Preparation and Characterization of Plasma Membranes from Bovine Corpus Luteum*

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SUMMARY

A rapid method for preparing plasma membranes from bovine corpus luteum is described. Various fractions are isolated by differential centrifugation and discontinuous sucrose density gradients. The plasma membrane fractions have a density in sucrose of 1.16 and 1.18. The yield is 0.5 to 0.75 mg per g of corpus luteum. This fraction is characterized by electron microscopy, enzymatic assay, and binding properties of 125I-luteinizing hormone.

The plasma membrane fraction is free of nuclei and mitochondria when examined by electron microscopy. It is mainly vesicular in nature, with a unit membrane structure evident in most sections. The cholesterol to phospholipid ratio is 0.72. A small amount of RNA is found to be associated with the plasma membrane but no DNA is detected.

5'-Nucleotidase and ouabain-sensitive ATPase are concentrated in the plasma membrane. No more than 3 to 8% contamination by mitochondria is indicated by the activity of the mitochondrial markers: cytochrome c oxidase and succinate dehydrogenase. The microsomal marker activities of glucose 6-phosphatase and glucose-6-P dehydrogenase are low, corresponding to 2 to 5% contamination by microsomes.

The binding of 125I-luteinizing hormone to the plasma membrane fraction is 20 times greater than its binding to the homogenate. The binding of 125I-luteinizing hormone to nuclei, mitochondria, and microsomes is low and parallels the 5'-nucleotidase activity of those fractions. It probably reflects their contamination by plasma membranes.

The initial event in luteinizing hormone action involves attachment to, and activation of, a hormone-specific receptor of the target cell. Receptor activation then triggers a series of reactions culminating in enhanced steroidogenesis. Localization, isolation, and characterization of the LH receptor is of fundamental importance for understanding the mechanism of action of this hormone. In order to determine the localization of the LH receptor site, we have analyzed the 125I-LH-binding capacity of different subcellular fractions obtained from bovine corpus luteum. The plasma membrane fraction contained most of the binding activity.

We have designed a reproducible procedure for the isolation of plasma membrane from bovine corpus luteum. By this method, large amounts of starting material, i.e., plasma membranes, can be available for the isolation of the receptor site of LH.

EXPERIMENTAL PROCEDURE

Materials

Glucose 6-phosphate, ouabain, and AMP type III were obtained from Sigma Chemical Co., St. Louis, Mo. ATP (disodium salt) was obtained from Schwarz-Mann Co., Orangeburg, N. Y. NADPH, NADH, NAD+, horse heart cytochrome c, and lactoperoxidase (grade B) were obtained from Calbiochem, San Diego, Calif. Sucrose was obtained from Merek Co., Rahway, N. J. Vestopal W was obtained from Polysciences, Inc., Warington, Pa. 125I Na carrier-free was obtained from New England Nuclear, Boston, Mass.

Highly purified preparations of ovine LH were prepared by the method of Papkoff et al. (1) and were further purified by chromatography on diethylaminoethyl cellulose to remove contaminating thyroid-stimulating hormone as described by Pierce and Carsten (2). Biological activity measured by 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 15 to 30 milliunits per mg. Contamination by growth hormone, follicle-stimulating hormone, adrenocorticotropic hormone, or melano-cyte-stimulating hormone could not be detected.

Chemical Determinations

Protein concentration was determined according to the method of Lowry et al. (3) with bovine serum albumin as standard. Particles (membranes, mitochondria, etc.) were solubilized in 0.2 M sodium hydroxide. Inorganic phosphate was measured by a modification (4) of the method of Burton et al. (5). One milliliter of 10% trichloracetic acid was added to the reaction mixture to stop the reaction. Then, 1.2 ml of a freshly made reagent consisting of 0.212 g of ammonium molybdate 4H2O, dissolved in 20 ml of 1.15 M H2SO4 and 1.465 g of FeSO4 7H2O, were added. The absorbance of the samples was read at 700 nm. Inorganic phosphate standards were prepared with KH2PO4 to contain between 0.1 and 1 μmole of phosphate. Phospholipids were measured according to the method of Chen.

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1 The abbreviation used is: LH, luteinizing hormone.

