Immunofluorescent Localization of Rat Luteinizing Hormone* (33510)

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It has been shown previously by immunodiffusion, agglutionation-inhibition and neutralization studies that antibodies to human chorionic gonadotropin (anti-HCG) cross-react with human luteinizing hormone (LH) (1–3). Subsequently, anti-HCG has been used with immunofluorescent techniques to localize LH in sections of human pituitary glands (2, 4–7), and homologous anti-LH sera have been used to localize LH in pituitary glands of other species (8, 9). Since widespread immunologic species cross-reactivity had been demonstrated for other hormones (10), it seemed possible that anti-HCG might cross-react with rat LH. If such a cross-reaction existed, this antiserum could be used with the fluorescent antibody technique to localize LH in the pituitary gland of the rat.

Materials and Methods. Antiserum. As reported previously (11), antiserum to HCG was prepared in adult rabbits by repeated subcutaneous injection of commercial HCG emulsified in complete Freund's adjuvant. The antiserum was absorbed with normal human serum and an extract of male urine until a single precipitin band was obtained on immunodiffusion with preparations containing HCG. This antiserum was precipitated three times with ammonium sulfate at half saturation to obtain a crude globulin fraction. The anti-HCG globulin was then conjugated with fluorescein isothiocyanate and absorbed with activated wood charcoal and human liver powder to reduce nonspecific staining (12). Fluorescein-labeled normal rabbit globulin (fl-NRG) was prepared similarly.

Rat LH. Pituitary glands from adult, female rats which had been ovariectomized 60 days prior to sacrifice were extracted three times in acetone and dried. The acetone-dried powder was dispersed in distilled water and the extract lyophilized.

Rat pituitary glands. Four groups of adult female rats were used; three groups were treated in a manner presumed to alter the LH content of the pituitary gland. The treated rats were: (i) adult female rats which had been ovariectomized 4 weeks prior to sacrifice; (ii) adult female rats which had received one intramuscular injection of 10 mg of testosterone cyclo-pentyl propionate (Depo-Testosterone-Upjohn) per week for 4 weeks; and (iii) adult female rats which had been treated with estrogen in the form of a 1.25 mg of cholesterol pellet containing 25% diethylstilbestrol implanted subcutaneously 25 days prior to sacrifice. Some pituitary glands from each group were stained by the fluoresent antibody technique and the remainder were assayed for LH activity by the ovarian ascorbic acid depletion (OAAD) assay (13).

Pituitary glands which were used for histological study were fixed in Bouin's fluid for 18 hr, washed in multiple changes of 0.01 *M* phosphate buffered saline at pH 7.0 (PBS) for 3 days at room temperature, and embedded in paraffin. Adjacent 4 μ serial sections were stained with (a) hematoxylin and eosin, (b) fluorescein-labeled anti-HCG (fl-anti-HCG), and (c) fl-NRG or fl-anti-HCG absorbed with its antigen. Coverslips were mounted with a mixture of nine parts glycerin to one part PBS. To determine whether cells thought to contain LH were periodic acid–Schiff (PAS) positive, fluores-

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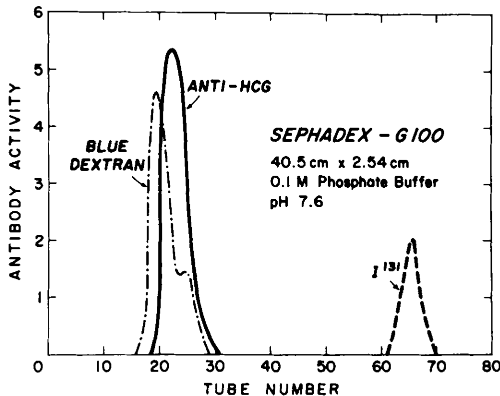

FIG. 1. Characteristics of reagents and Sephadex G-100 column. Rat LH (not shown) was eluted in the region between tubes 31 to 37.

cent-positive cells were photographed, the coverslips were removed, and the slides were restained by the PAS procedure. The identical cells in the same sections were then relocated and photographed for the second time.

