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# ESTROGEN RECEPTORS IN UTERINE PLASMA MEMBRANE

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SUMMARY

In order to assess the subcellular distribution of estrogen-binding components in their native state, plasma membrane and other cell fractions were prepared by quantitative methods from uterine cells in the absence of [$^3$H]-estradiol-17$\beta$ ($E_2\beta$). Cells isolated from uteri of ovariectomized rats were disrupted with minimum homogenization in buffered isotonic sucrose with $CaCl_2$ and fractionated using isotonic media throughout. Activities of succinate dehydrogenase and acid phosphatase were concentrated in the mitochondria:lysosome (M + L) fraction. Glucose-6-phosphatase occurred predominantly in the microsome-rich (P) fraction. Alkaline phosphatase and 5'-nucleotidase were found principally in P and crude nuclear (N) fractions, with N also retaining 96% of DNA. Determinations of specific [$^3$H]-$E_2\beta$ binding to cell fractions at equivalent protein levels were conducted by equilibration for 2 h at 4°C. Binding-sites for $E_2\beta$ were present in N > P > M + L > 105,000 *g* supernates (S). However, by using alternative homogenization procedures known to elicit lysis, fragmentation, and stripping of cell structures, $E_2\beta$ binding-sites as well as 5'-nucleotidase, a plasma membrane marker enzyme, both occurred predominantly in S. Using the more conservative cell disruption and fractionation procedures, plasma membrane subfractions (F2) with densities of 1.13–1.16 were partially purified, principally as smooth membrane vesicles and large "ghosts", by further centrifugation of N in a discontinuous sucrose density gradient. Activity of 5'-nucleotidase in F2 was enriched to 12 times that of the homogenate. Specific binding-sites for $E_2\beta$ were concentrated in F2 to 23 times homogenate levels. Such binding of $E_2\beta$ in F2 was saturable, with an association constant of $4.3 \times 10^{10}\ M^{-1}$. At saturation, $E_2\beta$ receptors in F2 correspond to 2.0 pmol/mg membrane protein. Ligand specificity of [$^3$H]-$E_2\beta$ binding to F2 was established by negligible competition by 200-fold molar excess of unlabelled estradiol-17$\alpha$, cortisol, testosterone, or progesterone, whereas $E_2\beta$ and diethylstilbestrol were effective inhibitors. Specific binding of $E_2\beta$ to F2 at 4°C was blocked by prior exposure of membranes to trypsin or to 60°C, but remained essentially undiminished by extraction of membranes with either severely hypotonic or high-salt buffers. In controlled experiments, it was found that only 6–9% of [$^3$H]-$E_2\beta$-labelled cytosol components became associated with F2 during 2 h incubation at 4°C; the bulk of such adsorbed material was readily extracted by high-salt buffers. Thus, enrichment of F2 in $E_2\beta$-binding activity cannot be attributed to gross entrapment or adsorption of cytosolic components. These data indicate that high-affinity membrane binding-sites with specificity for $E_2\beta$ must be further considered in investigations of the uterine cell recognition of and response to the hormone.

## INTRODUCTION

Estrogens are accumulated and retained in responsive cells by interactions with extranuclear macromolecules which possess high affinity and specificity for the hormone [1, 2]. Estrogen binding facilitates transfer of receptor protein to the nuclear chromatin, where the complex is believed to promote expression of the phenotypic effects [1–5].

It is not established with certainty whether the extranuclear receptor in its native state occurs as such in the cytosol or is associated with some cellular structure [2, 6–10]. The pioneering studies of Gorski and associates [cf. 1, 5, 6] indicated that the bulk of estradiol-17$\beta$ ($E_2\beta$) that became specifically bound when responsive organ segments were incubated with the hormone at 0–4°C occurred in association with high-speed supernatant fractions obtained after homogenization. However, Noteboom and Gorski[6] also identified some specific $E_2\beta$ binding in mitochondrial and microsomal fractions. In more recent work, additional evidence for the occurrence of binding components with high affinity and specificity for $E_2\beta$ in particulate fractions of target cells has been presented [7–9, 11–13]. Using an affinity-binding procedure [10], we have also found that substantial numbers of intact cells isolated from endometrium, but not those from intestinal epithelium, bind to estrogen immobilized by covalent linkage to an inert support. Collectively, such data have raised the question of whether the widely reported predominance of receptors for $E_2\beta$ in the cytosol fraction of target cells might have resulted from inadvertent extraction of native hormone receptors by homogenization procedures which elicited extensive damage to cellular structures [2, 7–10, 14].

In essentially all biochemical investigations of the cellular localization of $E_2\beta$ binding-sites, data adequate to permit evaluation of the relative purity of the resulting cellular fractions and the integrity of cellular organelles [cf. 15] are lacking. The present work seeks

to determine the distribution and interrelationships of estrogen-binding components in plasma membrane and other cellular fractions obtained after controlled homogenization of uterine cells. As recommended by de Duve [16] and others [15], we have adopted an analytical approach to subcellular fractionation and provide in the present report a balance sheet, wherein are given the values of several marker enzyme activities and biochemical constituents as well as specific hormone-binding sites, as these are related to the levels of the corresponding components in the homogenate.

## MATERIALS AND METHODS

### *Isolation of uterine cells*

Female rats (60–80/experiment) of an inbred Sprague–Dawley strain were ovariectomized at 6 wk of age and approx. 160 g body wt. The animals were then kept for 3 wk in a low-steroid environment under controlled conditions of light and temperature [14]. On the day of experiment, the animals were lightly anesthetized with Nembutal (sodium pentobarbital, 5 mg/100 g body wt), administered subcutaneously. Uteri were rapidly freed from mesometrial fat and connective tissue, excised, and rinsed of superficial blood in Ringer solution (see below). The organs were finely minced with razor blades at 4°C and then incubated at 37°C with 200 mg collagenase (Type I; Worthington Biochemical Corp., Frehold, N.J.)/100 ml $Ca^{2+}$-, $Mg^{2+}$-free Ringer with 10 mg soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO)/100 ml. Ringer solution lacking divalent cations was composed of 136.9 mM NaCl, 2.7 mM KCl, 1 mM sodium pyruvate, and buffered at pH 7.4 with 8.1 mM $Na_2HPO_4$ and 1.5 mM $KH_2PO_4$. After 20 min, the medium was made 0.6 mM with $CaCl_2$ and 0.5 mM with $MgCl_2$, and incubation was continued for 30 min [17]. The preparation was then filtered through 64-$\mu$m nylon mesh (Nitex; Tobler, Ernst and Traber, Inc., Elmsford, N.Y.) with the aid of 8 volumes of ice-cold $Ca^{2+}$-, $Mg^{2+}$-free Ringer. Filtered cells were sedimented at 100 *g* for 5 min at 4°C. Cells were then resuspended in divalent cation-free Ringer, filtered through 1 layer of 35-$\mu$m nylon mesh to remove debris, and resedimented. The resultant cells were suspended in serum-free medium consisting of Earle's balanced salt solution [18] enriched with $1 \times 10^{-8}$ M highly purified bovine insulin (Eli Lilly and Co., Indianapolis, IN), 0.1% (w/v) albumin (Pentex; Miles Laboratories, Elkhart, IN), Minimum Essential Medium amino acids (Grand Island Biological Co., Grand Island, NY), 10 mM glucose, 1 mM sodium pyruvate, and 50 $\mu$g Gentamicin (Schering Corp., Kenilworth, N.J.)/ml, and incubated 10 h in a humidified atmosphere of 5% $CO_2$ in air at 37°C. All vessels used for these preparations were polyethylene or polypropylene. All materials and instruments were obtained sterile or autoclaved, and solutions were filter-sterilized. At least 95% of all cells used excluded 0.05% nigrosin during 5–10 min incubation in complete Ringer at 22°C.