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et al. (6) and cholesterol by the method of Glick et al. (7). A factor of 35 was used to convert micrograms of phosphorous to micrograms of phospholipid.

RNA was determined by the method of Fleck and Monro (8) and DNA by the method of Burton (9).

**Enzyme Assays**

Enzymatic activities were assayed according to the following methods: total ATPase activity and ouabain-sensitive ATPase activity by a modification (4) of the method of Bonting et al. (5); 5'-mononucleotidease as described by Song and Bodansky (10); glucose-6-phosphatase by the method of de Duve et al. (11); cytochrome c oxidase according to the method of Cooperstein and Lazarow (12); NADH cytochrome c reductase according to the method of Phillips and Langdon (13); and glucose-6-P dehydrogenase according to the method of Kelly et al. (14).

**Iodination Procedure**

Iodination of LH was accomplished using lactoperoxidase as the catalytic agent (15, 16). 125I-LH contained an average of 1 atom of iodine per molecule of hormone. The iodination of 1 tyrosyl residue per molecule of LH had no deleterious effect on the antigenicity of the molecule when it was analyzed by quantitative precipitin test and no effect on the binding of 125I-LH to antibody insolubilized by coupling to agarose (15, 16). The biological activity of the 125I-luteinizing hormone was indistinguishable from native luteinizing hormone when analyzed by the ovarian ascorbic acid depletion test or by its steroidogenic activity by a modification (4) of the method of Bonting et al. (5); 5'-mononucleotidease as described by Song and Bodansky (10); glucose-6-phosphatase by the method of de Duve et al. (11); cytochrome c oxidase according to the method of Cooperstein and Lazarow (12); NADH cytochrome c reductase according to the method of Phillips and Langdon (13); and glucose-6-P dehydrogenase according to the method of Kelly et al. (14).

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**I-II-binding Assay**

Binding of LH to the different subcellular fractions was done as described by Gospodarowicz (15).

**Preparation of Plasma Membrane Fraction**

Corpora lutea from dairy cows of mixed breeds were used. The duration of pregnancy was estimated from the crown-rump length of the fetus, and corpora lutea corresponding to a fetal age of 10 to 20 cm were taken. Ovaries were collected as soon as possible following slaughter and transported in ice-cold 0.9% NaCl solution to the laboratory where they were immediately dissected free from adventitial tissues. Luteal cells were scraped from the theca using a razor blade. Four corpora lutea yielded 18 to 22 g of tissue. Sucrose solutions were made with 0.025 M Tris, pH 7.5 at 8°, and 1 mM CaCl₂ (Buffer A). Solutions for the sucrose gradients were prepared on the basis of the percentage by weight of sucrose, and the final values were checked at 25°C in an Abbe-3L refractometer (Bausch and Lomb, Inc., Rochester, N. Y.). Homogenizations were performed in a Dounce glass homogenizer (size C from A. H. Thomas, Philadelphia, Pa.). Calculation of the volumes of all fractions obtained from sucrose gradients were made from values for the weight and the density of the fraction. The density (d) of each sample at 5°C was obtained from the Handbook of Biochemistry (17) after measuring its sucrose concentration in the refractometer. All centrifugations were performed at 2°C.