Demonstration of immunochemical cross-reactivity. Although attempts to neutralize the biological activity of rat LH with this antiserum were unsuccessful, the possibility remained that anti-HCG reacted with rat LH resulting in the formation of soluble complexes which retained biological activity. If this were true, antibody-LH complexes would have an effective molecular size greater than that of LH alone, and would show a different elution profile with gel filtration chromatography. To test this hypothesis the elution characteristics of the reagents and rat LH were first determined on a column of Sephadex G-100 using 0.1 *M* phosphate buffer at pH 7.6. Three-ml aliquotes were collected, selectively pooled, and assayed by the OAAD method to determine the LH activity in the column effluent. Next the fraction rich in rat LH was mixed separately with both antiserum and normal rabbit serum. The two mixtures were then independently fractionated on the column and 3-ml aliquotes were again collected, similarly pooled, and assayed for LH activity.

Results. Figure 1 shows the characteristics of the reagents and the Sephadex G-100 column used in the study. Anti-HCG, as measured by its ability to react with radioactively-labeled HCG, was eluted just behind 2 million molecular weight blue dextran which is completely excluded from Sephadex G-100. Although not shown in the figure, rat LH was eluted in the region between tubes 31 and 37.

The LH activity in the column effluent when the LH-NRG mixture and the rat LH-anti-HCG mixture were fractionated separately is summarized in Fig. 2. The solid bars (LH-antiserum mixture) indicate that most of the activity was found in the region between tubes 18–24, the same region in which anti-HCG was found (Fig. 1). Thus one can conclude that LH does react with anti-HCG, forming soluble, biologically active complexes. The clear bars (LH-NRS mixture) show that practically all the LH activity was found in the region between tubes 31–37, the same region in which hormone was found when it was passed through the column in the absence of additional protein. This indicates that LH does not react with nonimmune rabbit serum.

To determine whether cytoplasmic fluorescence in sections stained with fl-anti-HCG indicated the presence of LH, adjacent serial

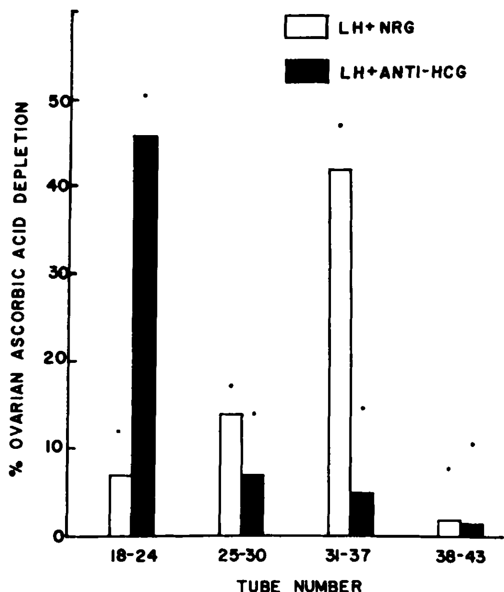

FIG. 2. LH activity in the column effluent after separate fractionation of LH plus NRS, and LH plus anti-HCG; dots represent one standard error.

