Single Step Purification of Ovine Luteinizing Hormone by Affinity Chromatography*

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SUMMARY

Affinity chromatography has been applied to the problem of specifically removing luteinizing hormone from sera used to supplement tissue culture media. Horse or fetal calf sera, passed through a column of an anti-luteinizing hormone immunoglobulin fraction linked covalently to agarose, were specifically depleted in their content of luteinizing hormone as seen by radioimmunoassay and by their effect on cultures of ovarian cells dependant on luteinizing hormone for their growth.

Affinity chromatography has also been applied to the one step isolation of luteinizing hormone from a partially purified or crude extract of ovine pituitary glands. Partially purified extracts or crude extracts of pituitary glands, passed through a column of an anti-luteinizing hormone immunoglobulin fraction linked covalently to agarose, were depleted in their luteinizing hormone content. Elution of the column by 6 M guanidine HCl pH 1.5 resulted in the recovery of the hormone which was further characterized by chemical and biological analysis as well as its electrophoretic pattern. It was demonstrated that affinity chromatography can be used to isolate preparations of highly homogeneous ovine luteinizing hormone in one step.

Affinity chromatography has also been applied to the problem of removing specific hormones from tissue culture medium (11). Cultures of ovarian cells dependent on LH are grown in media supplemented with sera (11). To study the trophic effect of LH on these cells, it was mandatory that the serum added to the media did not contain any LH. Affinity chromatography in this case offered the advantage that the LH could be specifically removed from the serum used to supplement the growth medium without removing other factors necessary for either cell survival or growth (12-14).

This report presents the results of these studies.

EXPERIMENTAL PROCEDURE

Materials and Methods

Materials

Analytical grade reagents were used in all experiments. Ovine pituitary glands were obtained from Reynolds Co., Melbourne (Australia). Female New Zealand white albino rabbits, 3 months old, were obtained from a local source. Sepharose 4B was from Pharmacia. Guanidine HCl, ultrapure, was from Schwarz-Mann, 131I was from Cambridge Nuclear Co., and 125I was from Tracerlab. NIH-LH-S17 was a gift from the Endocrine Study Section.

Antiserum Preparation

A modified version of the immunization procedure of Mougial and Li (15) and Li et al. (16) was used. Recipient animals were albino rabbits of approximately 3 kg. Each animal received a total of 10 mg of antigen. The antigen used was a highly purified preparation of ovine LH. One-milligram portions were dissolved in 0.9% NaCl solution, emulsified in Freund's adjuvant, and injected in two separate subcutaneous locations and intraperitoneally. The animals were rested 2 weeks between a second and third identical series of injections. A special LH-alum suspension was prepared by mixing 10 mg of LH in 6 ml of PBS with 3.2 ml of 10% alum in water. An amount of 1 N NaOH, (1.2 ml) was added and followed with 0.6 ml of 0.4 N NaO1 to bring the pH to 7. One week after the third series of injections, 0.5 ml of the alum suspension was given to each animal intravenously and intraperitoneally. The animals were bled 1 week after the last booster dose. The serum was separated and stored without preservative. Booster injections of highly purified ovine LH were given on the day following the venesection and the...
bleeding-booster procedure was repeated at weekly intervals for 5 to 10 bleedings.

Antisera to a k type light chain was prepared by the same method. The k type light chain was produced by a transplantable Balb/c mouse myeloma tumor P3, and was purified from urine of tumor bearing mice by the method of Melchers et al. (17). The antigen was a fraction from the DEAE-cellulose column that was shown by starch gel electrophoresis to contain light chains mostly as monomers with a trace of dimers.

**Antiserum Analysis**

The rabbit antiserum was characterized by quantitative precipitin test using the method of Li et al. (16), by immunoelectrophoresis according to the method of Scheidegger (18), by double diffusion as described by Ouchterlony (19), and by radiimmunooassay.