Tissues were diluted with 7 volumes of 0.25 M sucrose, and homogenized in a Dounce homogenizer with five strokes of a loosely fitting pestle (size A). The suspension was filtered through two layers of cheesecloth to remove the connective tissue and centrifuged in a clinical centrifuge (IEC) at 400 × g for 4 min. The supernatant was collected. Microscopic examination of the supernatant revealed cell fragments and nuclei with no intact cells. The pellet, which contained numerous cells and cell aggregates, was resuspended in 8 volumes of 0.25 M sucrose buffer and homogenized in a Dounce homogenizer with eight strokes of a tightly fitting pestle (size B). The homogenate was centrifuged as described. The supernatant was decanted and pooled with the first supernatant and the pellet put aside for the preparation of nuclei. The pooled supernatants were centrifuged at 700 × g for 10 min (Sorvall RC2B centrifuge with SS 34 rotor). The pellet was discarded and the supernatant centrifuged under the same conditions at 9000 × g for 15 min. The supernatant was put aside for the preparation of the endoplasmic reticulum. The pellet was homogenized in 20 ml of 0.25 M sucrose buffer in a Dounce homogenizer with two strokes of a loosely fitting pestle. Ten milliliters of homogenate were layered on a discontinuous sucrose density gradient made by layering in a 30-ml IEC polycarbonate tube, 5 ml of 50% sucrose (d: 1.23), 8 ml of 40% sucrose (d: 1.18), 8 ml of 35% sucrose (d: 1.16), and 5 ml of 30% sucrose (d: 1.14). The gradients were centrifuged at 66,000 × g for 90 min (IEC preparative centrifuge model J60 with a SI 110 rotor). At the end of the centrifugation, four layers were obtained (Fig. 1). The first (top) layer (F₁) consisted mostly of endoplasmic reticulum. The second layer (F₂) and the third (F₃) were mostly plasma membranes while the fourth layer (F₄) was made up of mitochondria (Fig. 1). F₂ and F₃ were collected separately with a syringe, diluted 1:2 with Buffer A, and centrifuged at 100,000 × g for 45 min (Beckman model L preparative ultracentrifuge, with an S 30 rotor). Supernatants were discarded and the pellets from both the F₂ and F₃ fractions were resuspended in 0.25 M sucrose with two strokes of a loosely fitting pestle and layered on a discontinuous sucrose density gradient identical with the one used for the original homogenate. They were centrifuged as already described for 90 min at 60,000 × g. Two bands were obtained with F₃, one main band (F₃) corresponding to the interface (d: 1.16, 1.18), and a minor band (F₃) at the interface (d: 1.14, 1.16), and 5 ml of 30% sucrose (d: 1.14). The gradients were centrifuged at 66,000 × g for 90 min (IEC preparative centrifuge model J60 with a SI 110 rotor). The supernatant was put aside for the preparation of nuclei. The layers at the interfaces were collected with a syringe, diluted 1:5 with Buffer A, and centrifuged at 100,000 × g for 60 min in an S 30 rotor (Beckman model L). The pellets were then collected separately, resuspended in Buffer A, homogenized with two strokes of a loosely fitting pestle, and either frozen at −20°C or kept in an ice bath at 0°C.

**Preparation of Other Subcellular Fractions**

To study the binding of 125I-LH to the different subcellular components, preparations of nuclei, mitochondria, and endoplasmic reticulum were made.

**Nuclei**—Homogeneous populations of nuclei were prepared by the method of Chauvoe et al. (18), as modified by Maggio et al. (19). As starting material, the 400 × g pellet obtained with the crude homogenate of corpus luteum was used. Microscopic examination of the final preparation did not reveal contamination by intact cells, cell debris, cytoplasm, or mitochondria.

**Mitochondria**—Layer F₂ (d: 1.18, 1.23) obtained by discontinuous sucrose density gradient was collected, diluted 1:3 with Buffer A, and centrifuged (Beckman ultracentrifuge model L) for 60 min at 100,000 × g in an S 30 rotor. The supernatant was discarded and the pellet homogenized in 0.25 M sucrose with two strokes of a loosely fitting pestle. The homogenate was
then layered on a discontinuous sucrose density gradient similar to the one used for the isolation of plasma membrane fractions and centrifuged in the SS 110 rotor of an IEC preparative ultracentrifuge at 66,000 × g for 90 min. The layer at the interface (d: 1.18, 1.23) was collected and centrifuged in a Beckman ultracentrifuge model L as already described. The final pellet was resuspended in 0.25 M sucrose buffer.

Endoplasmic Reticulum—The 9,000 × g supernatant obtained during the preparation of the plasma membrane was centrifuged at 100,000 × g for 180 min (Beckman preparative ultracentrifuge, model L, S 40 rotor). The pellet was resuspended in 0.25 M sucrose buffer and then layered on a discontinuous sucrose gradient similar to the one used for the preparation of the plasma membrane. It was then centrifuged at 66,000 × g for 90 min (IEC preparative ultracentrifuge, SS 110 rotor). The layer at the interface (d: 1.14, 1.16) was collected and centrifuged in a Beckman ultracentrifuge model L as already described. The final pellet was resuspended in 0.25 M sucrose buffer.

