Purification of rabbit endometrial plasma membranes from receptive and non-receptive uteri*

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Summary. We have developed a method for isolation of plasma membranes from rabbit endometrium, with high yield and purification. Endometrial homogenates are precipitated with calcium chloride and the resulting supernatant is fractionated by centrifugation in a self-forming gradient of 20% Percoll. Before fractionation, the intact luminal epithelial surface was labelled with $^{125}$I-labelled soyabean agglutinin. Between buoyant densities of 1.015 and 1.017 g/ml, a discrete peak of surface label was obtained, which coincided with activities for 5'-nucleotidase and alkaline phosphatase, enzyme markers for the plasma membrane. This peak was well separated from the majority of cellular protein, and from marker enzyme activities for mitochondria and microsomes (NADH cytochrome C reductase) and lysosomes (acid phosphatase). Electron microscopy of the purified membranes showed membrane sheets and vesicles free from other cellular organelles. Analysis of detergent-soluble membrane proteins, fractionated by concanavalin A-affinity chromatography, revealed differences in the protein pattern of membranes from uteri of rabbits receptive (Day 6 of pregnancy) and non-receptive (Day 3) for implantation. The method will be useful for generation of immunological and affinity probes for surface antigens involved in ovoimplantation.

Introduction

Implantation of the mammalian blastocyst involves apposition and adhesion of the blastocyst and endometrial surfaces and, finally, invasion of the trophoblast into the epithelium (Enders & Schlafke, 1971, 1975; Enders, 1976). Implantation can occur only at a specific stage in pregnancy (Chang, 1950; Finn, 1977). In the rabbit, the endometrium reaches maximum receptivity to adhesion with the blastocyst between 6 and 7 days post coitum, as shown by the failure of competent blastocysts to implant when transferred to the uterus of asynchronously pseudopregnant rabbits (Chang, 1950). The endometrial epithelium contributes significantly to the lack of uterine receptivity resulting from hormonal deprivation or excess (Nilsson, 1967; Psychoyos, 1973; Beier, 1974). These observations suggest that the endometrial epithelium undergoes an hormonally regulated change in its acquisition of adhesiveness to the blastocyst.

There have been studies of pregnancy stage-specific changes in various properties of the uterine luminal (Nilsson, 1974; Hewitt, Beer & Grinnell, 1979; Denker, 1982; Anderson & Hoffman, 1984; Ricketts, Scott & Bullock, 1984; Winterhager, Busch & Kuhnel, 1984) and outer trophectodermal (Carollo & Weitlauf, 1981; Chavez & Enders, 1981, 1982; Johnson & Calarco, 1980; Nilsson & Hjerten, 1982; Nilsson, Näsland & Curman, 1980) cell surfaces before implantation and at implantation. None, however, has demonstrated changes involved in blastocyst–endometrial interaction and the biochemical basis of this interaction is not understood.

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The plasma membrane is a complex structure, with many polypeptide constituents, which regulates cellular metabolism, differentiation and growth. Glycoproteins on plasma membranes are postulated to be important in cell–cell recognition, adhesion and fusion (Moscona, 1963; Stanley, 1981; Nicolson, 1982; Damsky, Richa, Solter, Knudsen & Buck, 1983). In general, the characterization of plasma membrane structure has been hampered by its complexity and also by difficulties in purifying plasma membranes from cells of different origin. This paper reports a method for the purification of endometrial plasma membranes from the rabbit and a preliminary analysis of solubilized membrane proteins at receptive and non-receptive stages of pregnancy.

**Materials and Methods**

*Chemicals.* Chorionic gonadotrophin (hCG) was purchased from Organon, Inc., West Orange, NJ. Phenylmethylsulphonylfluoride (PMSF), p-nitrophenylphosphate (p-NPP), α-naphthol phosphate (α-NP), Fast Garnet GBC-salt, cytochrome C (type III from horse heart), 1,4-piperazinediethanesulphonic acid (Pipes), and molecular weight standard proteins were obtained from Sigma Chemical Company, St Louis, MO. Percoll density marker beads and concanavalin A–Sepharose 4B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Osmium tetroxide and Spurr low-viscosity embedding medium were from Ladd Research Inc., Burlington, VT; 50% glutaraldehyde was purchased from Electron Microscopy Sciences, Ft Washington, PA, and proxylene oxide from Eastman Kodak, Rochester, NY. Ultra-pure chemicals for polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA. All other chemicals were analytical grade from Fisher Scientific Company, Fairlawn, NJ.