**Quantitative Precipitin Test**—Increasing amounts of LH in solution in 0.1 ml of PBS were added to 0.3 ml portions of antiserum. The mixture was diluted to 0.6 ml with PBS, incubated at 37° for 1 hour, and then for 18 hours at 4°. The precipitates were collected by centrifugation, washed three times with cold PBS, and dissolved in 0.2 ml of 0.2 M KOH. Protein was determined by the method of Lowry et al. (20) and the amounts of antibody and antigen precipitated were calculated using a standard curve of the absorbancy of rabbit IgG and LH. The sera contained 1.9 to 2.5 mg of anti-LH per ml and precipitated 280 to 300 μg of LH per ml at equivalence.

**Double Diffusion Test**—Precipitin tests by double diffusion in 1% agar in PBS were done at room temperature for 24 hours. Concentrations of LH tested were 1 and 0.5 mg per ml. Approximately 25 μl of solution filled a well. Under these conditions, reaction of sera with LH yielded a single precipitin line.

**Immunoelectrophoresis**—Electrophoresis was done in 1% agar in PBS were done at room temperature for 24 hours. Concentrations of LH tested were 1 and 0.5 mg per ml. Approximately 25 μl of solution filled a well. Under these conditions, reaction of sera with LH yielded a single precipitin line.

**Radioimmunooassay**—When analyzed by radioimmunooassay, the sera showed less than 0.01% cross-reaction against ovine follicle-stimulating hormone or ovine thyrotropin.

**Iodination of LH**

Iodination of highly homogeneous preparation of ovine LH was carried out as described by Hunter (21), utilizing light iodination to insure minimal damage to the hormone. LH, 1 nm, was incubated with 2.2 mCi of 125I (1 nm based on an activity of 17 Ci per mg at 100% enrichment) and 2 nm of chloramine T. The ovine LH was subsequently purified by chromatography on a Whatman CF 11 cellulose column (1 x 0.4 cm) (22) followed by gel filtration on Sephadex G-200. 125I-LH had a final activity of 30 Ci per mm and when assayed by the quantitative precipitin test, showed the same equivalence zone as native LH. Radioactivity, 90% to 95%, precipitated in the form of 125I-LH-IgG complexes.

**Hormone Preparation**

**Crude Extract**—Ten grams of pituitary tissue were homogenized in 20 ml of PBS in a vortex blender at 0° and then agitated for 2 hours at the same temperature. Nuclei and mitochondria were removed from the homogenate by centrifugation at 12,000 g for 10 min. Ribosomes and membranes were removed by sedimentation at 150,000 g for 3 hours. The supernatant was collected and used as crude extract.

The protein concentration measured by the method of Lowry et al. (20), was 60 μg per ml. The concentration of LH measured by quantitative precipitin test was 3 μg per mg of proteins.

**Partially Purified Extract**—Partial purification was performed by the method of Pankoff et al. (10). Following the addition of (NH₄)₂SO₄ to 0.5 saturation, the precipitate was collected by centrifugation, suspended in 0.2 m K₂HPO₄, and heated to 55-60° for 3 min. The suspension was then immediately chilled in an ice bath for 5 min and then dialyzed for 24 hours. The dialysate was collected and the precipitate which formed during dialysis was separated by centrifugation. The supernatant fluid was lyophilized and used as the partially purified extract. The concentration of LH measured by the quantitative precipitin test was 50 μg per mg of protein.

**Purified LH**—Highly purified preparations of ovine LH were obtained using the method of Pankoff (10), and were further purified by chromatography on diethylaminoethyl cellulose to remove contaminating thyroid-stimulating hormone, as described by Pierce and Carsten (29). The biological activity measured by the ovarian ascorbic acid depletion assay was 2.75 units per mg (95% confidence limits 2.1 to 3.7) referred to the standard NIH-LH-S17. Thyroid-stimulating hormone activity amounted to 5 to 20 milliunits per mg. Contamination by other pituitary hormones such as growth hormone, follicle-stimulating hormone, and adrenocorticotropic hormone, or melanocyte-stimulating hormone could not be detected. The LHβ subunit was isolated by counter current distribution as described by Pankoff and Samy (24) and modified by Reichert et al. (25).