Electron Microscopy—Aliquots of the different membrane fractions diluted 10-fold with Veronal acetate buffer, pH 7.4, were centrifuged at 12,000 × g for 20 min (SR2B Sorvall centrifuge, rotor RSA). The supernatants were discarded and the pellets washed with Veronal acetate buffer, pH 7.4. They were fixed with 2% OsO₄ in this buffer, then dehydrated by rapid passage through an acetone series and embedded in Vestopal W. The pellets were embedded in gelatin capsules and allowed to polymerize at 60° for 48 hours. Sections of 200 to 300 Å were cut with glass knives on an LKB ultramicrotome and collected on 300-mesh copper grids coated with nitrocellulose. Sections were then stained with 1 to 2% uranyl acetate for 5 to 10 min and double stained with Reynolds lead citrate for 5 min. The sections were scanned with an Hitachi H11D electron microscope.

RESULTS

Purification of Plasma Membrane

Rationale

Plasma membrane fractions have previously been isolated from tissues such as liver (20-24), kidney (25), intestine (26, 27), bladder (28), as well as from single cells, such as those of HeLa (29), L (30) cell cultures, and Ehrlich ascites tumors (31). With few exceptions, the methods designed to isolate enzymatically active fractions are derived from the procedure originally employed by Neville (21). This involves gentle homogenization of the tissue to avoid extensive fragmentation of plasma membranes, repeated low speed centrifugation of the homogenate to sediment plasma membranes along with nuclei and cell debris, separation of plasma membranes from the other components of the nuclear pellet by gradient centrifugation, and purification of membranes either by repeated washing or by further gradient centrifugation.

For the corpus luteum we followed the same general procedure as Neville with less satisfactory results. First, the concentration of mitochondria in steroidogenic organs such as the corpus luteum is much higher than in the liver, and the numerous washing steps which serve to free the liver preparation from mitochondrial contamination had to be eliminated due to large membrane losses. Secondly, corpus luteum requires more vigorous homogenization than liver and the luteal cells fragment into smaller particles. Also, the low content in desmosomes of the luteal cells (32) results upon homogenization in a vesicular rather than a long sheet-like structure. Those vesicles require higher gravitational forces to sediment, resulting in a greater degree of mitochondrial contamination which can best be eliminated by discontinuous sucrose density gradient centrifugation.

Finally, homogenization of the corpus luteum in the presence of isotonic sucrose and 1 mM CaCl₂ offers definite advantage compared to the aqueous media used by Neville (21) and Emmerlot et al. (22). This media preserves the structural integrity of mitochondria and nuclei and reduces the contamination of plasma membrane fraction with DNA or mitochondrial outer membranes.

In the present study, we have assumed that 5′-nucleotidase is the most specific marker for the plasma membrane (10, 33, 34). A preliminary investigation of the 5′-nucleotidase activity in different fractions obtained from a 400 × g supernatant of bovine corpus luteum homogenate indicates (Table I) that 50% of the activity sediments between 700 and 9000 × g. On the basis of those results, the purification of the plasma membrane is effected using as starting material a 700 to 9000 × g cut of crude homogenate of corpus luteum.

Discontinuous sucrose density gradient resolved this fraction into four components (Fig 1). The first one at the top of the sucrose layer (d: 1.14) was mostly composed of microsomes and cell sap, as determined by enzyme assay. The layer at the interface 1.14, 1.16 is primarily plasma membrane contaminated with microsomes. The layer at the interface (d: 1.16, 1.18) is mostly made of plasma membrane contaminated with mitochondria, and the layer at the interface 1.18, 1.23 was mostly mitochondria. To eliminate the microsomes and the mitochondria trapped in the layer sedimenting at the interface 1.14, 1.16 and 1.16, 1.18, a second centrifugation on discontinuous sucrose density gradient is performed. Two fractions are obtained which contained plasma membranes, one sedimenting at the interface (d: 1.14, 1.16), and the second one at the interface (d: 1.16, 1.18).