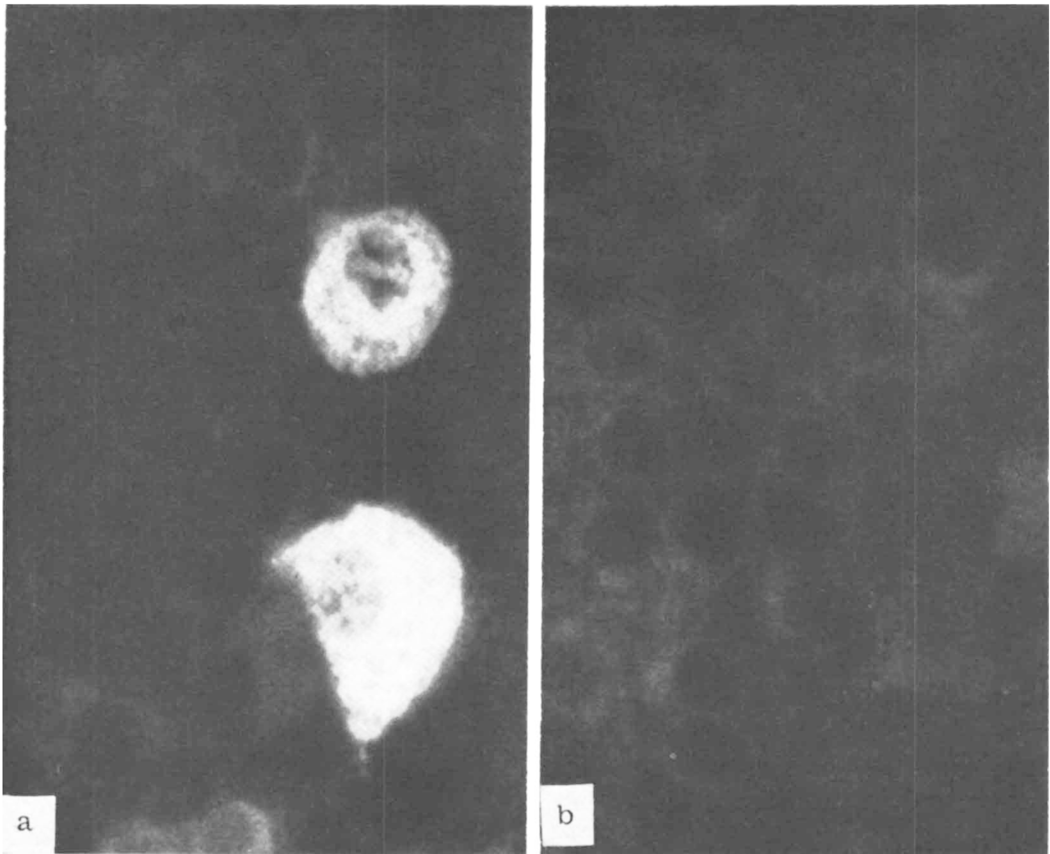

FIG. 3a. Two fluorescent-positive cells in a pituitary gland from a normal female rat stained with fl-anti-HCG. (b) The adjacent serial section stained with fl-NRS.

control sections were stained with either fl-NRG or fl-anti-HCG absorbed with its antigen. These control sections were all devoid of specific fluorescence (Fig. 3b). Nonlabeled anti-HCG blocked subsequent staining by fl-anti-HCG, whereas pretreatment with nonlabeled NRG had no appreciable effect on subsequent staining by fl-anti-HCG. In addition, fl-anti-HCG did not stain tissues other than pituitary including uterus, ovary, intestine, liver, kidney, brain, adrenal, spleen, lung, and heart. Accordingly, it was concluded that specific cytoplasmic fluoresecence represented localization of the reaction between anti-HCG and the immunologically cross-reactive portion of rat LH.

Positive cytoplasmic fluorescence (Fig. 3a) was found in isolated and occasionally clustered parenchymal cells in sections of pituitary glands from each of the untreated male and female rats. Most fluorescent-positive cells appeared to be diffusely stained although some appeared stippled and had a granular pattern. The resolution of the immunofluorescent technique was insufficient to determine whether this stippling was due to staining of cytoplasmic granules or areas between granules. Positive cells were distributed through the pars distalis of the adenohypophysis but the majority were located in the periphery of the gland. The fluorescent-positive cells were PAS positive, but the intensity of the histochemical staining bore no correlation with the intensity of fluorescence. In general, cells containing LH were stained lightly with PAS and were coarsely and sparsely granulated. The majority of cells which were stained intensely by PAS did not react with fl-anti-HCG.

