Partial purification and characterization of oestrogen receptors in subfractions of hepatocyte plasma membranes

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To assess the subcellular distribution of oestrogen-binding components in their native state, plasma membrane and other cell fractions were prepared from hepatocytes in the absence of [^{3}H]oestradiol-17β. Cells from livers of ovariectomized rats were disrupted, with submaximal homogenization in buffered isotonic sucrose with $CaCl_2$ and proteinase inhibitor, and fractionated by using isotonic media. Fractions were characterized by determinations of enzyme activities, biochemical constituents and ligand binding. Specific binding of 2 nm-[^{3}H]oestradiol-17β to intact cells and their fractions was detemined after equilibration for 1.5 h at 4°C. More than 92% of the radioactivity from representative preparations was verified as authentic oestradiol by thin-layer chromatography. Activities of plasma-membrane marker enzymes as well as binding sites for oestrogen and for wheat germ agglutinin were present principally in particulate fractions, rather than in 105000g-supernatant fractions. However, by using alternative homogenization procedures (i.e. hypotonic media), known to fragment and strip structural components, oestradiol-binding sites and activities of plasma-membrane marker enzymes were distributed predominantly into cytosol. By using the more conservative procedures, plasma membranes of low ($\rho = 1.13$–1.16) and high ($\rho = 1.16$–1.18) density were purified from crude nuclear fractions. A second low-density subfraction of plasma membrane was prepared from microsome-rich fractions. Activities of plasma-membrane marker enzymes were enriched to about 28 and four times that of the homogenate in plasma membranes of low and high density respectively. Binding sites for wheat germ agglutinin and oestradiol were concentrated in low-density plasma membranes to 46–63 times that of the homogenate. Specific binding of oestrogen in low-density plasma membranes purified from crude nuclei was saturable, with an apparent association constant of 3.5 nm. At saturation, such oestradiol receptors corresponded to 526 fmol/mg of membrane protein. A Hill plot showed a moderate degree of positive co-operativity in the interaction of hormone with plasma membranes. Specific binding of [^{3}H]oestradiol-17β was reduced by a 200-fold molar excess of unlabelled oestradiol-17β, oestriol or diethylstilbestrol, but not by oestradiol-17α, cortisol, testosterone or progesterone. Binding was also blocked by prior exposure of membranes to trypsin or to 60°C, but remained essentially undiminished by extraction of membranes with either hypotonic or high-salt buffers. Extraction with 0.1% (v/v) Triton X-100 partially solubilized the oestrogen-binding component(s) of plasma membranes. Particle-free extracts were resolved on 5–20% (w/v) sucrose density gradients with either 0.01 m- or 0.4 m-KCl, and the fractions were analysed by adsorption to hydroxyapatite. In low-salt gradients macromolecule-bound oestrogen sedimented at predominantly 7.4 S and binding was 1560 times that of the homogenate. Under high-salt conditions oestradiol-binding activity occurred at both 3.6 S and 4.9 S.

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Nuclear transfer of oestrogen-binding sites was investigated by incubating intact hepatocytes with 0.1 nM-oestradiol for 10 min at 22°C. A profound increase in oestrogen binding in nuclei was found to be coupled with a marked loss of binding in membranes, as compared with controls at 4°C. Collectively, these results indicate that components of hepatocyte plasma-membrane may be integral to the recognition of and response to oestrogen.

Exposure of mammalian liver to oestrogen is known to elicit several phenotypic effects. These include enhanced chromatin template activity (Hamilton, 1968), RNA synthesis (Church & McCarthy, 1970), biosynthesis of specific plasma proteins (Miller & Griffin, 1975; Eisenfeld et al., 1976), oxidative metabolism of cholesterol (Pietras & Szego, 1979a), alterations in the activity of (Na+,K+-activated)-ATPase and in bile flow (Davis et al., 1978), and increased incidence of tumours (Schenken & Burns, 1943; Edmondson et al., 1976). As in other cellular targets of oestrogen (see King and Mainwaring, 1974; Gorski & Gannon, 1976; Thrall et al., 1978), extranuclear macromolecules with high affinity for binding this hormone occur in liver (Smirnova et al., 1974; Viladiu et al., 1975; Chamness et al., 1975; Eisenfeld et al., 1976; Beers & Rosner, 1977; Pietras & Szego, 1977, 1979a). Binding to these receptors appears to initiate transfer of the hormone to chromatin (Eisenfeld et al., 1976).