*Animals.* Adult, New Zealand White rabbits, weighing 3–3.5 kg, were mated to fertile males and injected i.v. immediately after mating (Day 0) with 50 I.U. hCG. Uteri were excised, under anaesthesia with pentobarbitone sodium, on Day 3 (non-receptive) or Day 6 (receptive) of pregnancy. The uteri were flushed with 20 ml Tris-buffered saline (TBS; 10 mM-Tris–HCl, pH 7.4, 0.15 M-NaCl) and pregnancy was confirmed by counting corpora lutea and by the presence of embryos in the flushings of oviducts or uteri. The uteri were placed on ice, slit longitudinally, and

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Scraping of endometrial cells + PMSF

Polytron

Homogenate

Ca²⁺ aggregation

2000 g, 10 min

Supernatant S₁

26 000 g, 30 min

Pellet P₁

Supernatant S₂

Pellet P₂

Washed

20% Percoll-gradient
in buffered 0.9% NaCl

26 000 g, 30 min

Plasma membranes
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**Text-fig. 1.** Flow-sheet of purification procedure for endometrial plasma membranes. For details see text.
the endometrium scraped off with a microscope slide. Each membrane preparation contained endometrial scrapings from 3–4 animals at each of the two stages of pregnancy.

**Purification of plasma membranes.** All the purification steps were carried out at 2–4°C. The purification procedure (Text-fig. 1) is based on that used by Yakymyshyn, Walker & Thompson (1982) for the isolation of intestinal brush border membranes. The endometrial suspension was centrifuged at 500 g for 5 min and the pellet was resuspended in 18 ml TBS to which 180 µl 100 mM-PMSF in methanol had been added. The tissue was homogenized using a Polytron homogenizer at speed setting 6 for 15 sec, after which CaCl$_2$ was added to 10 mM and the mixture was rotated slowly for 10 min. After centrifugation at 2000 g for 10 min, the supernatant (S$_1$) was separated and centrifuged at 26 000 g for 30 min. The pellet (P$_2$) was washed once with TBS and resuspended in 0.5 ml TBS using 5 strokes of a Teflon pestle. The resuspended sample was layered on the surface of 8.5 ml 20% Percoll in TBS and centrifuged at 26 000 g for 30 min in a Beckman Ti75 rotor. A blank tube to establish the background of Percoll in subsequent enzyme assays contained TBS instead of the sample. A control tube with known density marker beads in 20% Percoll was run with the sample and blank tubes; the gradient was calibrated by measuring the distance of each band of beads from the bottom of the tube. The sample and blank tubes were fractionated from top to bottom using an Autodensiflo (Buchler Instruments) apparatus.

**Enzyme and protein assays.** In the enzyme assays, the reaction was followed under zero order kinetics and the hydrolysis rates were linear with time and enzyme concentration. All assays were carried out in duplicate at 37°C. Blanks were determined from samples to which substrate was added after the incubation had ended.

Alkaline phosphatase (EC 3.1.3.1) activity was assayed using p-NPP as the substrate. The standard assay mixture consisted of 0.3 ml 0.1 M-Tris-HCl, pH 9.5, containing 8.4 mM-MgCl$_2$, 0.1 ml substrate solution to give a final concentration of 1 mM, and 0.1 ml of sample. The reaction was stopped by adding 0.8 ml 0.1 M-NaOH. Absorbance at 410 nm was read after 10 min and enzyme activity was determined from a standard curve constructed with p-nitrophenol.