### *Isolation of plasma membrane subfractions*

With the exception of a series of experiments to test the effect of different homogenization conditions on the distribution of estrogen-binding and other components in major cell fractions (see below), plasma

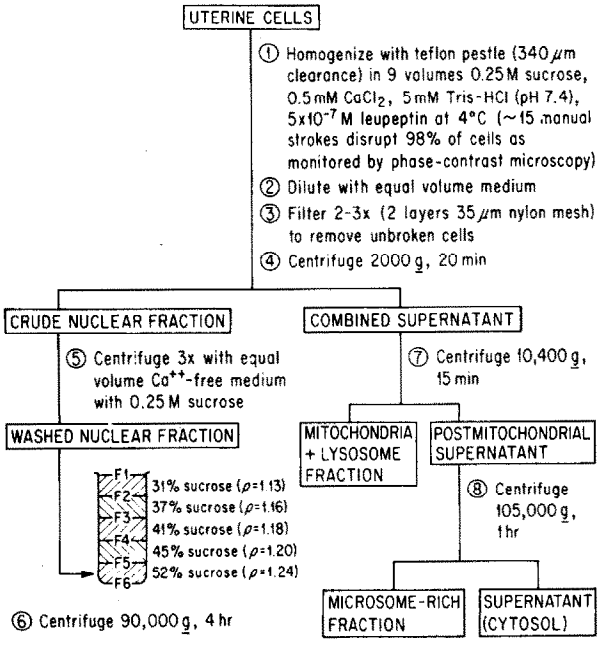


Fig. 1. Flow diagram for homogenization and fractionation of isolated uterine cells (protocol 7). See text for additional details.

membrane and other subcellular fractions were prepared at 4°C from the isolated uterine cells essentially by the method of Berman *et al.*[19] with the slight modification introduced by Yamamoto *et al.*[20]. A summary of major steps in the isolation scheme is shown in Fig. 1. Cells were homogenized in 9 volumes of 0.25 M sucrose: 0.5 mM $CaCl_2$: $5 \times 10^{-7}$ M leupeptin (acetyl- and propionyl-L-leucyl-L-leucyl-L-arginal; Protein Research Foundation, Osaka, Japan): 5 mM Tris–HCl buffer, pH 7.4. Approximately 15 manual up-and-down strokes with a ball-shaped Teflon pestle in a Dounce homogenizer (~0.34 mm clearance) were just sufficient to disrupt 98% of the uterine cells [cf. 21], as systematically monitored in each experiment by phase-contrast microscopy. The homogenate was then diluted with an equal volume of medium and filtered 2–3 times through two layers of 35-$\mu$m nylon mesh to remove all unbroken cells. The filtrate was then centrifuged at 2000 *g* for 20 min to sediment the crude nuclear fraction. This fraction was washed three times with an equal volume of 0.25 M sucrose prepared in $Ca^{2+}$-free medium (i.e., 5 mM Tris–HCl buffer: $5 \times 10^{-7}$ M leupeptin), and finally suspended in 7 ml of 52% (w/w; $\rho = 1.24$) sucrose in $Ca^{2+}$-free medium. The following four discontinuous sucrose density layers were introduced above the latter suspension: from bottom to top, 7 ml of 45% ($\rho = 1.20$), 7 ml of 41% ($\rho = 1.18$), 7 ml of 37% ($\rho = 1.16$) and 9 ml of 31% ($\rho = 1.13$) sucrose in $Ca^{2+}$-free medium. Gradients were centrifuged in an SW-27 rotor at 90,000 *g* for 4 h at 4°C using a Beckman Model L5-75 ultracentrifuge. After centrifugation, fractions at $\rho < 1.13$ (F1), $\rho = 1.13$–$1.16$ (F2), $\rho = 1.16$–$1.18$ (F3), $\rho = 1.18$–$1.20$ (F4), $\rho = 1.20$–$1.24$ (F5) and the sediment (F6) were each collected, diluted with $Ca^{2+}$-free medium, and washed twice by centrifugation at 22,500 *g* for 20 min. All fractions were then suspended in 0.25 M sucrose in $Ca^{2+}$-free medium to about 1 mg protein/ml and stored at 4°C. Analyses for hormone binding and enzyme activities were conducted within 6 and 24 h, respectively.

*Preparation of mitochondria:lysosome, microsome and particle-free supernatant fractions*

The combined supernatants and washes of the crude nuclear pellet (N) were centrifuged twice at 10,400 *g* for 15 min, yielding a mitochondrial-lysosomal sediment (M + L). The postmitochondrial supernatants were combined and centrifuged at 105,000 *g* for 1 h, yielding a microsome-rich pellet (P) and a supernatant (S; i.e., cytosol). All fractions were diluted with 0.25 M sucrose in $Ca^{2+}$-free medium to about 1 mg protein/ml and stored at 4°C.

*Determination of specific [$^3$H]-estradiol-17$\beta$ binding in cell fractions*

To determine specific $E_2\beta$ binding in the several cellular fractions, incubations were begun with the addition of [2,4,6,7,16,17-$^3$H]-estradiol-17$\beta$ (152 Ci/mmol; New England Nuclear, Boston, Mass.) at 4°C. A 200-fold molar excess of unlabelled $E_2\beta$ (Schering) was present with [$^3$H]-$E_2\beta$ in paired samples for determination of displaceable binding [cf. 10, 22]. In related competitive binding experiments, a 200-fold molar excess of unlabelled estradiol-17$\beta$, diethylstilbestrol, cortisol, progesterone (all from Schering), or testosterone (Sigma) was added with [$^3$H]-$E_2\beta$ to additional paired samples as appropriate. After 2 h of equilibration with Vortex stirring at 10-min intervals, samples were diluted with two volumes of ligand-, $Ca^{2+}$-free medium with 0.25 M sucrose at 4°C. Particulate fractions were then sedimented and washed once by centrifugation to remove unbound $E_2\beta$. The washed sediments were solubilized at 4°C with 0.1 N NaOH:0.1% (v/v) Triton X-100 (Rohm & Haas Co., Philadelphia, PA). To separate bound from free $E_2\beta$ in particle-free supernatant fractions [23], one volume of supernatant was mixed with a pellet of dextran-coated charcoal (Norit A), obtained by centrifugation of two volumes of 0.5% charcoal and 0.05% dextran in 0.25 M sucrose in $Ca^{2+}$-free medium. After 10 min of equilibration with stirring in an ice bath, the dextran-coated charcoal was removed from the supernatant fraction by centrifugation. Samples were collected for determination of [$^3$H] by liquid scintillation counting in Biofluor (New England Nuclear) and for analyses of protein by the method of Lowry *et al.*[24]. In initial experiments, hormone bound to particulate and soluble fractions was extracted with methanol and ether as described by Beers and Rosner[25] and analyzed by thin layer chromatography with benzene:methanol [26]; more than 95% of the [$^3$H] label migrated with authentic $E_2\beta$.