**Preparation of Immunoadsorbant**

Immunoadsorbant preparation techniques varied with the anti-LH titer of the serum used. Serum samples were designated low titer if LH specific IgG concentrations were less than 800 μg per ml of serum. Sera with more concentrated titer were called high titer.

1. Preparation with High Titer Antiserum—

**Concentration Step**—Ammonium sulfate concentration of IgG was performed by adding (NH₄)₂SO₄ to 95 ml of serum, containing 180 mg of anti-LH specific IgG, until 40% saturation was reached. The precipitate formed was collected, dissolved in 20 ml of buffer containing 0.6 m NaCl and 0.2 m potassium phosphate at pH 7.2, and dialyzed overnight against the same buffer. The protein concentration at that step was 780 mg of which 140 mg were IgG specific against LH as determined by the quantitative precipitin test. It was able to precipitate 16 mg of LH at equivalence.

**Coupling to Sepharose 4B**—The IgG fraction was coupled to Sepharose 4B using Cuatrecassas' (3) modification of Porath's method (2). Fifty grams of hydrated Sepharose were activated with cyanogen bromide for 16 min at pH 11 using 250 mg per g of wet Sepharose. The mixture was then washed with 1 liter of the phosphate-0.35% NaCl solution buffer used above at 0°. The activated Sepharose was then mixed with the IgG fraction at a ratio of 15 mg per g of wet Sepharose and stirred at 4° for 12 hours. The gel was washed with the same buffer until no further protein was eluted and then in rapid succession washed three times with 300 ml of 6 m urea, 0.1 m phosphate buffer at pH 7.3, and 6 m guanidine HCl at pH 1.5. The total yield of the coupling step was 95%. The anti-LH IgG Sepharose had a capacity of 250 μg of LH per g of wet Sepharose. It was stored in PBS, 10-4 m mercaptoethanol, at 4°.

2. Preparation with Low Titer Antiserum—Instead of using the
of 250 pg of LH per ml of anti-LH IgG Sepharose was routinely obtained with high titer antisera (Fig. 1).

Adsorption and Elution of LH from Anti-LH IgG Sepharose Column

Trace amounts of $^{3}H$-LH in PBS were mixed with various quantities of LH in solution in PBS. Graded concentrations of $^{3}H$-LH were added to 0.3 ml of fetal calf serum and applied on small columns (1 x 0.4 cm) of anti-LH IgG Sepharose equilibrated with PBS. The eluent was collected in 0.1-ml fractions, mixed with 15 ml of Bray's solution containing 4% Cab-O-Sil and counted in a liquid scintillation vial (Nuclear Chicago model Unilux II). The column was washed with PBS until no more radioactivity was eluted and 6 M guanidine HCl effluent was neutralized at pH 7.3 and dialyzed overnight against 0.6 M NaCl, 0.2 M potassium phosphate pH 7.2. The IgG fraction was coupled to the Sepharose 4B as described for the high titer serum with a ratio of 15 mg of IgG per g of wet Sepharose. Immunoadsorbants prepared with high and low titer antisera were used with similar results in the adsorption of the LH present in horse or fetal calf serum.

Preparation with IgG Anti-P4 Light Chain—The IgG fraction of the antisera raised against P4 $\alpha$ light chain was concentrated by (NH$_4$)$_2$SO$_4$ precipitation and coupled to Sepharose 4B as described earlier with a ratio of 15 mg of IgG per g of wet Sepharose.