Characterization of Two Isolated Plasma Membrane Fractions

Morphology—Fig. 2A illustrates the most common and characteristic structural feature of the fraction found at the interface between densities 1.14 to 1.16. The material consists mainly of fragments of membranes having a triple layered structure (Fig. 2B) and vesicles. These vesicles very likely originate from projections or infolding of the limiting membranes of the luteal cells in specialized regions of the cell surface. In some cases, fibrillar material is attached to the membranes. This may be

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Proteins</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
<td>2800</td>
<td>100</td>
</tr>
<tr>
<td>P₁ (700 × g)</td>
<td>3.3</td>
<td>200</td>
<td>2</td>
</tr>
<tr>
<td>P₁ (2,000 × g)</td>
<td>9.0</td>
<td>800</td>
<td>27.7</td>
</tr>
<tr>
<td>P₂ (6,000 × g)</td>
<td>12</td>
<td>530</td>
<td>23</td>
</tr>
<tr>
<td>P₃ (12,000 × g)</td>
<td>19.7</td>
<td>107</td>
<td>7.6</td>
</tr>
<tr>
<td>P₄ (100,000 × g)</td>
<td>7.3</td>
<td>800</td>
<td>22</td>
</tr>
<tr>
<td>S₁ (100,000 × g)</td>
<td>0.21</td>
<td>780</td>
<td>0.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> 400 × g supernatant.
<sup>b</sup> P₁ pellet; S, supernatant.
FIG. 1. A, centrifugation of a crude preparation of plasma membrane through discontinuous density gradient solution of sucrose. Twelve milliliters of a 700 to 9,000 X g pellet resuspended in 0.25 M sucrose are layered in a polycarbonate tube over a discontinuous sucrose density gradient prepared as described in the text. The tube is photographed after it had been centrifuged at 66,000 X g for 60 min. The subcellular components separated in four fractions according to their buoyant density. F2 and F5 part of the basement membrane which stays associated with the plasma membrane during the purification procedure. Partially disorganized lipofuscin granules are observed, but no mitochondria, rough endoplasmic reticulum vesicles, or nuclear fragments are seen. In the fraction found at the interface between 1.16 to 1.18, membrane fragments showing the same morphological features as described above are present (Fig. 3). Very few mitochondria are present.

The fraction found at the interface between densities 1.18 to 1.23 consists mainly of mitochondria with very few large vesicles present. These results suggest that the fractions found at the interfaces 1.14 to 1.16 and 1.16 to 1.18 contained mostly plasma membranes. Enzymatic properties support this.

**Biochemistry**—Tables II and III record the specific activities of the various enzymatic activities found in the final plasma membrane fractions. Of these, 5'-nucleotidase and ouabain-sensitive ATPase activities are found in the plasma membrane, while Mg\textsuperscript{2+}-dependent ATPase is present in plasma membrane and mitochondria. Succinate dehydrogenase and cytochrome c oxidase are present mainly in mitochondria; their activity in the plasma membrane Fractions F1 and F11 accounts for a cross-contamination of 8% in F11 and 1% in F1 based on the succinate dehydrogenase activity and 7.5% in F11 versus 3% in F1 based on the cytochrome c oxidase activity. The activity of glucose 6-phosphatase and glucose-6-P dehydrogenase, which are microsomal markers, are low in the plasma membrane fraction (Table III) and accounted for a cross-contamination of no more than 2 to 7% by the microsomal fraction. The glucose 6-phosphatase are composed of plasma membranes, F1 of mitochondria, B, the rerun of Fraction F1 under conditions identical with those mentioned above. Plasma membranes are found in Fractions F1 and F11. C, the rerun of Fraction F1. Plasma membranes are found in F1 and F11.

FIG. 2. A, representative field of membrane Fraction I (X 15,000). At this level the fraction is mainly composed of membranes either paired or organized into large vacuoles. The fine fibrillar material interspersed among the membranes comes from cytoplasmic filaments or basement membrane associated with either adhering granules or adhering maculae (desmosomes). Partially disorganized lipofuscin granules (arrow) can be observed. Mitochondria or mitochondrial fragments are absent. B, representative field of membrane Fraction I at a magnification of 120,000-fold. The unit membrane structure (arrow) of the plasma membrane is clearly visible.
Fig. 3. A, electron micrograph of membrane Fraction II (X 60,000). Numerous vesicles and lipofuchsine granules can be seen. The unit membrane structure is clearly visible in B (X 120,000) and in the vesicle shown in C (X 120,000).