*Analyses of enzyme activities and DNA in plasma membrane and other cellular fractions*

Activity of 5'-nucleotidase (EC 3.1.3.5) in the several cell fractions was determined by the method of Touster *et al.*[27]; $P_i$ liberated was analyzed by the method of King[28]. Analyses for alkaline phosphatase (EC 3.1.3.1) were done as described in Pietras[29]. Succinate dehydrogenase (EC 1.3.99.1) activity was determined according to Pennington[30], and glucose-6-phosphatase (EC 3.1.3.9) by the method of Hübscher and West[31]. Acid phosphatase (EC 3.1.3.2) was analyzed as described by Szego *et al.*[32]. Specific activities of enzymes are given as nmol/min/mg protein. Relative specific activity represents the specific activity of enzyme in a given fraction in relation to that in the homogenate. DNA was determined by the method of Hill and Whatley[33].

*Preparation of membranes for electron microscopy*

Membranes were fixed for 1 h at 4°C by suspension in 3% glutaraldehyde buffered at pH 7.3, sedimented by centrifugation at 22,500 *g* for 20 min and then washed with glutaraldehyde-free buffer at the centrifuge. Samples were postfixed in osmium tetroxide and embedded in Epon. Thin sections were mounted,

Table 1. Summary of the various homogenization conditions used to determine distribution of 5′-nucleotidase and binding of estradiol-17$\beta$ in uterine cell fractions*

| Protocol | Homogenization medium | Homogenizer (No. of strokes†) |
|---|---|---|
| 1 | 5 mM Tris–HCl (pH 7.4)<br>1.5 mM EDTA | Glass–glass<br>(13) |
| 2 | 5 mM Tris–HCl (pH 7.4)<br>1.5 mM EDTA | Teflon–glass<br>(26) |
| 3 | 5 mM Tris–HCl (pH 7.4)<br>1.5 mM EDTA<br>0.25 M sucrose | Teflon–glass<br>(33) |
| 4 | 5 mM Tris–HCl (pH 7.4)<br>0.5 mM $CaCl_2$<br>0.25 M sucrose | Glass–glass<br>(17) |
| 5 | 5 mM Tris–HCl (pH 7.4)<br>0.5 mM $CaCl_2$<br>0.25 M sucrose | Teflon–glass<br>(35) |

* Isolated uterine cells were suspended in 4 volumes of the indicated homogenization media at 4°C. Subsequent preparation of N, M + L, P and S fractions was carried out using the corresponding homogenization medium. However, in protocols 4 and 5, the media used after homogenization contained no additional $CaCl_2$.

† Mean number of manual strokes required to disrupt ~100% of cells, as monitored by phase-contrast microscopy.

stained with uranyl acetate and lead citrate, and observed by electron microscope [34].

## RESULTS

### *Effects of diverse homogenization methods on yield of enzyme activity and estradiol-17$\beta$ binding in major fractions of uterine cells*

The effects of different homogenization conditions on yield of protein, 5′-nucleotidase, a valid plasma membrane marker enzyme for uterus [35, 36], and specific $E_2\beta$-binding in major subfractions of uterine cells were investigated first. In two sets of paired experiments, isolated cells were disrupted and fractionated by one of the five different protocols specified in Table 1. As recommended in conventional methods of tissue homogenization for studies of estradiol binding [cf. 37, 38], essentially 100% of cells were disrupted in this series of experiments. After homogenization, the general scheme for isolation of crude nuclear (N), mitochondria:lysosome (M + L), microsome-rich (P), and cytosol (S) fractions (cf. Fig. 1) was used with the medium specified for each protocol (see Table 1).

The results of these initial experiments are presented in Table 2. The distribution of protein among the major cell fractions prepared from the homogenates of protocols 1–5 shows little variation. In contrast, distribution of the activity of 5′-nucleotidase exhibits marked variation which is most apparent in cytosol fractions, with cytosolic activity in protocol 1 > 2 > 3 > 4 > 5. Since the occurrence and relative specific activity of 5′-nucleotidase in the crude nuclear and microsome-rich fractions, where the bulk of plasma membrane cosediments [15, 19], follows a reverse order among these five protocols, it appears that the plasma membrane marker enzyme is redistributed from particulate to soluble fractions in protocol 1 ≫ 5 (Table 2). Likewise, specific $E_2\beta$ binding, which occurs predominantly in cytosol fractions when prepared by protocol 1, is minimal in cytosol obtained by protocol 5. The bulk of $E_2\beta$ binding is associated with particulate fractions in the latter method (Table 2). Thus, homogenization procedures which are less conservative of membrane integrity, as evaluated by independent determination of 5′-nucleotidase in cell fractions, elicit higher levels of specific $E_2\beta$-binding in cytosol.

To minimize the aberrant redistribution of cellular macromolecules due to excessive homogenization, Plagemann[21] has recommended that homogenization should be limited to disruption of only 98% of the total cell population. This latter criterion was utilized in two additional sets of paired experiments shown in Fig. 2. Cells were disrupted (1) by suspension in four volumes of 5 mM Tris–HCl (pH 7.4) with 1.5 mM EDTA, followed by manual glass–glass homogenization (shaded bars, protocol 6; Fig. 2), or (2) by the method indicated in Fig. 1 (clear bars, protocol 7; Fig. 2). After homogenization, unbroken cells were removed by filtration (see Fig. 1). It is important to note that, with the exception indicated, homogeniza-

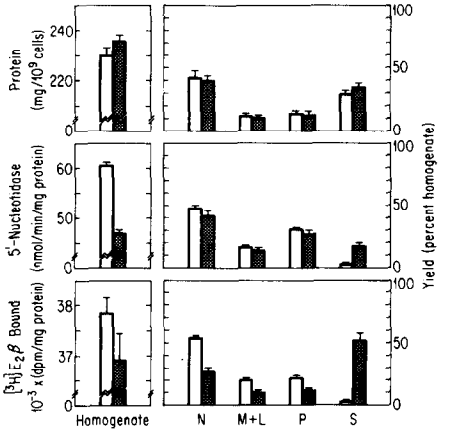


Fig. 2. Effect of homogenization conditions on yield of protein, 5′-nucleotidase activity and specific binding of [$^3$H]-estradiol-17$\beta$ in major fractions of disrupted uterine cells. In *protocol 6* (shaded bars), cells were suspended in 4 volumes of hypotonic medium consisting of 5 mM Tris–HCl (pH 7.4) with 1.5 mM EDTA. In accord with the recommendation of Plagemann[21], about 98% of the cells were disrupted at 4°C with a glass–glass homogenizer (Kontes) using 7–10 manual strokes. The same homogenization medium was utilized throughout subsequent steps in the otherwise standard fractionation procedure (Fig. 1). In *protocol 7* (clear bars), isolated uterine cells were homogenized and fractionated according to the scheme presented in Fig. 1. The mean ± SE of data from three paired experiments is shown. Total recoveries of protein, 5′-nucleotidase activity and specific $E_2\beta$ binding in crude nuclear (N), mitochondrial-lysosomal (M + L), microsome-rich (P) and cytosol (S) fractions ranged from 97–101% of that in the initial homogenates.