Determination of Capacity of Anti-LH IgG Sepharose

Trace amounts of $^{3}H$-LH in PBS were mixed with various quantities of LH in solution in PBS. Graded concentrations of $^{3}H$-LH were added to 0.3 ml of fetal calf serum and applied on small columns (1 x 0.4 cm) of anti-LH IgG Sepharose equilibrated with PBS. The eluent was collected in 0.1-ml fractions, mixed with 15 ml of Bray's solution containing 4% Cab-O-Sil and counted in a liquid scintillation vial (Nuclear Chicago model Unilux II). The column was washed with PBS until no more radioactivity was eluted and 6 M guanidine HCl effluent was neutralized at pH 7.3 and dialyzed overnight against 0.6 M NaCl, 0.2 M potassium phosphate pH 7.2. The IgG fraction was coupled to the Sepharose 4B as described for the high titer serum with a ratio of 15 mg of IgG per g of wet Sepharose.

Adsorption and Elution of LH from Anti-LH IgG Sepharose Column

Trace amounts of $^{3}H$-LH were added to the crude extract or the partially purified extract of pituitary glands dissolved in PBS (final concentration 10 mg per ml). The extract was then layered on and passed through a column (15 x 1.1 cm) of anti-LH IgG Sepharose at room temperature. The column was extensively washed with PBS to remove all nonspecifically adsorbed proteins and then successively by 4 M MgCl$_2$, 0.2 M potassium phosphate pH 7.2, and 6 M guanidine HCl pH 1.5.

In order to monitor the kinetics of adsorption and elution of the $^{3}H$-LH, the radioactivity of 0.2-ml portions of the column effluent was determined as described. The fractions containing the $^{3}H$-LH were then mixed, neutralized with NaOH to pH 7, and dialyzed against PBS or NH$_4$HCO$_3$, 0.2 M overnight. The LH was then kept in solution and its concentration was measured by the method of Lowry et al. (20) using purified LH as a standard. When amino acid analysis was to be performed, samples of LH were dialyzed against distilled water and lyophilized. When serum instead of pituitary extract was adsorbed on the column of anti-LH IgG Sepharose, the flow rate was reduced from 100 to 20 ml per hour to allow enough time for the specific binding of LH to IgG to occur despite the presence of other proteins at high concentration.

Polyacrylamide Gel Electrophoresis

LH was analyzed by disc gel electrophoresis at pH 4.5 (29). Cross linked 12% gels were used. It was also analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis as described by Weber and Osborn (30) with slight modifications (31).

Amino Acid and Carbohydrate Analysis

Duplicate samples of LH (500 pg at a final concentration 1 mg per ml) were hydrolyzed in 6 N HCl in evacuated sealed tubes at 110° for 24 hours. They were evaporated to dryness over NaOH in a desiccator. Analyses were performed with a Spinco 120B amino acid analyzer by the method of Spackman et al. (32).

The amounts of glucosamine and galactosamine were determined by chromatographic separation and ninhydrin reaction utilizing the standard resins in the Spinco 120B amino acid analyzer. Glycoprotein samples were hydrolyzed in 4 N HCl...
in evacuated sealed glass tubes at 110° for 6 hours. Determination of the hexosamine content was also carried out with the method of Galt and Bermann (33) using galactosamine HCl as a standard.

**Biological Assay and Radioimmunological Assay**

Luteinizing hormone activity was assayed by the ovarian ascorbic acid depletion assay (34) and expressed in terms of NIH-LH-S17. One milligram of this reference preparation was considered to have 1 unit of LH activity.

The immunological reactivity of ovine LH was examined in the homologous ovine LH-anti-ovine LH system. Radioimmunoassay of LH was performed as described by Niswender et al. (35). The antiserum (Rabbit 15) has been described elsewhere (35). The ovine LH anti-serum was used at an initial dilution of 1:25,000. Purified ovine LH for iodination (LER 2056-C2) was supplied by Dr. L. E. Reichert, Jr. (Emory University, Atlanta, Georgia). Radioiodination of LH was performed by the method of Greenwood et al. (36) as modified and described in detail by Niswender et al. (37). Thiosulfate free NaI was used at a concentration of greater than 800 mCi per ml. Duplicate standard curves were obtained with each assay using NIH-LH-S17 at concentrations between 0.1 and 25 ng. The mean of the duplicate values was used for each sample. Values which correspond to a displacement of 125I-LH from 10 to 50% were selected.