**TABLE II**

**Phosphatase activity of different subcellular fractions**

Results are the mean of determination of five separate membrane preparations. Standard errors do not exceed 5% of the mean. The units are as follows: 5'-nucleotidase, sodium and potassium ion-activated ATPase (Na⁺-K⁺ATPase), and Mg-ATPase, micromoles of P₁ liberated per 10 min; glucose 6-phosphatase, micromoles of P₁ liberated per hour.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>5'-Nucleotidase</th>
<th>Glucose 6-phosphatase</th>
<th>Na⁺-K⁺ATPase</th>
<th>Mg⁺-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>12</td>
<td>0.20</td>
<td>0.3</td>
<td>1.18</td>
</tr>
<tr>
<td>Plasma Membrane F₁</td>
<td>108</td>
<td>0.20</td>
<td>0.3</td>
<td>1.18</td>
</tr>
<tr>
<td>Plasma Membrane F₂</td>
<td>135</td>
<td>0.09</td>
<td>0.1</td>
<td>1.34</td>
</tr>
<tr>
<td>Mitochondria F₃</td>
<td>15</td>
<td>ND</td>
<td>ND</td>
<td>0.83</td>
</tr>
<tr>
<td>Microsomes</td>
<td>10</td>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Units per mg of protein.
* Not detectable.

**TABLE III**

**Reductase activity of different subcellular fractions**

Results are the mean of determination of five separate membrane preparations. Standard errors do not exceed 5% of the mean. The units are ΔE per min per mg of protein.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Succinate dehydrogenase</th>
<th>Cytochrome c oxidase</th>
<th>NADH cytochrome c reductase</th>
<th>Glucose-6-P dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Membrane F₁</td>
<td>0.036</td>
<td>0.070</td>
<td>0.045</td>
<td>0.1</td>
</tr>
<tr>
<td>Plasma Membrane F₂</td>
<td>0.240</td>
<td>0.180</td>
<td>0.163</td>
<td>0.150</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>3.33</td>
<td>2.4</td>
<td>4.2</td>
<td>0.011</td>
</tr>
<tr>
<td>Microsomes</td>
<td>0.020</td>
<td>0.040</td>
<td>0.6</td>
<td>2.0</td>
</tr>
</tbody>
</table>

No DNA was found to be present under conditions of the diphenylamine reaction (Table IV) and only a very low level of RNA (0.1 to 1 µg per mg of membrane protein) was present. This probably reflects the low content in RNA of the luteal cells. Our yield for the plasma membrane fraction was tested in 12
various steps of isolation. Even liver plasma membrane, which is finally isolated by isopyknic binding at the interface of sucrose the technique of homogenization and the media employed in membranes preparations varied from 1.18 to 1.14 according to fraction. Furthermore, if the 5'.nucleotidase activity in the microsomes..

Nuclei

Microsomes.

90°; nonspecific adsorption to filter and 10°; to cellular fragments. the amount that is not displaced by high concentrations of native LH. Nonspecific binding is 0.4% of the input and represents 90% nonspecific adsorption to filter and 10% to cellular fragments. The 5'-nucleotidase activity is expressed in micromoles of Pi per mg of protein per 10 min reaction and per mg of protein.

## TABLE V
### Binding of $^{125}$I-LH to Different Subcellular Fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>$^{125}$I-LH bound</th>
<th>5'-Nucleotidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenatea</td>
<td>22,400</td>
<td>15</td>
</tr>
<tr>
<td>Plasma Membrane (F₁ and F₃₁)</td>
<td>456,280</td>
<td>166</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>15,300</td>
<td>13</td>
</tr>
<tr>
<td>Nuclei</td>
<td>15,600</td>
<td>12</td>
</tr>
<tr>
<td>Microsomes</td>
<td>39,000</td>
<td>16</td>
</tr>
</tbody>
</table>

a Homogenate: 400 × g supernatant.

different fractionations and was found to average 0.5 to 0.75 mg of protein per g wet weight of corpus luteum.