Table 2. Effects of various conditions of cellular disruption on distribution of protein, 5′-nucleotidase and specific binding of [$^3$H]-estradiol-17$\beta$ among the cell fractions*

| Determination | Homogenization protocol* | Homogenate (units) | Subcellular fraction (% homogenate) N | M + L | P | S | Recovery (%) | Subcellular fraction (relative specific activity) N | M + L | P | S |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Protein | 1 | 26.6† | 36.6 | 8.6 | 12.4 | 38.7 | 96.3 | | | | |
| | 2 | 23.7 | 40.0 | 9.8 | 10.9 | 36.4 | 97.1 | | | | |
| | 3 | 24.8 | 37.9 | 10.9 | 12.1 | 34.4 | 95.3 | | | | |
| | 4 | 25.5 | 40.0 | 11.4 | 14.3 | 31.4 | 97.1 | | | | |
| | 5 | 26.8 | 40.5 | 9.3 | 12.4 | 37.5 | 99.7 | | | | |
| 5′-Nucleotidase | 1 | 55.8‡ | 22.9 | 6.6 | 10.3 | 59.8 | 99.6 | 0.63 | 0.77 | 0.82 | 1.54 |
| | 2 | 67.1 | 33.6 | 8.5 | 15.0 | 38.2 | 95.3 | 0.84 | 0.87 | 1.37 | 1.05 |
| | 3 | 65.2 | 38.9 | 10.2 | 15.1 | 30.8 | 95.0 | 1.01 | 0.94 | 1.33 | 0.89 |
| | 4 | 60.2 | 40.5 | 14.7 | 20.3 | 25.1 | 100.6 | 1.01 | 1.28 | 1.42 | 0.80 |
| | 5 | 66.5 | 47.3 | 13.8 | 29.1 | 9.1 | 99.3 | 1.17 | 1.48 | 2.35 | 0.13 |
| Estradiol-17$\beta$ binding | 1 | 32.2§ | 15.2 | 0.5 | 6.2 | 78.1 | 100.0 | 0.42 | 0.06 | 0.49 | 2.02 |
| | 2 | 32.5 | 25.0 | 4.2 | 7.0 | 62.9 | 99.1 | 0.62 | 0.43 | 0.64 | 1.73 |
| | 3 | 34.0 | 32.2 | 9.0 | 13.4 | 47.2 | 101.8 | 0.85 | 0.82 | 1.19 | 1.37 |
| | 4 | 34.7 | 41.2 | 12.1 | 19.4 | 27.2 | 99.9 | 1.03 | 1.05 | 1.36 | 0.86 |
| | 5 | 36.8 | 44.5 | 12.2 | 22.1 | 20.4 | 99.2 | 1.10 | 1.31 | 1.80 | 0.54 |

* Homogenization and fractionation were conducted at 4°C, under conditions specified in Table 1. The mean values of data from two sets of paired experiments are shown.
† mg/$10^8$ cells.
‡ nmol/min/mg protein.
§ Specific binding of $2 \times 10^{-9}$ M [$^3$H]-$E_2\beta$, expressed as $10^{-3} \times$ (d.p.m./mg protein).

Table 3. Distribution of protein, enzymes, and specific [$^3$H]-estradiol-17$\beta$ binding in major fractions of uterine cells*

| Determination (No. of experiments) | Homogenate (units) | Subcellular fraction N | M + L | P | S | Recovery (%) |
|---|---|---|---|---|---|---|
| | | | (% homogenate) | | | |
| Protein (4) | 240† ±25 | 43.1 ±3.1 | 11.3 ±1.9 | 12.7 ±0.3 | 30.3 ±0.8 | 97.4 ±3.1 |
| Succinate dehydrogenase (3) | 11.7‡ ±0.8 | 10.2 ±0.3 | 72.1 ±2.7 | 10.5 ±3.9 | 3.3 ±2.7 | 96.1 ±2.4 |
| Glucose-6-phosphatase (3) | 2.9‡ ±0.8 | 12.9 ±1.9 | 11.9 ±2.4 | 65.1 ±2.1 | 6.3 ±1.1 | 96.2 ±2.8 |
| Acid phosphatase (3) | 17.9‡ ±1.2 | 12.8 ±3.4 | 63.4 ±0.2 | 16.6 ±2.9 | 1.3 ±1.2 | 94.1 ±2.6 |
| Alkaline phosphatase (3) | 37.1‡ ±2.3 | 49.6 ±4.4 | 19.6 ±1.1 | 24.8 ±0.1 | 1.1 ±0.0 | 95.1 ±4.0 |
| 5′-Nucleotidase (4) | 70.4‡ ±3.0 | 46.9 ±4.2 | 15.6 ±1.4 | 29.2 ±0.4 | 3.3 ±0.1 | 95.0 ±2.6 |
| Estradiol-17$\beta$ binding (4) | 34.6§ ±2.5 | 56.4 ±3.7 | 20.6 ±0.9 | 22.4 ±3.3 | 1.5 ±0.6 | 100.9 ±3.4 |

* Cells were disrupted and separated into crude nuclear (N), mitochondrial-lysosomal (M + L), microsome-rich (P), and cytosol (S) fractions by methods described in Fig. 1. All data are given as mean ± SE.

† mg/$10^9$ cells.

‡ nmol/min/mg protein.

§ Specific binding of $2 \times 10^{-9}$ M [$^3$H]-E$_2\beta$; reported as $10^{-3}$ × (d.p.m./mg protein).

tion conditions used in protocols 6 and 7 correspond with those in protocols 1 and 5, respectively. However, disruption of only 98% of cells in the present series of experiments (as opposed to 100% of cells in protocols 1 and 5) requires a significantly lesser number of homogenization strokes than those necessary in the previous protocols. The results show a greater degree of localization of 5′-nucleotidase in particulate fractions of protocols 6 and 7 (Fig. 2) as compared to protocols 1 and 5 (Table 1). Nevertheless, and notwithstanding the diminished force required in protocol 6 vs. its counterpart (protocol 1 in Table 1), the atypical appearance of a substantial portion of 5′-nucleotidase in cytosol remains characteristic of samples prepared in hypotonic buffer ($P < 0.001$, protocol 6 vs. protocol 7). Similarly, the distribution of specific E$_2\beta$ binding-sites in cytosol is greater in fractions obtained by protocol 6 than in those prepared by protocol 7 ($P < 0.001$). In consideration of the more characteristic distribution of 5′-nucleotidase in particulate fractions of the homogenate after utilization of protocol 7 [cf. 15, 35, 36], the latter method was used in all further experiments to study the distribution of enzyme activities and E$_2\beta$ binding-sites in uterine cell fractions.