It is not established with certainty whether the extranuclear oestrogen receptor in its native state occurs as such in the cytosol or is associated with some cellular structure. The initial investigations of Gorski et al. (1968) indicated that the bulk of oestradiol-17β that became specifically bound when uterine segments were incubated with the hormone at 0–4°C was associated with high-speed supernatant fractions obtained after homogenization. However, Noteboom & Gorski (1965) also identified specific oestradiol-binding sites in mitochondrial and microsomal fractions. Numerous independent reports on the occurrence of binding components with high affinity and specificity for oestrogen in particulate fractions of responsive cells have been reviewed by Szego & Pietras (1980). Such data have raised the question of whether the widely reported predominance of receptors for oestradiol in the cytosolic fraction of target cells might have resulted from inadvertent extraction of native receptors for the hormone by homogenization procedures, which elicited extensive damage to cellular structures (cf. Little et al., 1972; Jensen et al., 1974; Szego, 1974; Pietras & Szego, 1979b).

The present work seeks to determine the distribution and interrelations of oestrogen-binding components in plasma membrane and other cellular fractions obtained after controlled homogenization of rat hepatocytes. As recommended by de Duve

(1967) and others (DePierre & Karnovsky, 1973; Evans, 1978), we have adopted an analytical approach to subcellular fractionation and provide in the present report a balance sheet for the distribution of several marker and other enzyme activities and biochemical constituents, as well as ligand binding.

A preliminary report of a portion of these findings has been presented elsewhere (Pietras et al., 1978).

Materials and methods

Isolation of hepatocytes

Livers were excised from five to eight female Sprague–Dawley rats that had been ovariectomized at 6 weeks of age and maintained for 3 weeks in a low-steroid environment (Szego, 1974). Cells, predominantly of the parenchymal type, were isolated by the method of Howard et al. (1973) as modified by Pietras & Szego (1979a). However, in the present experiments, soya-bean trypsin inhibitor (0.1 mg/ml; Sigma Chemical Co., St. Louis, MO, U.S.A.) was added to all perfusion and incubation media containing collagenase (2 mg/ml; type I, Worthington Biochemical Corp., Freehold, NJ, U.S.A.), as recommended by Wisher & Evans (1977). The resultant isolated cells were then suspended in serum-free, chemically-defined medium consisting of Earle's balanced salt solution (Earle et al., 1943) enriched with highly purified (1×10^{-8} M), crystalline bovine insulin (Eli Lilly and Co., Indianapolis, IN, U.S.A.), 0.1% albumin (Pentex: Miles Laboratories, Elkhart, IN, U.S.A.), minimum essential medium amino acids (Grand Island Biological Co., Grand Island, NY, U.S.A.), 10 mM-glucose, 1 mM-sodium pyruvate and 50 μg of Gentamicin (Schering Corp., Kenilworth, NJ, U.S.A.) per ml, and incubated for 10 h in a humidified atmosphere of 5% CO_2 in air at 37°C. At least 95% of all cells used in these experiments excluded the dye, nigrosin, at a final concentration of 0.05% in complete Ringer solution (cf. Pietras & Szego, 1979a) during 5–10 min of incubation at 22°C.

Isolation of plasma membrane subfractions

Plasma membrane and other subcellular fractions were prepared at 4°C from the isolated hepatocytes essentially by the method of Berman et al. (1969) with the slight modification of Yamamoto et al. (1973). A summary of major steps in the

isolation scheme is shown in Fig. 1. In brief, cells were homogenized in 9 vol. of 0.25 M-sucrose (ultra-pure, density-gradient grade; Schwarz/Mann, Becton, Dickinson & Co., Orangeburg, NY, U.S.A.) with 0.5 mM-CaCl$_2$, 0.5 μM-leupeptin (acetyl- and propionyl-L-leucyl-L-leucyl-L-arginal; Peptide Institute, Osaka, Japan) and 5 mM-Tris/HCl buffer (pH 7.4). Approx. 35–40 manual strokes (i.e., total forward and return) with a ball-shaped Teflon pestle in a Dounce homogenizer (approx. 0.15 mm clearance) were sufficient to disrupt the liver cells, as systematically monitored in each experiment by phase-contrast microscopy. As recommended by Plagemann (1969), the cellular preparation was disrupted with the minimum homogenization required to break about 98% of the cells.