**RESULTS**

**Adsorption of LH Present in Sera (Fig. 1)—**Fetal calf serum or horse serum containing graded concentrations of LH mixed with traces of 125I-LH was passed through a small (1 ml) anti-LH IgG Sepharose column (Fig. 1). At low concentrations of LH (up to 250 μg), 5 to 10% of the radioactivity, passed through the column unadsorbed. Elution of the column with 6 M guanidine HCl resulted in a 92% recovery of the input as assessed from the elution pattern of the 125I-LH. The binding capacity remained constant even when large quantities of LH (up to 1 mg) were applied. When coupling of the anti-LH IgG to the Sepharose was carried out at pH 7.2, the capacity of the column amounted to 80% of the theoretical capacity of the IgG for LH (assuming that 2 moles of LH binds 1 mole of IgG). This confirms the original observation of Custaeas (3), that the binding capacity of IgG is increased by coupling at low pH. When coupling of the anti-LH IgG was carried out in 0.2 M bicarbonate buffer pH 8.9, only 20% of the theoretical binding capacity was obtained.

Adsorption of the LH normally present in sera was routinely done by adding trace amounts of 125I-LH to the sera and recycling it twice through the anti-LH IgG Sepharose column using a 10-ml column for 100 ml of serum. After two cycles, 90% of the radioactivity had disappeared. The LH content in the sera assayed by immunoassay before adsorption was 4 to 10 ng per ml. After adsorption, no LH activity could be detected although levels of 0.1 ng per ml would be detected by this method. The selective adsorption of LH was tested using an ovarian cell line dependent on LH for its growth (11). These cells maintained in normal serum or serum adsorbed with anti-P, IgG Sepharose grew at a normal rate. When serum passed through an anti-LH IgG Sepharose column was substituted for the unadsorbed serum, little or no growth was observed; however, when NIH-LH-B7 was added to the media, the growth of the cells resumed demonstrating that the missing factor in the adsorbed serum could be replaced by a preparation of bovine LH (11). No other factors necessary for growth and cellular differentiation aside from LH (11) were removed from the sera since the growth of 3T3 cells (11) was unaffected when they were maintained in adsorbed serum as compared to unadsorbed serum.

**Purification of LH Present in Partially Purified Extract of Pituitary Glands—**When 60 mg of partially purified extract of pituitary glands, to which trace amounts of 125I-LH had been added, was applied to a column (15 X 1.1 cm) of Sepharose, most of the protein passed through the column unadsorbed together with very little radioactivity. If the column was then eluted with 4 M MgCl2, 0.2 M potassium phosphate pH 7.2, a small peak of protein did appear but was not associated with any 125I-LH. When 6 M guanidine HCl was applied to the column, a peak of proteins containing 95% of the 125I-LH was eluted (Fig. 2). A yield of 2.7 mg of LH was obtained from 60 ml of a partially purified extract.

To test the efficiency of the binding of LH to the column and to make certain that native LH did not behave differently from 125I-LH as far as its immunologic properties were concerned, the different eluants were subjected to double diffusion in agar (Fig. 3). No LH was detected in the first peak as compared to the input (Fig. 3A). In contrast, LH was detected in the guanidine HCl fraction as compared to the first peak (Fig. 3B). The MgCl2 eluant did not contain any LH (Fig. 3C) while the various fractions of the guanidine HCl peak contained LH (Fig. 3, D and E). In contrast, no LH could be detected in the various fractions of the first peak (Fig. 3F).