Acid phosphatase (EC 3.1.4.1) activity was assayed using a-NP as substrate. The reaction mixture consisted of 0.1 ml 0.1 M-sodium acetate buffer, pH 3.5, 0.1 ml substrate solution to give a final concentration of 1 mM and 0.1 ml sample. After incubation for 60 min, the liberated a-naphthol was coupled with 0.1 ml 10% (w/v) Fast Garnet GBC in distilled water, to which 10% (v/v) Tween 20 was added to disperse the colour product, and stabilized by adding 0.5 ml 1 M-sodium acetate buffer, pH 4.2. The absorbance was read at 540 nm and a-naphthol was used as the standard.

5'-Nucleotidase (EC 3.1.3.5) activity was assayed by the method of Bodansky & Schwartz (1963). The reaction was stopped after 60 min with 25% (v/v) trichloracetic acid and the precipitate was collected by centrifugation. The liberated inorganic phosphate was determined by the method of Fiske & Subbarow (1925). The intensity of the blue colour was measured at 660 nm. Potassium dihydrogen phosphate was used as the standard. Tartrate was included in the assay to inhibit acid phosphatase activity (Michell & Hawthorne, 1965). At neutral pH, non-specific phosphatase activity accounts for less than 5% of the hydrolysis of 5'-AMP (Song & Bodansky, 1967).

NADH cytochrome C reductase (EC 1.6.2.1) was assayed by the method of Fleischer & Fleischer (1967). The final volume of the reaction was 1.2 ml and sample volume varied from 10 to 50 µl. The increase in optical density at 550 nm during incubation for 5 min was measured spectrophotometrically. The extinction coefficient for cytochrome C was given by the company as 29.5 mm$^2$·cm$^{-1}$.

**Protein concentration** of the samples was determined according to Lowry, Rosebrough, Farr & Randall (1951). Samples from Percoll fractions were heated at 100°C for 20 min with 0.1 M-NaOH before protein assay. For samples prepared for polyacrylamide gel electrophoresis, protein was determined by the Lowry method as modified by Bensadoun & Weinstein (1976). Rabbit serum albumin was used as the standard.

**Electron microscopy.** Plasma membrane preparation was centrifuged at 105 000 g for 2 h to
pellet Percoll. Supernatant and a layer of membranes covering a hard Percoll pellet was transferred into another tube and centrifuged again at 105,000g for 1 h. The resultant supernatant was discarded and the pelleted membranes fixed in 7 ml 3% (v/v) glutaraldehyde in 0.1 M-Pipes buffer, pH 7.3, by gentle vortexing. The mixture was centrifuged immediately at 105,000g for 1 h. The membranes were allowed to fix overnight. The fixative was removed and the pellet was rinsed in 0.1 M-Pipes buffer, pH 7.3 for 1 h. Post-fixation was then performed for 1 h in 4% (w/v) osmium tetroxide in 0.2 M-Pipes buffer, pH 7.3, followed by dehydration in an ethanol series and treatment with propxylene. The pellets were embedded in propylene:Spurr medium (1:1, v/v) and, finally, in 100% Spurr medium for 2 h. Sections were cut and stained in lead citrate solution. Electron micrographs were obtained with a Jeol-100C transmission electron microscope.

Surface labelling. Soyabean agglutinin was iodinated with 125I using Iodogen catalyst, as described previously (Ricketts et al., 1984). The excised uterus was inverted and both ends of each horn were ligated. The exposed luminal surface was washed in 5 changes of 25 ml TBS and labelled by incubation in 5 ml TBS containing 250 µl 125I-labelled soyabean agglutinin (106 c.p.m./ml) for 60 min on ice. The tissue was removed, washed again in TBS, and the endometrium was scraped off. After homogenization, the specific radioactivity was about 30 c.p.m./µg total protein.