If the fluorescent-positive cells contained

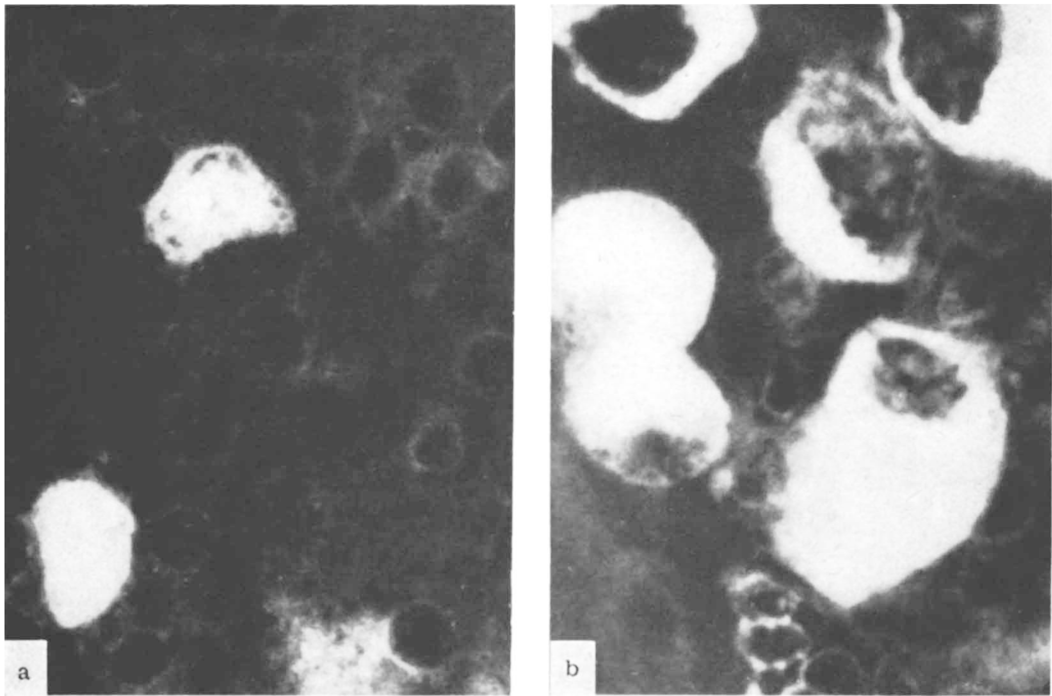

FIG. 4a. Fluorescent-positive cells in a pituitary from an untreated animal. (b) Hypertrophic fluorescent-positive cells in a pituitary gland from an ovariectomized rat. Both slides were photographed at same magnification.

rat LH one would expect a direct correlation between fluorescence as seen on histological sections and LH content as measured by the OAAD assay. The results of such a study are summarized in Table I. The LH content of the pituitary glands from the group which received no treatment was equivalent to 6.0 μg of NIH-LH-S5/mg of acetone dried pituitary powder. The size of the fluorescent-positive cells was arbitrarily called "normal" and their frequency was "moderate." Pituitary glands from overiectomized animals showed a 3.5-fold increase in LH concentration as compared to the control groups. The fluorescent-positive cells in these glands were hypertrophic with a diameter 2 to 3 times that of similar cells in nontreated animals and were increased in number (Fig. 4). Pituitary glands from animals treated with either testosterone or estrogen had approximately 1/10 the LH concentration of the control group. The size of the fluorescent-positive cells was normal, but there were very few such cells in these pituitary glands. Although the concentration of FSH in the pituitary glands from the testosterone-treated animals

TABLE I. Correlation of Pituitary Gland Concentration of LH with Immunofluorescence in Treated and Untreated Rats.

Treatment	Relative potency (μg/mg)[a]	95% C.L.	λ	Size of fl+ cells	No. of fl+ cells
None	6.0	4.2 – 8.8	.183	Normal	Normal
Ovariectomy	21.0	14.1 –31.3	.175	Enlarged	Increased
Estrogen	0.4	.28– .71	.213	Normal	Decreased
Testosterone	0.6	.42– .92	.179	Normal	Decreased

[a] Expressed as μg equivalents of NIH-LH-S5 per mg of dry wt. of pituitary gland.

in this study was not determined, pitutary glands from animals treated in a similar manner contained high levels of FSH (>200 μg of NIH-FSH-S-1/mg of pituitary powder) (14).

Discussion. The positive correlation between pituitary LH concentration and immunofluorescence suggests that the fluorescent-positive cells contain rat LH. Although attempts to neutralize rat LH with anti-HCG were unsuccessful, anti-HCG was shown to cross-react with rat LH. The soluble complexes formed in this cross-reaction were of interest since they retained biological activity. Whether the antigen–antibody complex per se possessed biological activity or whether the complex was first dissociated in the test animal was not determined.

Special note should be made of the method employed for fixation of rat LH. The procedure used was the only method by which fluorescent positive cells could be consistently demonstrated in the rat pituitary gland when stained with fl-anti-HCG. Other methods of fixation including formalin, cold acetone, and freeze–drying were unsatifactory.