The drastic elution conditions resulted in the dissociation of LH into its two subunits. In order to reconstitute LH, it was necessary to pool the fractions containing the hormone immediately after elution of the column, neutralize them and dialyze them overnight against PBS or 0.2 M bicarbonate buffer pH 8.3

![Fig. 2. Elution pattern of LH present in a partially purified extract of pituitary glands.](https://www.jbc.org/figure/2-fig2.png)
Only if these precautions were taken could significant biological and immunological activity be retained (Tables I and II). Dialysis against water and lyophilization both resulted in a drastic drop of biological activity.

The LH preparation thus obtained was further characterized by polyacrylamide gel electrophoresis at pH 4.5 (Fig. 4) which shows the purification achieved. The pattern of LH eluted by guanidine HCl was similar to that of highly purified LH (Fig. 4A) and similar results were obtained with sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 4B).

Further evidence of the purity of the LH was obtained from determination of the amino acid and carbohydrate compositions (Table III) which showed that the hormone preparation obtained by affinity chromatography was indistinguishable from the preparation obtained by conventional methods.

**Table I**

<table>
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<th>Preparations</th>
<th>Assay</th>
<th>Relative Potency</th>
<th>95% confidence limits</th>
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<td>2.4</td>
<td>1.88-3.23</td>
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<tr>
<td></td>
<td>2</td>
<td>2.3</td>
<td>1.62-3.15</td>
</tr>
<tr>
<td>Guanidine HClb</td>
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<td>1.4</td>
<td>0.82-2.16</td>
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<td>0.8</td>
<td>0.43-1.23</td>
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<td>1.3</td>
<td>0.82-1.97</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.3</td>
<td>0.78-1.86</td>
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<tr>
<td></td>
<td>3</td>
<td>1.2</td>
<td>0.75-2.83</td>
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* Expressed in terms of NIH-LH-S17.

**Table II**

<table>
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<th>Preparation</th>
<th>Immunological Activity</th>
<th>95% confidence limits</th>
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<tbody>
<tr>
<td>LH</td>
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<td>1.65-2.15</td>
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<tr>
<td>Guanidine HClb</td>
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<td>1.66-2.07</td>
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<tr>
<td>Guanidine HClc</td>
<td>1.41</td>
<td>1.21-1.62</td>
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</table>

* Measured by radioimmunoassay and expressed in terms of NIH-LH-S17.

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In order to obtain satisfactory purification of LH from a crude extract of pituitary glands, we found it necessary to start with a 150,000 g supernatant. If such a step was omitted, a wide variety of biological structures such as ribosomes containing nascent chains of LH or membrane fragments on which LH is adsorbed will bind to the column, thus resulting in poor purification.

**Table III**

<table>
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<tr>
<th>Preparation</th>
<th>Immunological Activity</th>
<th>95% confidence limits</th>
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<td>LH</td>
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<td>1.65-2.15</td>
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<td>Guanidine HClb</td>
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<tr>
<td>Guanidine HClc</td>
<td>1.41</td>
<td>1.21-1.62</td>
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</table>

* Obtained from a partially purified extract.

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![Fig. 3](http://example.com/fig3.png)

**Fig. 3.** A double diffusion study of the binding and elution pattern of LH present in a partially purified extract of pituitary gland run through an anti-LH IgG Sepharose column (elution pattern shown in Fig. 2). Antigens are in the outer wells. In clockwise order starting from the top are: A, LH 50 µg; unadsorbed peak 10 mg, unadsorbed peak 1 mg, LH 100 µg, input 10 mg, input 1 mg; B, LH 50 µg, guanidine HCl peak 500 µg, guanidine HCl peak 50 µg, LH 100 µg, unadsorbed peak 10 mg, unadsorbed peak 1 mg; C, MgCl₂ peak, Fractions 31, 32, 33, 34, 35, LH 60 µg; D, guanidine HCl peak, Fractions 52, 53, 54, 55, 56, LH 50 µg; E, guanidine HCl peak, identical to D but diluted 1:5 with PBS; F, unadsorbed peak, Fractions 8, 9, 10, 11, 12, LH 50 µg.