Solubilization and affinity chromatography. The membrane fractions were pooled from the Percoll gradient and proteins were solubilized by adding an equal volume of twice concentrated extraction buffer (100 mM-sodium acetate, 40 mM-Tris-Cl buffer, pH 5-0, containing 2% (v/v) Triton X-100 and 2 mM-EDTA). After rotation for 4 h, Percoll was removed by centrifugation and the solubilized protein suspension was dialysed against 20 volumes of 20 mM-Tris-Cl buffer, pH 7-0, containing 0.5 M-NaCl, 1 mM-MnCl2 and 1 mM-CaCl2 with two changes in 24 h. Lectin affinity chromatography was carried out by mixing the dialysed sample with 1 ml concanavalin A-Sepharose 4B, previously equilibrated with the dialysis buffer, and agitating the mixture for 2 h. After centrifugation for 2 min in a microfuge, the supernatant containing unbound proteins was removed and the affinity resin was washed with dialysis buffer (10 x 1 ml). The washings were combined with the unbound material. Bound proteins were eluted from the resin by washing with 1 M-α-methyl-d-mannoside in dialysis buffer (10 x 1 ml). The bound and unbound fractions were dialysed against 20 volumes of 50 mM-ammonium bicarbonate with three changes in 24 h and then freeze-dried.

Polyacrylamide gel electrophoresis. Electrophoresis in polyacrylamide–sodium dodecyl sulphate (SDS; 10% (w/v) acrylamide, 0.05% (w/v) N,N'-methylene-bis-acrylamide, 0.1% (w/v) SDS, pH 8.8) slab gels was performed according to the method of Porzio & Pearson (1977), using a stacking gel of 4:3% acrylamide, 0.1% N,N'-methylene-bis-acrylamide, 0.1% SDS, pH 6.8 (Laemmli, 1970). Freeze-dried samples were dissolved in a sample buffer of 10% (v/v) glycerol, 5% (v/v) mercaptoethanol, 3% SDS in 63 mM-Tris–Cl buffer, pH 5.8 (Laemmli, 1970) and heated at 97°C for 4 min. Samples of 10–40 µl containing 15–20 µg protein were used for electrophoresis.

Myosin (M, 200 000), β-galactosidase (M, 116 000), phosphorylase b (M, 97 000), bovine serum albumin (M, 67 000), ovalbumin (M, 43 000), carbonic anhydrase (M, 34 000) and soyabean trypsin inhibitor (M, 21 000) were used as marker proteins. After stacking for 1.5 h (25 mA/slab gel), the current was increased to 40 mA. After electrophoresis the gel was agitated in 50% (v/v) methanol for 12 h and proteins were stained with the silver-staining method of Wray, Boulikas, Wray & Hancock (1981).

Results

Purification of plasma membranes

To monitor the luminal plasma membrane through the fractionation procedure, the intact epithelial surface was first labelled by binding 125I-labelled soyabean agglutinin. Preliminary
Text-fig. 2. Percoll-density gradient fractionation of endometrial plasma membranes: (a) distribution of surface label (125I-labelled soyabean agglutinin) and total protein; (b) distribution of enzyme activities for 5'-nucleotidase and NADH-cytochrome C reductase in the same gradient.

Text-fig. 3. Distribution of total protein and of enzyme activities for alkaline phosphatase, acid phosphatase, and NADH cytochrome C reductase after fractionation of endometrial membranes on a Percoll gradient.
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<th>Homogenate</th>
<th>Pellet P₁</th>
<th>Supernatant S₂</th>
<th>Pellet P₂</th>
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<td>Yield</td>
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<tr>
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<td>—</td>
<td>—</td>
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</tr>
<tr>
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<td>50</td>
<td>2.1 ± 3.9</td>
<td>20</td>
</tr>
<tr>
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<td>1.7 ± 2.7</td>
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<tr>
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<td>—</td>
<td>60</td>
<td>—</td>
<td>17</td>
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*Mean ± pooled s.e.m. (d.f. = 8).  
N.D. = not detectable.
Fig. 1. Transmission electron micrograph of purified plasma membranes after Percoll-density gradient centrifugation. × 18 225.