### Binding of $^{125}$I-LH to Different Subcellular Fractions

Since polypeptide hormones have been reported to have their receptors localized in the plasma membrane (35, 39), we have studied the binding of $^{125}$I-LH to plasma membrane preparations and compared it to the other subcellular fractions. As shown in Table V, the highest specific activity is obtained with the plasma membrane fractions, which shows a 20-fold increase over the crude homogenate; no difference can be seen between the two plasma membrane Fractions F₁ and F₃₁. Binding also occurs with mitochondrial, nuclear, and microsomal fractions; however, their specific activity is lower than that of the plasma membrane fraction. Furthermore, if the 5'-nucleotidase activity in the mitochondrial, nuclear, and microsomal fractions reflects contamination by plasma membrane fragments which can bind $^{125}$I-LH, then the mitochondria, nuclei, and microsomes of luteal cells have no receptors for LH.

### DISCUSSION

With the method used, two plasma membrane fractions are obtained. One sediments at the interface of sucrose density 1.14 to 1.16, and the other at the interface 1.16 to 1.18. A similar difference in density for the plasma membranes isolated from Ehlich ascites carcinoma (31), eye lens fiber (40), and rat liver (41, 42) has been described. The densities of the plasma membranes preparations varied from 1.18 to 1.14 according to the technique of homogenization and the media employed in various steps of isolation. Even liver plasma membrane, which is finally isolated by isopyknic binding at the interface of sucrose densities 1.16 and 1.18, can be resolved in a continuous sucrose gradient in two major subfractions of densities 1.12 and 1.21 when dispersed in a tightly fitting Dounce homogenizer (41, 42). The lighter liver plasma membrane fraction is mainly vesicular in structure, while the heavier subfraction retains the membrane sheets and structure characteristic of the original plasma membrane fraction. Similarly, in our method the initial homogenization of the corpus luteum is done with a loosely fitting Dounce homogenizer, followed by homogenization with a tightly fitting Dounce homogenizer. The yield of plasma membrane isolated from corpus luteum is in the range of 8% based on the 5' nucleotidase activity of those fractions and compares reasonably well with the yield obtained from other organs (33, 39, 43-46) when isotonic sucrose is used as an homogenization medium. The yield in various procedures is important but must be considered in the light of the purity of the preparation obtained. Assays for the marker enzymes indicate that the purity of our plasma membrane fractions is at least 85% and the present method should, even with a relatively low yield of 8%, be valuable.

Our ability to obtain purified plasma membrane fractions and other subcellular fractions has allowed us to localize the receptor sites for LH in the luteal cell. These receptor sites are in the plasma membranes. LH does not bind significantly to other subcellular structures. These results are in contrast to those of Coulon et al. (47) and Rao et al. (48) who find that LH or human chorionic gonadotropin bind to subcellular structures such as mitochondria, proteins of the cytosol, and microsomes. Whether this binding is nonspecific and reflects the degree of denaturation of $^{125}$I-LH or iodinated human chorionic gonadotropin during the iodination or whether it reflects the contamination of other subcellular structures by plasma membrane cannot be determined.

Our results agree with those of Rajaniemi and Vanha-Perttula (49) who found, using pseudopregnant rat ovaries, that the luteal LH receptor is in particle preparations which are presumably plasma membranes.

**Note Added in Proof**—DePierre and Karnovsky have recently published a review of methods for the characterization and isolation of plasma membranes of mammalian cells (50). In this review advantages and disadvantages of different methods of plasma membrane preparation are discussed.

### Acknowledgments
I express appreciation to Mrs. P. Gospodarowicz and to Mr. P. Curtiss for excellent technical assistance and to Ms. M. Bajak for the electron micrographs. I thank the Talone Meat Co. for providing the bovine corpora lutea. Without their cooperation this work would not have been possible. I also thank Dr. K. Jones for his invaluable assistance and constructive criticism of the manuscript drafts.

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