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![Fig. 4](http://example.com/fig4.png)

**Fig. 4.** A, polyacrylamide gel electrophoresis at pH 4.5; 1, partially purified extract of pituitary gland (500 µg); 2, guanidine HCl fraction (50 µg); 3, guanidine HCl fraction (100 µg). Electrophoresis for 90 min, 8 ma per tube, staining with Amido schwarz 1% in 7% acetic acid solution. B, sodium dodecyl sulfate polyacrylamide gel electrophoresis; 1, partially purified extract of pituitary gland (500 µg); 2, guanidine HCl fraction (15 µg); 3, guanidine HCl fraction (50 µg). Electrophoresis 300 min, 8 ma per tube, staining with Coomassie brilliant blue.
made for losses due to decomposition.

hydrolysis in 4

ovine system was 1.9 units per mg (Table II). In contrast, LH as measured by radioimmunoassay using a homologous ovine-

by A&&y Chromatography-The biological activity of the native ovine LH purified by the method of Papkoff et al. (10) was 2.4 units per mg (Table I) and its immunological activity was also found to be lower than that of native ovine LH.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>LHa</th>
<th>Guanidine HCl LH* partially purified extract</th>
<th>Guanidine HCl LH* crude extract</th>
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<tr>
<td>Lysine*</td>
<td>11</td>
<td>10.2</td>
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<tr>
<td>Glutamic acid</td>
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<tr>
<td>Proline</td>
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<td>21.5</td>
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<tr>
<td>Glycine</td>
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<td>10.9</td>
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<td>Alanine</td>
<td>12.1</td>
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<tr>
<td>Cystine (half)</td>
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<td>Methionine</td>
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<td>Isoleucine</td>
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<td>Leucine</td>
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<td>Tyrosine</td>
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<td>Glucosamine*</td>
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<tr>
<td>Galactosamine*</td>
<td>3.2</td>
<td>3</td>
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</table>

* Residues per 28,300 molecular weight (38).

Method of hydrolysis described in the text. Values obtained at 24-hours hydrolysis in 6 N HCl. No corrections have been made for losses due to decomposition.

Galactosamine and glucosamine values obtained at 6-hours hydrolysis in 4 N HCl.

When a 150,000 g supernatant of crude extract of pituitary gland (920 mg of protein) to which trace amounts of 3H-LH had been added, was applied to an anti-LH IgG Sepharose column (15 x 1.1 cm), similar results (see Fig. 5) to those obtained with a partially purified preparation were observed. Most of the proteins passed through the column unadsorbed, in association with very little radioactivity. No LH could be detected by double diffusion in agar. When the column was washed with 4 M MgCl2, 0.2 M potassium phosphate pH 7.2, a small peak of protein devoid of radioactivity was eluted. When 6 M guanidine HCl pH 1.5 was applied to the column, 90% of the radioactivity emerged with a peak of protein which was identified as LH by polyacrylamide gel electrophoresis at pH 4.5 (Fig. 6A), by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 6B) and by double diffusion in agar. Amino acid and carbohydrate analyses confirmed the homogeneity of the LH preparation (Table III). A yield of 280 µg of LH was obtained from 100 mg of a crude extract.

Biological and Immunological Activities of Ovine LH Purified by Affinity Chromatography—The biological activity of the native ovine LH purified by the method of Papkoff et al. (10) was 2.4 units per mg (Table I) and its immunological activity as measured by radioimmunoassay using a homologous ovine-ovine system was 1.9 units per mg (Table II). In contrast, LH purified by affinity chromatography, whether from crude extract or from a partially purified preparation, had half the biological activity of the native ovine LH. This was mostly due to the conditions of elution which dissociated the LH into its two subunits. When the subunits were allowed to recombine during dialysis against 0.2 M NH4HCO3 or PBS, no more than 50% recombination occurred as already reported (20). The immunological activity of the recombined subunits was also found to be lower than that of native ovine LH.