(Facing p. 480)
Fig. 2. Electrophoresis in 10% acrylamide–SDS gels of solubilized proteins from purified endometrial plasma membranes, before and after separation on concanavalin A-Sepharose 4B. $M_r = \text{molecular weight standards (} \times 10^{-3}\text{). Arrows indicate proteins of 84 000 (84K), 55 000 (55K), and 38 000 (38K) present in total proteins and concanavalin A-unbound proteins of receptive-stage membranes, and proteins of 30 000 (30K) and 78 000 (78K) present in concanavalin A-bound proteins of receptive (R) and non-receptive (NR) stages, respectively.}
experiments showed that soyabean agglutinin gave the highest binding of radioactivity of the iodinated lectins tested in 6-day uteri. The Percoll gradient gave a peak of radioactivity well separated from the bulk of the protein in the gradient (Text-fig. 2a). This peak contained the majority of the 5'-nucleotidase activity, a plasma membrane enzyme marker, although some of this activity evidently aggregated with the protein peak, which contained high levels of NADH-cytochrome C reductase activity, an enzyme marker for mitochondrial (and also microsomal) membranes (Text-fig. 2b).

Additional enzyme markers were used to confirm the separation of the plasma membranes. Alkaline phosphatase activity, a second plasma membrane marker, was coincident with the peaks of surface label and 5'-nucleotidase activity, and was well separated from acid phosphatase activity, a lysosomal marker, and from NADH-cytochrome C reductase activity and total protein peak (Text-fig. 3).

Thus a plasma membrane fraction, identified by a surface label and by two marker enzymes, was obtained at a density of 1.015–1.017 g/ml, well separated from the majority of cellular proteins and from marker enzyme activities for mitochondria, microsomes, and lysosomes. Table I shows the yield and fold-purification of the plasma membranes at different stages of fractionation. All of the surface label (87 000 c.p.m.) was recovered in the purified fraction, with a 45-fold enrichment of specific radioactivity (c.p.m./μg protein) over the starting homogenate. Plasma membrane marker enzyme activity was recovered in 25% yield for 5'-nucleotidase, with 42-fold purification, and in 41% yield for alkaline phosphatase, with 44-fold purification. Enzyme activity for intracellular organelles (acid phosphatase or NADH-cytochrome C reductase) was not detectable in the purified membrane fraction.

The purity of the membrane preparation was further assessed by transmission electron microscopy of the pelleted pooled fractions from the Percoll gradient. Plate 1, Fig. 1, shows that the purified plasma membrane preparation is composed mainly of membrane vesicles and sheets. No other cellular organelles were recognized. Some dark granular material was thought to represent residual Percoll, which was present in small amounts even after extensive washing of the pellet. Nondescript electron-dense material, commonly called 'fuzz', is also present but its nature is unknown.

**Analysis of soluble membrane proteins**

About 75% of the solubilized proteins did not bind during concanavalin A–Sepharose 4B affinity chromatography. The electrophoretic patterns of solubilized membrane proteins from receptive or non-receptive endometria are shown in Pl. 2, Fig. 2. Comparison of the lanes of proteins at the two stages before or after concanavalin A–Sepharose 4B chromatography showed some reproducible differences. Among the total proteins before chromatography, proteins of \( M, 84\,000 \) (84K), 55 000 (55K) and 38 000 (38 K) were evident only at the receptive stage. These proteins did not bind to concanavalin A–Sepharose 4B (Pl 2, Fig. 2). Enrichment of proteins by the affinity chromatography revealed further differences. Among the bound proteins, one of \( M, 30\,000 \) (30K) was specific for the receptive stage, whereas a protein of \( M, 78\,000 \) (78K) was evident only at the non-receptive stage (Pl. 2, Fig. 2). Other differences may be noted but were not seen in every preparation.

**Discussion**

Purification of endometrial plasma membranes from the rabbit at receptive and non-receptive stages was achieved by repeated centrifugation and a self-forming Percoll gradient. We chose to start with endometrial scrapings, rather than perform cell separation as in our earlier work (Ricketts, Hagensee & Bullock, 1983), to avoid enzymic treatment and consequent degradation of membrane glycoproteins (Lotan & Nicolson, 1979; Nicolson, 1982). Several marker enzymes were used to assess the purity of the plasma membrane preparation. The yield and the final enrichment
of the plasma membrane marker enzymes (41% and $44.3 \pm 2.7$-fold for alkaline phosphatase, respectively; 25% and $42.4 \pm 3.9$-fold for S'-nucleotidase, respectively) compare favourably with results reported by others for human intestine (Schmitz et al., 1973; Hauser, Howell, Dawson & Bowyer, 1980), rabbit intestine (Yakymyshyn et al., 1982), rat kidney (Wilfong & Neville, 1970) or hamster fibroblast (Gahmberg, 1971) plasma membranes.