Various investigators using histochemical techniques have noted different distributions for the gonadotropin containing cells in the rat pituitary gland. Purves and Griesbach (15) using a PAS stain, were able to differentiate two types of gonadotrophs—"folliculotrops and interstitiotrops." The interstitiotrops, which presumably contained LH, were described as having a central location, often adjacent to the pars intermedia while folliculotrops, or FSH containing cells, were concentrated at the periphery of the gland. Rennels (16), and Hildebrand *et al.* (17), using different staining techniques, were also able to distinguish two functional types of gonadotrops but they found the distribution of cells opposite to that described by Purves and Griesbach. The distribution of the LH containing cells as demonstrated by immunofluorescent technique is in agreement with that reported by these latter authors, namely, peripheral.

It is unlikely that the fluorescent positive cells contained predominantly FSH, and not LH, for there is no evidence that anti-HCG antiserum cross-reacts with rat FSH. Although the pituitary glands from the animals treated with testosterone or diethylstibestrol, had low concentrations of LH, the pituitary glands from the testosterone-treated animals had high concentrations of FSH. Both groups of pituitary glands,however, showed few fluorescent positive cells thus indicating that the anti-HCG serum did not cross-react with rat FSH. The distribution and characteristics of the cells containing FSH were not determined and will be the subject of future study.

Summary. Anti-HCG cross-reacted with rat pituitary LH resulting in the formation of soluble complexes which were biologically active as measured by the OAAD bioassay. Pituitary glands from castrated rats, presumably containing elevated levels of LH, showed an increased number of hypertrophic fluorescent positive cells. Conversely, pituitary glands from rats which had received either diethylstibestrol or testosterone cyclopentylpropionate for 1 month, treatments expected to result in lowering of pituitary LH content, showed minimal fluorescence. These observations strongly suggest that the immunofluorescent-positive cells contain rat LH.

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A Study of the Lipids of the Rat Aorta During Induced Calcification (33511)

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Gilman *et al.* (1) produced calcification in the aortas of rats by administering massive oral dosages of vitamin D_2. This model of induced ectopic calcification has been used by Fleisch *et al.* (2), Irving *et al.* (3) and others to study factors controlling the calcification process. As had been consistently observed before in normal calcifying tissue (4–7), Irving *et al.* (3) found that calcified areas of the rat aortas, so produced, were strongly Sudan black positive, whereas normal aortas were only weakly stained. This model system seemed useful for the study of lipids in calcifying tissue because calcification could be induced under carefully controlled conditions. Any obvious changes in the lipid composition accompanying the aortic calcification should be of significance in elucidating the role of lipids in the calcification process.

Materials and Methods. Two experiments were performed in which 150–200 g female rats of the Wistar strain were given large oral dosages of vitamin D_3 for 5 consecutive days, the control groups receiving none. The animals were sacrificed on the fourth day after the final dosage of vitamin D_3. In the first experiment 75,000 IU of vitamin D_3/kg of body weight per day were given; in the second experiment 75,000 IU/rat per day were administered. The aortas (from the aortic arch to the bifurcation into the iliac arteries) were dissected free, collected in a solution of 0.9% NaCl at 4°, carefully cleaned of adhering tissue, split lengthwise, rinsed three times with fresh cold saline, blotted free of excess moisture, weighed. Lipids were extracted from the pooled fresh tissues with choloroform–methanol (8) using five consecutive extractions, the first of which lasted overnight; the rest allowing 1–2 hr contact between tissue and solvent. The aortas were then dried under N_2, weighed, and decalcified with 100 ml of 0.5 *M* EDTA pH 8.0, at 4° for 24 hr. The decalcified aortas were rinsed repeatedly with cold (4°) distilled water to remove the EDTA, blotted free of excess moisture and reextracted with chloroform–methanol five times as before. To insure complete removal of lipids, the tissues were reextracted five more times with chloroform–methanol–conc. HCl, 200:100:1 (v/v) (9). Lipid extracts were purified by previously published methods (10). Phospholipids were analyzed using paper chromatography (11); nonpolar lipids being analyzed by the method of Amenta (12) using silica gel–loaded glass-fiber paper (Gelman Instrument Co., Ann Arbor, Michigan, chromatography media I.T.L.C., type SA, 20 × 20 cm) with solvent systems adapted from the method of Freeman and West (13).

For chemical analysis, cleaned aortas from six rats of each experimental group were blotted free of excess moisture, weighed, freeze dried, and reweighed, moisture content being determined by difference. Calcium and inorganic phosphorus were analyzed from