*Distribution of protein, enzymes, and specific [$^3$H]-estradiol-17$\beta$ binding-sites in major fractions of uterine cells*

Table 3 presents results of more extensive analyses of the cellular distribution of protein, E$_2\beta$ binding-sites, and several marker enzymes with the present method of cell disruption (cf. Fig. 1; protocol 7, Fig. 2). Activities of succinate dehydrogenase and acid phosphatase are concentrated in the mitochondria:lysosome fraction. Glucose-6-phosphatase occurs predominantly in the microsome-rich fraction. Alkaline phosphatase and 5′-nucleotidase activities are found largely in the crude nuclear and microsome-rich fractions. The crude nuclear fraction also accounts for 96 ± 2% ($n = 4$) of the DNA in the homogenate. Moreover, in confirmation of our initial findings (see above), binding-sites for E$_2\beta$ are present in N > P > M + L > S (Table 3).

*Characterization of plasma membrane preparations and associated cellular fractions*

Crude nuclear sediment is generally contaminated with plasmalemmal as well as microsomal membrane components released during cell disruption [cf. 15, 19]. Further fractionation of the nuclear sediment was achieved by isopycnic centrifugation in a discontinuous sucrose density gradient (see Fig. 1). The resultant bands at $\rho < 1.13$ (F1), $\rho = 1.13$–$1.16$ (F2), $\rho = 1.16$–$1.18$ (F3), $\rho = 1.18$–$1.20$ (F4) and $\rho = 1.20$–$1.24$ (F5) and the semipurified nuclear sediment (F6) were collected, washed at the centrifuge and analyzed for E$_2\beta$ binding and enzyme activities (Table 4). Partially purified nuclei (F6) exhibit no specific binding-sites for E$_2\beta$ and little activity of the predominantly extranuclear enzymes shown in Table 4. F4 and F5 represent a mixture of particulate material with no enrichment of plasma membrane marker-enzymes or binding-sites for E$_2\beta$. The highest specific activities of 5′-nucleotidase and alkaline phosphatase, both valid plasma membrane marker enzymes [15, 35], occur predominantly in F2 and F3, and to a lesser extent in F1 (Table 4). Clearly, the greater enrichment of these two activities occurs in F2 with relative specific activities averaging 12.3 ± 1.4 and 8.5 ± 1.9 for 5′-nucleotidase and alkaline phosphatase, respectively. By interpolation from concen-

Table 4. Distribution and relative specific activities of protein, enzymes, and specific [$^3$H]-estradiol-17$\beta$ binding in plasma membrane and associated subfractions of uterine cells*

| Measurement (No. of experiments) | Homogenate (units) | Subfraction of crude nuclear pellet (% homogenate) F1 | F2 | F3 | F4 | F5 | F6 | Subfraction of crude nuclear pellet (relative specific activity) F1 | F2 | F3 | F4 | F5 | F6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Protein (4) | 240† | 0.6 | 1.1 | 2.4 | 6.3 | 10.8 | 18.9 | | | | | | |
| | ±25 | ±0.1 | ±0.2 | ±0.5 | ±2.7 | ±2.9 | ±1.4 | | | | | | |
| Succinate dehydrogenase (3) | 11.7‡ | 0.0 | 0.1 | 2.1 | 1.2 | 3.6 | 2.7 | 0.00 | 0.14 | 0.52 | 0.76 | 0.23 | 0.20 |
| | ±0.8 | ±0.0 | ±0.1 | ±0.7 | ±0.4 | ±0.4 | ±0.5 | ±0.00 | ±0.05 | ±0.22 | ±0.18 | ±0.05 | ±0.07 |
| Glucose-6-phosphatase (3) | 2.9‡ | 0.4 | 0.6 | 2.2 | 2.5 | 5.2 | 2.4 | 0.50 | 0.51 | 0.77 | 1.15 | 0.33 | 0.29 |
| | ±0.8 | ±0.1 | ±0.0 | ±0.4 | ±0.4 | ±0.3 | ±0.3 | ±0.10 | ±0.01 | ±0.14 | ±0.13 | ±0.04 | ±0.04 |
| Acid phosphatase (3) | 17.9‡ | 0.3 | 0.5 | 4.1 | 2.5 | 4.0 | 0.8 | 0.48 | 0.49 | 1.01 | 1.10 | 0.26 | 0.10 |
| | ±1.2 | ±0.2 | ±0.3 | ±1.5 | ±0.5 | ±0.8 | ±0.2 | ±0.12 | ±0.12 | ±0.44 | ±0.43 | ±0.10 | ±0.05 |
| Alkaline phosphatase (3) | 37.1‡ | 0.5 | 9.2 | 8.6 | 6.7 | 6.4 | 1.5 | 2.97 | 8.61 | 4.52 | 1.02 | 0.40 | 0.09 |
| | ±2.3 | ±0.1 | ±0.5 | ±1.1 | ±0.4 | ±0.3 | ±0.5 | ±0.20 | ±0.24 | ±0.70 | ±0.18 | ±0.06 | ±0.05 |
| 5′-Nucleotidase (4) | 70.4‡ | 1.8 | 14.0 | 20.1 | 7.4 | 5.3 | 1.0 | 2.96 | 12.33 | 8.45 | 1.06 | 0.34 | 0.11 |
| | ±3.0 | ±0.3 | ±2.5 | ±4.2 | ±2.4 | ±0.2 | ±0.4 | ±0.20 | ±1.38 | ±1.86 | ±0.09 | ±0.10 | ±0.05 |
| Estradiol-17$\beta$ binding (4) | 34.6§ | 0.0 | 26.9 | 24.3 | 0.4 | 0.0 | 0.0 | 0.00 | 23.20 | 13.70 | 0.04 | 0.00 | 0.00 |
| | ±2.5 | ±0.0 | ±3.4 | ±4.0 | ±0.4 | ±0.0 | ±0.0 | ±0.00 | ±3.10 | ±3.20 | ±0.03 | ±0.00 | ±0.00 |

* Fractions 1–5 and the nuclear sediment (F6) were obtained by discontinuous sucrose density gradient centrifugation of the washed crude nuclear fraction (see Fig. 1). All data are given as mean ± SE.

† mg/$10^9$ cells.

‡ nmol/min/mg protein.

§ Specific binding of $2 \times 10^{-9}$ M [$^3$H]-E$_2\beta$; reported as $10^{-3} \times$ (d.p.m./mg protein).