To aggregate endoplasmic reticulum, basolateral membrane, lysosomes and mitochondria, MgCl$_2$ (Hauser et al., 1980) and CaCl$_2$ (Malathi, Preiser, Fairclough, Mallett & Crane, 1979; Yakymyshyn et al., 1982) have been used. Yakymyshyn et al. (1982) compared the effect of MgCl$_2$ and CaCl$_2$ and showed that with CaCl$_2$ there was higher yield and greater specific activities of enzyme markers, less contamination with lysosomes and basolateral membranes, and the plasma membranes were separated as a sharper band on Percoll gradient. In this study, CaCl$_2$-aggregation was used and the enzyme marker determinations showed that about 60% of NADH cytochrome C-reductase and acid phosphatase activities were eliminated by this step, together with about 50% of S'-nucleotidase activity. As well as being present in plasma membranes, S'-nucleotidase occurs also in significant amounts in intracellular membranes (Neville & Kahn, 1974). Alkaline phosphatase activity concentrated mostly in the membrane pellet although 40% was lost in the CaCl$_2$-precipitate. Yakymyshyn et al. (1982) reported that 40% Percoll resulted in higher yield of membranes than glycerol or sucrose gradients. In this work, 20% Percoll gave the best result in yield and purity of endometrial plasma membranes. Electron microscopy verified the absence of other cell organelles in the purified plasma membrane preparation.

Enriched localization of glycoproteins in surface membranes of cells has been demonstrated by high content of protein-bound fucose and glucosamine in isolated plasma membranes (Gahmberg, 1971), by fractionation of cell surfaces with plant agglutinin (Nicolson, 1972) and by labelling cell surface carbohydrates (Gahmberg & Hakomori, 1973; Lotan & Nicolson, 1979). Plasma membrane glycoproteins have also been purified by lectin affinity chromatography (see Lotan & Nicolson, 1979). In this study luminal plasma membrane proteins were labelled with iodinated lectins in situ before the purification of plasma membranes. The enrichment of $^{125}$I in the membrane fraction (45%) was in good agreement with the plasma membrane enzyme marker studies. The higher yield of label (100%) than of marker enzymes indicates the greater specificity of the lectin marker for the membrane surface.

Proteins solubilized from purified plasma membranes were separated on concanavalin A-Sepharose 4B affinity chromatography into bound and unbound proteins. Bound proteins would have glucose or mannose on their polypeptide backbone, whereas unbound proteins would have other sugars or inaccessible glucose and mannose residues, or could be non-glycosylated. Electrophoresis of membrane total protein subunits before concanavalin A-Sepharose 4B separation, revealed three proteins (84K, 55K and 38K) which were specific to the receptive stage. These proteins were present among receptive-stage unbound proteins after concanavalin A-Sepharose 4B chromatography. In our previous work (Ricketts et al., 1984), using surface iodination of isolated or cultured rabbit uterine epithelial cells, we detected a protein also of $M_r$ 38 000 that contributed to discrimination of the receptive from the non-receptive epithelial surface. Further differences could be seen among the concanavalin A-bound proteins. Protein 30K was present in the receptive stage, whereas Protein 78K was missing at this stage. These differences could not be detected amongst the total proteins because there were several proteins with similar molecular weights.

This study supports the suggestion that acquisition of receptivity is influenced by changes in protein composition of endometrial plasma membranes (Finn, 1977; Ricketts et al., 1984). The change from non-receptive to receptive may involve a shift in number, distribution or conformation of some of the molecules on the surface. The present method does not resolve, of course, whether these stage-specific proteins are of epithelial or stromal origin, and does not identify specific luminal surface proteins. Purification of endometrial plasma membranes by this method will be useful for studies of stage-specific changes and for generation of immunological and affinity probes for specific surface membrane components.
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References


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