The homogenate was diluted with an equal volume of medium and filtered two to three times through two layers of nylon mesh of 35-μm pore size (Nitex; Tobler, Ernst, and Traber Inc., Elmsford, NY, U.S.A.) to remove all unbroken cells. The filtrate was centrifuged at 1600 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., 0.25 M-sucrose/5 mM-Tris/HCl buffer (pH 7.4)/ 0.5 μM-leupeptin], and suspended in 4 ml of the same medium. The suspension was introduced above the following four discontinuous sucrose-density layers (all, w/w): from bottom to top, 10 ml of 45 ($\rho = 1.20$), 10 ml of 41 ($\rho = 1.18$), 8 ml of 37 ($\rho = 1.16$) and 5 ml of 31% ($\rho = 1.13$) sucrose in Ca^{2+}-free medium. All gradients were centrifuged in a Spinco SW-27 rotor at 22 500 rev./min (67 000 g at $r_{av.}$) for 2 h at 4°C with a Beckman model L5-75 ultracentrifuge.

After centrifugation of gradient I (see Fig. 1), a partially purified plasma-membrane fraction at 31–37% sucrose ($\rho = 1.13$–1.16) was collected, diluted with Ca^{2+}-free medium, and washed twice by centrifugation at 16 800 g for 20 min. A portion of the resultant sediment was saved and the remainder
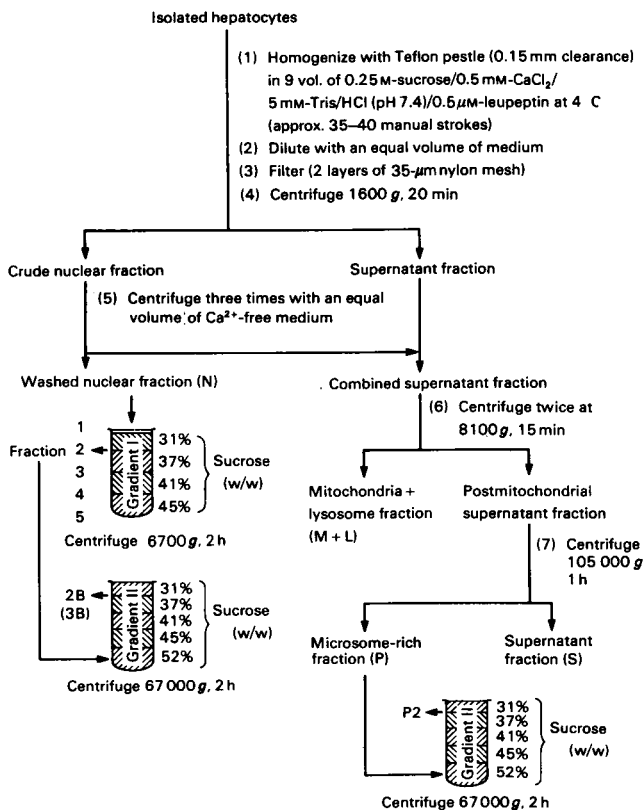

Fig. 1. *General scheme for fractionation of hepatocytes isolated from livers of ovariectomized rats*
A complete description of the fractionation procedure is provided in the text. It is to be noted that, in independent experiments, Fraction 3 (gradient I, 37–41% sucrose), as well as Fraction 2 (31–37% sucrose) shown in the Figure, was purified further by flotation through gradient II, yielding Fraction 3B (37–41% sucrose); all, w/w.

was suspended in 7 ml of 52% (w/w; $\rho = 1.24$) sucrose in Ca^{2+}-free medium. Four layers of sucrose in Ca^{2+}-free medium at concentrations of 45 (7 ml), 41 (7 ml), 37 (7 ml) and 31% (9 ml) were then introduced above the latter suspension. Gradient II was then centrifuged at 67000 g for 2 h, and the purified plasma-membrane fraction at 31–37% sucrose ($\rho = 1.13$–1.16) was removed and washed by centrifugation as above. Whole homogenate and membrane fractions were each diluted with Ca^{2+}-free medium to about 1 mg of protein per ml and stored at 4°C. Hormone binding and enzyme activity analyses were performed on each sample within 6 and 24 h respectively.