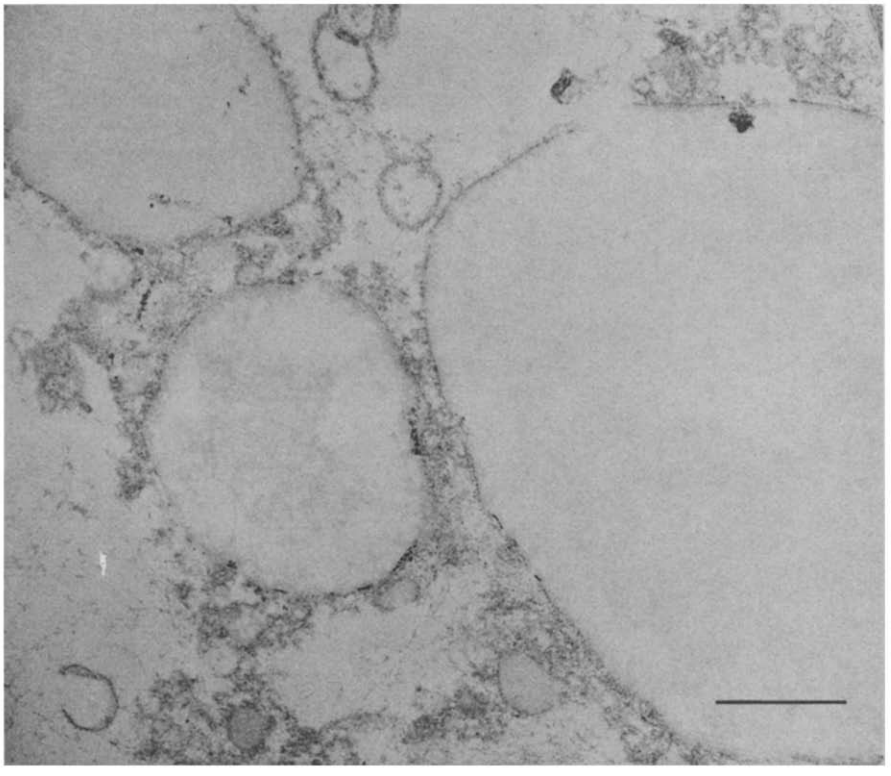

Fig. 3. Electron micrograph of plasma membrane preparation F2 from isolated uterine cells. It is evident that F2 consists primarily of smooth membrane vesicles and large "ghosts". Occasional electron-dense vesicles are also present, but mitochondria and nuclei are not detected. Micrographs were prepared through the courtesy of Dr. W. Jann Brown. Magnification 35,000 × ; bar represents 0.5 $\mu$m.

trations in the mitochondria:lysosome fraction (Table 3), F2 has a relative specific activity of 0.014 for succinate dehydrogenase, indicating a mitochondrial contamination of 1.4%. Similar calculations for glucose-6-phosphatase activities suggest that F2 is composed of microsomal material to the extent of 10.6%.

The above enzymic analyses confirm a predominantly plasma membrane origin of F2 and are consistent with morphologic evidence obtained by electron microscopy (Fig. 3). The latter studies show that F2 consists principally of large "ghosts" and smooth membrane vesicles [ref. 7, 35, 39]. Cross-sectional views of trilaminar membrane and apparent planar surfaces of membrane were observed. Stained material, possibly myofibrillar elements as noted in independent preparations of uterine membrane [7, 40], was also associated with some vesicles. No contamination by mitochondria or nuclei was detected, but electron-dense vesicles were occasionally present.

*Specific binding of [$^3H$]-estradiol-17$\beta$ to plasma membrane fractions*

Specific binding-sites for $E_2\beta$ occur in plasma membrane subfractions F2 and F3 (Table 4). Of the two fractions, F2, composed of membrane components with densities of 1.13–1.16, is the more selectively enriched to 23 times that of the homogenate. About 27% of total cellular $E_2\beta$ binding is recovered in F2.

Since entrapment of cytosol inside plasma membrane vesicles or adsorption of soluble proteins to plasma membranes could account for a substantial portion of the $E_2\beta$ binding detected in F2, a series of control experiments was instituted to investigate these potential artifacts. The results in Table 5 show that $E_2\beta$ binding to F2 is not substantially reduced by extraction of the latter with hypotonic buffer (i.e., 5 mM Tris–HCl:1.5 mM EDTA), physiological saline (0.9% NaCl), or high-salt buffer (5 mM Tris–HCl:1.5 mM EDTA:0.4 M KCl). After such extraction, the level of specific $E_2\beta$ binding relative to membrane protein is essentially unchanged, or even slightly enhanced due to selective removal of protein (i.e., 5–7%; Table 5). On the other hand, the addition of a low concentration of the detergent, Triton X-100, to the latter mixture was effective in removing a substantial portion of membrane-bound radioactivity as well as membrane protein from the preparation (Table 5). This observation suggests either that the detergent promoted extraction of [$^3$H]-$E_2\beta$ *per se* [cf. 41], or that binding-sites together with hormone may be susceptible to leaching under these conditions. This question is being pursued in additional experiments to be reported elsewhere.

To test further for inadvertent adsorption of [$^3$H]-$E_2\beta$ to the F2 membrane subfraction, cytosol prepared by protocol 1 (see Table 1) was labelled by

Table 5. Effect of diverse extraction conditions on retention of [$^3$H]-estradiol-17$\beta$ bound to plasma membrane subfraction F2*

| Extraction conditions | [$^3$H]-$E_2\beta$ Specifically bound d.p.m. | Membrane protein $\mu$g (Per cent of control†) | [$^3$H]-$E_2\beta$ Specifically bound d.p.m./$\mu$g membrane protein |
|---|---|---|---|
| 0.9% NaCl | 96.1 (2) | 93.9 (2) | 102.3 (2) |
| 5 mM Tris–HCl: 1.5 mM EDTA | 95.2 (2) | 93.0 (2) | 102.4 (2) |
| 5 mM Tris–HCl: 1.5 mM EDTA: 0.4 M KCl | 94.9 ± 2.4 (3) | 95.1 ± 3.1 (3) | 99.8 ± 0.7 (3) |
| 5 mM Tris–HCl: 1.5 mM EDTA: 0.4 M KCl: 0.1% Triton X-100 | 52.2 + 8.6 (3) | 84.2 + 4.4 (3) | 61.4 ± 7.8 (3)‡ |

* Samples of membrane (~120 $\mu$g protein) were incubated with $2 \times 10^{-9}$ M [$^3$H]-$E_2\beta$ for 2 h at 4°C, sedimented and washed at the centrifuge with 5 mM Tris–HCl (pH 7.4) in 0.25 M sucrose, as described in the text. They were then extracted under the conditions indicated in the Table for 1 h at 4°C, with repeated resuspensions at 10-min intervals with a Vortex stirrer. Specific binding of [$^3$H]-$E_2\beta$ is given as per cent of appropriate control samples extracted for 1 h with 5 mM Tris–HCl (pH 7.4) in 0.25 M sucrose.

† Specific binding of [$^3$H]-$E_2\beta$ averaged 704 ± 86 d.p.m./$\mu$g protein (~120 $\mu$g/sample) at 4°C.