DISCUSSION

Affinity chromatography has already been shown to be a powerful tool for the purification of proteins (4). However, until recently only limited reports have appeared on the application of this technique to the purification of pituitary hormones, one of the reasons being the stringent experimental conditions required to elute the hormones from the column which resulted in their denaturation. This approach has proved satisfactory for the purification of insulin (4, 5) and has been found useful for the separation of prolactin from growth hormone, although no attempts to recover the growth hormone were made (6). Provided that the pooled fraction obtained by elution with 6 M guanidine HCl was immediately adjusted from pH 1.5 to pH 7.3, that dialysis was done against NH4HCO3, 0.2 M or PBS instead of distilled water, and that the LH was kept in solution rather than lyophilized, this method of purification can also be applied to LH. One of the difficulties we have encountered in preparing the immunoadsorbants arose from the necessity to start with an antiserum with a high titer of IgG directed against LH. When sera with low titers of antibody were used, nonspecific adsorption of protein became too large as compared to the specific adsorption of LH, and made a preliminary concentration step of the LH-specific IgG necessary. This step being tedious, we found it easier to start with a high titer antiserum which could only be obtained by boosting the animals with alum-precipitated LH.
that it does not have immunological sites which cross-react with anti-LH IgG.

A distinct advantage of this technique is that it makes it possible to isolate LH from animals, such as the rat, from which pituitary glands can be obtained only in limited supply. Since ovine and rat LH share common antigenic determinants, purification of rat LH using anti-LH IgG Sepharose should be possible on a microscale. A further advantage of this technique is that it yields follicle-stimulating hormone with low LH immunological activity. Using an immunosorbant column, we have been able to isolate follicle-stimulating hormone with as little as 0.005% LH contamination as determined by radioimmunoassay. This degree of contamination is the lowest recorded so far and lies at the limit of sensitivity of the radioimmunoassay.

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FIG. 6. A, polyacrylamide gel electrophoresis at pH 4.5; 1, 150,000 X g supernatant of pituitary gland (500 µg); 2, guanidine HCl fraction (100 µg); 3, guanidine HCl fraction (200 µg). Electrophoresis for 90 min at 8 ma per tube. Staining with Amido black 150,000 X g supernatant of pituitary gland (500 µg); 2, guanidine HCl fraction (100 µg); 3, guanidine HCl fraction (200 µg). Electrophoresis for 90 min at 8 ma per tube. Staining with Coomassie brilliant blue.

The second problem encountered in the affinity chromatography of LH was the extremely stable binding of LH to the antibody. Even 4 mM MgCl₂, which has been reported to dissociate antigen-antibody complexes (39), was inefficient. Elution with 6 mM guanidine HCl pH 1.5 resulted in the dissociation of LH into its subunits α and β. However, recombination of the two subunits during dialysis against PBS or NaH₂PO₄·H₂O, 0.2 M pH 8.3 did take place and a final preparation with 50% of the biological activity of the native LH was obtained (Table I). In contrast (Table II), the antigenicity of LH was less affected, this being mostly due to the fact that most of the antigenic sites of LH are in the β subunits and that LH does not need to be under its recombined form to be antigenic (26-28).

The practical implications of affinity chromatography of LH are numerous. Availability of LH-free serum has made it possible to follow the hormone-dependent growth response of a newly established rat ovarian cell line (11). Purification by affinity chromatography of LH from pituitary glands should also be helpful in establishing whether a pro-LH exists. So far we have been unable to observe components other than the α and β subunits of the hormone in the guanidine HCl eluant. In particular, no components heavier than the subunits or migrating as LH could be detected (Fig. 4B and Fig. 6B). This could mean either that pro-LH does not exist, that it is extremely labile, or

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