a buffer system.

Bioassay. The results of bioassay of ^{125}I LH-RH are shown in Table I. A linear dose-response relationship was found for both LH-RH and ^{125}I LH-RH, and there was no significant differences in the amount of rat hormones released by the iodinated preparation.

Membrane binding. Between 13% and 20% of the ^{125}I LH-RH was bound to the pituitary preparation, of which all but 3–5% was displaceable by 30 μg/ml of LH-RH. The binding was specific in that it was not displaced by LH, FSH, TSH, ACTH, TRH, LVP, Angiotensin II, and BSA. Scatchard plots showed the presence of two binding sites on the pituitary membrane with equilibrium constants of 5.4×10^9 liters/mole, and 1.7×10^6 liters/mole, respectively.

Discussion. Initial studies using chloramine-T iodination and G15 Sephadex column purification produced ^{125}I LH-RH which was not specifically bound to pituitary

membranes, only 75% of which was bound by excess antiserum. This could not be improved despite varying the amounts of reagents used in the iodination and using as little as 0.2 μg chloramine-T to iodinate 5 μg LH-RH. The lactoperoxidase–glucose–oxidase iodination method produced reliable iodination and minor changes to the procedure, described by Miyachi *et al.* (4) enabled the yield to be increased with reduced iodination time. Initial attempts to purify the ^{125}I LH-RH used 0.1 *M* ammonium acetate. In this system, the ^{125}I LH-RH emerged at an elution volume of 110–120 ml, and while 90% of the labeled hormone was bound by excess antibody, the fraction also contained 800–1000 pg/ml of unlabeled hormone. Progressive reduction of the concentration of eluant delayed the elution of ^{125}I LH-RH and enabled complete separation from the unlabeled hormone. This also allowed estimation of the specific activity of the ^{125}I LH-RH by immunoassay, and the calculated value is similar to the theoretical specific activity of mono-iodo-LH-RH (1600 μg/ml). The ^{125}I LH-RH retained biological activity, as has been shown for other monoiodo hormones insulin, ACTH and oxytocin (Freychet *et al.* 1971 [6]; Lefkowitz *et al.* 1970 [7]; Thompson *et al.* 1972 [8]), the iodinated hormone also bound to pituitary cell membrane with similar kinetics to those described by Grant *et al.* 1973 (9) using tritiated LH-RH.

The methodology described enables the preparation of stable ^{125}I LH-RH suitable for immunological, and membrane-binding studies which offers considerable advantages over tritiated material.

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