‡ Value significantly different from that of control at $P < 0.001$.

incubation with either $2 \times 10^{-10}$ M or $2 \times 10^{-9}$ M [$^3$H]-$E_2\beta$. Unbound hormone was removed by the dextran-coated charcoal procedure, and samples (~100 $\mu$g protein) of the cytosol so labelled with [$^3$H]-$E_2\beta$, $2 \times 10^{-10}$ M (14,694 d.p.m./mg protein), or $2 \times 10^{-9}$ M (76,080 d.p.m./mg), were incubated with samples of F2 (~250 $\mu$g protein), previously unexposed to $E_2\beta$. After 2 h incubation at 4°C with Vortex stirring at 10 min intervals, F2 was again sedimented, resuspended in 5 mM Tris–HCl (pH 7.4):0.25 M sucrose, and resedimented. Analyses of [$^3$H] in the pellets and supernatants revealed that only 6.1 ± 0.7% ($n = 3$) and 9.1 ± 0.9% ($n = 3$) of the initial cytosol radioactivity in the $2 \times 10^{-10}$ M and $2 \times 10^{-9}$ M samples, respectively, became associated with the final F2 sediment under these conditions (not shown). In addition, extraction of the latter [$^3$H]-$E_2\beta$:cytosol-exposed F2 sediments with 5 mM Tris–HCl:1.5 mM EDTA:0.4 M KCl reduced the apparent binding of cytosolic $E_2\beta$ receptors to F2 by 64 ± 5%. Collectively, these data indicate that the high $E_2\beta$-binding activity of F2 cannot be attributed to gross entrapment or adsorption of highly charged cytosol protein [3] with a capacity to bind $E_2\beta$.

Specific binding of $2 \times 10^{-9}$ M hormone to membranes (2 h, 4°C) is reduced to 3.9 × 2.0% ($n = 3$) and 0.3 ± 0.3% (n = 3) of controls ($P < 0.001$) by prior exposure of F2 (120 $\mu$g protein) for 1 h to bovine pancreatic trypsin (Sigma; 250 $\mu$g ≃ 2250 BAEE units) at 22°C or to heat denaturation at 60°C, respectively.

Binding of [$^3$H]-$E_2\beta$ by F2 was analyzed further in equilibrium binding experiments (Fig. 4). Samples of F2 were exposed to a series of [$^3$H]-$E_2\beta$ concentrations ranging from $5 \times 10^{-10}$ M to $4 \times 10^{-9}$ M. As shown in Fig. 4A, all samples with [$^3$H]-$E_2\beta$ alone (curve *x*) retain a greater amount of hormone than paired samples in which [$^3$H]-$E_2\beta$ was present together with a 200-fold molar excess of unlabelled hormone (curve *y*). The difference between the two curves, representing the specific binding of $E_2\beta$, is plotted in Fig. 4B. It is evident from the net retention

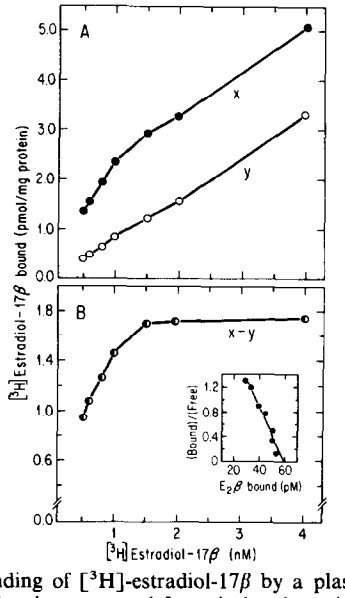


Fig. 4. Binding of [$^3$H]-estradiol-17$\beta$ by a plasma membrane subfraction prepared from isolated uterine cells of the ovariectomized rat. (A) Plasma membranes (F2) were incubated in $Ca^{2+}$-free medium with 0.25 M sucrose at approx. 60 $\mu$g membrane protein/2 ml for 2 h at 4°C with the concentrations of [$^3$H]-$E_2\beta$ given alone (●, curve *x*) or in the presence of a 200-fold molar excess of unlabelled $E_2\beta$ plus [$^3$H]-$E_2\beta$ (○; curve *y*). The latter curve shows an essentially linear increment in $E_2\beta$ binding with increasing hormone concentration ($r = 0.95$). (B) This curve shows the difference between the two curves in panel A and represents, according to the notation of Williams and Gorski[22], the specific binding of hormone by plasma membranes. A Scatchard plot of these data is shown as an inset in panel B. Each point represents the mean of two independent determinations.

Table 6. Ligand specificity of [$^3$H]-estradiol-17$\beta$ binding to plasma membrane subfraction F2 isolated from uterine cells

| Competing compound* | Specific [$^3$H]-estradiol-17$\beta$ binding (d.p.m./$\mu$g protein)† | (% paired control) |
|---|---|---|
| None (0.02% ethanol) | 821.7 ± 98.6 (3) | 100 ± 0 (3) |
| Estradiol-17$\beta$ | 0.0 ± 0.0 (3)‡ | 0 ± 0 (3)‡ |
| Estradiol-17$\alpha$ | 776.5 ± 85.4 (3) | 95 ± 2 (3) |
| Diethylstilbestrol | 238.3 ± 35.7 (3)‡ | 29 ± 2 (3)‡ |
| Progesterone | 850.6 (2) | 104 (2) |
| Testosterone | 812.8 (2) | 96 (2) |
| Cortisol | 733.0 (2) | 92 (2) |

* $4 \times 10^{-7}$ M, unless otherwise indicated.

† Only specific binding of $2 \times 10^{-9}$ M [$^3$H]-estradiol-17$\beta$ (defined as the difference in bound [$^3$H]-$E_2\beta$ between paired tubes, one of which contained a 200-fold molar excess of unlabelled competing compound and the other, $4 \times 10^{-7}$ M "cold" $E_2\beta$ throughout the experiment) at 4°C for 2 h is shown as mean ± SE ($n$).

‡ Value significantly different from that of paired vehicle control at $P < 0.001$.

so analyzed that binding of the hormone by plasma membranes is saturable. Scatchard analyses of specific [$^3$H]-$E_2\beta$ binding (see Fig. 4B inset) indicate that the association constant for the binding process is $4.3 \times 10^{10}$ M$^{-1}$. Total binding sites in F2 at saturation correspond to approx. 2.0 pmol $E_2\beta$ per mg membrane protein.

*Ligand specificity of [$^3$H]-estradiol-17$\beta$ binding to plasma membrane subfraction F2*

The ligand specificity of [$^3$H]-$E_2\beta$ binding to partially purified plasma membrane F2 was established by effective suppression, to 0 ± 0% and 29 ± 2% of initial value, respectively, by a 200-fold molar excess of unlabelled $E_2\beta$ or diethylstilbestrol (Table 6). In contrast, the extent of [$^3$H]-$E_2\beta$ binding by F2 was essentially uninfluenced by these levels of estradiol-17$\alpha$, progesterone, testosterone, or cortisol.

## DISCUSSION

The present studies provide additional evidence that a significant proportion (i.e., ~27%) of receptor components with high affinity and ligand specificity for binding $E_2\beta$ is concentrated in plasma membrane subfractions purified from isolated uterine cells of the ovariectomized rat. The occurrence in target cells of specific estradiol binding in several particulate fractions, including mitochondria [6], lysosome [9, 13], microsome [6–8, 42], mitochondria:microsome [12], and plasma membrane [43, 44], has been reported previously. In contrast, the external membranes of non-target epithelial cells from rat intestine do not display a capacity for binding estrogen [10].

The present findings are in apparent contrast to widely reported data that demonstrate occurrence of $E_2\beta$ binding predominantly in cytosolic fractions of responsive tissues [cf. 5, 37, 38]. This discrepancy is attributable, in part, to our application of homogenization and isolation procedures that differ significantly from those in general use for the preparation of cell fractions enriched in $E_2\beta$ binding components [37, 38]. The effectiveness of any cell fractionation scheme is limited by various artifacts which arise when cells are disrupted [15, 16]. It is to some extent unavoidable that shearing of plasma membranes and fragmentation of organelles, as well as entrapment of soluble material inside membrane vesicles or adsorption of soluble proteins onto membranes will occur. The present analytical approach to cell fractionation was instituted to minimize the loss of material intrinsic to particulate components and to account fully for the distribution of $E_2\beta$-binding activities in the several cell fractions. Disruption of a maximum of ~98% of isolated cells in a Teflon-glass homogenizer was used to prevent the aberrant redistribution of macromolecules known to be elicited by excessive cell homogenization [21]. Our use of isolated cell preparations as starting material, rather than organ segments, also significantly reduces the shearing and grinding forces required to break the cells. Previous workers have reported that the drastic homogenization necessary to disrupt tough muscular and connective tissue components of uterus results in very small fragments of plasma membrane, most of which is lost in postnuclear supernatant fractions at the first step of low-speed centrifugation [35]. Under hypotonic isolation conditions similar to those tested in protocols 1 and 2, nuclei, mitochondria and lysosomes are all known to lyse and fragment [15, 16, 45, 46]. Other investigators have also found marked differences in the constitution of proteins and enzyme activities from plasma membranes prepared by hypotonic versus isotonic isolation procedures [19]. Thus, the isolated uterine cells in the present study were disrupted in isotonic media and in the presence of divalent cations that promote the maintenance of the structural integrity of membranes [15, 19, 20].

Plasma membrane fractions (i.e., F2) prepared by our methods are well preserved by both morphologic and biochemical criteria. Significant recovery of large membrane "ghosts", the ideal goal in such preparations [15], as well as smaller smooth membrane

vesicles was attained with no detectable contamination by nuclei or mitochondria evident. The plasma membrane and other major cell fractions exhibit good yield and high enrichment of appropriate marker-enzymes.

The marked enrichment, to 23 times the homogenate, of specific $E_2\beta$ binding-sites in plasma membranes of uterine cells as presently demonstrated has not heretofore been reported. To investigate potential artifacts due to conditions of homogenization and processing which might give rise to this observation, we have attempted to extract $E_2\beta$ bound by F2 fractions with severely hypotonic buffers known to lyse whole cells as well as their membrane-bounded organelles [45, 46] and also with various media of high ionic strength that are known to promote the solubilization of proteins loosely associated with plasma membrane preparations [47]. The results indicate an apparent contamination of F2 with (or extraction from F2 of) only 5–7% soluble protein. However, 95% of specific [$^3$H]-$E_2\beta$ binding remains associated with the plasma membranes. Similarly, in mixing experiments, we find that only 6–9% of [$^3$H]$E_2\beta$-labelled cytosol components associate with plasma membranes at 4°C, and the bulk of such adsorption is readily extracted by high-salt buffer. Likewise, in experiments with microsomal membranes prepared from calf uteri, Jackson and Chalkley[8] have found that less than 10% of the input radioactivity associated with the [$^3$H]-$E_2\beta$:cytosol receptor–complex binds to membranes at 4°C. Such data indicate that no more than a small fraction of the high, though saturable and specific, binding-capacity of F2 for $E_2\beta$ may be attributable to cytosol protein entrapment or adsorption artifacts arising during cell disruption and fractionation. On the other hand, we have found that the nonionic detergent Triton X-100 is effective in extracting about 48% of plasmalemma-bound [$^3$H]-$E_2\beta$, concomitantly with solubilization of approximately 16% of membrane protein.

The sensitivity of the plasma membrane binding-component for $E_2\beta$ to trypsin and to 60°C heat, as well as its capacity to differentiate between stereoisomers of estradiol, suggest that the nature of the binder is at least in part protein. The binding constant (i.e., $4.3 \times 10^{10}\,M^{-1}$) for the interaction of $E_2\beta$ with plasmalemmal fraction F2 is high, as would be expected for a steroid-macromolecular association of high affinity [2, 3, 5]. The association constant for the binding of estradiol to its specific carrier protein in rat plasma is $5 \times 10^8\,M^{-1}$ [48], about two orders of magnitude lower than the plasma membrane binding constant in the present study.

Using criteria for specific ligand association as established by Williams and Gorski[22], the present data indicate that binding of $E_2\beta$ by cytosol fractions ranges from approx. 2% to 80% of total cellular binding, depending upon the choice of isolation protocol. It is critical to interpretation of the results with protocols 1–5 in the present work that the extent of 5′-nucleotidase extracted from particulate into cytosol fractions (i.e., 9–60% homogenate) parallels very closely the proportion of total cellular $E_2\beta$ binding that appears in cytosol (i.e., 20–78% homogenate). In the cases of both 5′-nucleotidase and $E_2\beta$ binding, elevation of activity in the cytosol fraction is accompanied by a corresponding loss in the other major cell fractions. Since 5′-nucleotidase activity is normally enriched only in particulate fractions and predominantly in plasma membranes [15], including those of uterus [35, 36], the present data conform to previous findings that use of hypotonic conditions [19, 45, 46] as well as excessive force [21, 35] to break cells elicits fragmentation of particulate fractions and the consequent redistribution of their components. Thus, extraction artifacts introduced during cell homogenization and fractionation must be minimized in efforts to localize the $E_2\beta$ binding-component in its native state.

Several questions of major significance for understanding the cellular action of $E_2\beta$ and other steroid hormones have been raised by the observations described here. Previous studies have suggested that a majority of native estradiol receptors in target cells are sequestered in (an) extranuclear compartment(s) apart from cytosol prior to cell homogenization [7–9, 14, 43, 44]. That suggestion gains additional support from the present findings. From the available evidence, it seems likely that binding-components specific for $E_2\beta$ are present in multiple cellular loci, perhaps in multiple molecular forms [7, 41]. It is noteworthy that cytosol counterparts of proteins native to the plasma membrane have recently been detected in kidney cells [49]. In addition, several cellular enzymes have been found to partition reversibly between distinct soluble and membrane-bound forms, with the distribution between these forms apparently regulated by specific metabolites [50]. Consideration of these new findings makes it tempting to speculate on potential parallels to the interaction of $E_2\beta$ with its cellular receptors. Therefore, it would appear fruitful to direct future research toward the investigation of a possible biosynthetic pathway or nuclear translocation circuit [2, 3, 5, 51] involving microsomal, mitochondrial, lysosomal and plasma membrane, as well as cytosol elements. Independent studies with other steroid hormones demonstrate the occurrence of saturable, high-affinity binding-sites for cortisol [52] and aldosterone [53] in plasma membrane fractions of liver and kidney, respectively, and the affinity-binding of amphibian oocytes to progesterone [54] or deoxycorticosterone [55] immobilized by covalent attachment to an inert support. Collectively, such data indicate that our understanding of the cellular mechanism of steroid hormone action will not be complete without further consideration of the role of cell membrane components in both the recognition of and response to hormone. It is hoped that the present report will promote efforts to undertake new approaches to these complex questions.

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