In some experiments, a membrane fraction sedimenting at 37–41% sucrose ($\rho = 1.16$–1.18) in gradient I, was purified further by flotation through gradient II, yielding Fraction 3B. In addition, plasma-membrane subfractions, which cosediment with microsomes (cf. Touster et al., 1970; Wisher & Evans, 1977), were prepared from the microsome-rich pellet by similar methods, yielding a fraction sedimenting at a density of 1.13–1.16 (see Fig. 1).

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

Liver mitochondria and lysosomes, microsomes and cytosol were prepared from the combined supernatants and washes of the crude nuclear pellet as outlined in Fig. 1. All fractions were diluted with Ca^{2+}-free medium, as appropriate, to approx. 1 mg of protein per ml and stored at 4°C until analysed (within the times indicated above).

Determination of specific [³H]oestradiol-17β binding in cell fractions

To determine specific oestradiol-17β binding in the several subcellular fractions, incubations were begun with the addition of [2,4,6,7,16,17-³H]oestradiol-17β (152 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) at 4°C. A 200-fold molar excess of unlabelled oestradiol-17β (Schering) was present with [³H]oestradiol in paired samples for determination of displaceable binding (cf. Williams & Gorski, 1973; Pietras & Szego, 1979a). In related competitive-binding experiments, a 200-fold molar excess of unlabelled oestradiol-17α, diethylstilbestrol, oestriol, cortisol, progesterone (all from Schering) or testosterone (Sigma) was added with [³H]oestradiol-17β to additional paired samples, as appropriate. After 1.5 h of equilibration with shaking, samples were diluted with 2 vol. of ligand-, Ca^{2+}-free medium at 4°C. Particulate fractions were then centrifuged at 16800 g for 20 min, except for microsomal samples, which were centrifuged at 105000 g for 1 h. The resultant pellets were resuspended in 2 vol. of the medium and sedimented

by centrifugation to remove unbound oestradiol. The washed sediments were solubilized with 0.1 M-NaOH and 0.1% (v/v) Triton X-100 (Rohm and Haas Co., Philadelphia, PA, U.S.A.). To separate bound from free oestrogen in particle-free supernatant fractions (cf. Korenman, 1975) 1 vol. of supernatant was mixed with a pellet of dextran-coated charcoal, which was removed from the supernatant fraction by centrifugation after 10 min of incubation in an ice bath (cf. Pietras & Szego, 1979b). Samples were collected for determination of ³H radioactivity by liquid-scintillation counting in Biofluor (New England Nuclear) and for analyses of protein by the method of Lowry et al. (1951).

Analysis of [³H]oestradiol-17β metabolism in liver cells

[³H]Oestradiol-17β at a final concentration of 1 nM (3.3×10^5 d.p.m./ml) was added in vitro to a 20 ml-suspension of approx. 10^8 cells in Ringer solution with 1 mM-sodium pyruvate. After 1.5 h of incubation with shaking at 4°C, cells were sedimented and washed twice by centrifugation with ice-cold, hormone-free medium. The cell pellets were then suspended in 9 vol. of ice-cold homogenization medium supplemented with 0.2 μM unlabelled oestradiol-17β (cf. Williams & Gorski, 1971; Jackson & Chalkley, 1974) and subjected to the controlled homogenization procedures described above. The resultant homogenates were then centrifuged at 105000 g for 1 h to yield a particle-free supernatant fraction. The sediment was resuspended in ice-cold medium and centrifuged at 105000 g for a second time. One volume of the combined cytosol and washings was incubated with 5 vol. of hydroxyapatite (analytical grade, Bio-Rad Laboratories, Richmond, CA, U.S.A.; cf. Erdos et al., 1970) at a concentration of 10 g per 100 ml of 50 mM-Tris/HCl and 10 mM-KH_2PO_4 (pH 7.2, 4°C). After 30 min of incubation with shaking at 4°C, hydroxyapatite and adsorbed protein (cf. Pavlik & Coulson, 1976) were sedimented by centrifugation at 600 g for 5 min and then washed four times with fresh buffer. The final hydroxyapatite sediment and the washed 105000 g-pellet (see above) represent the bound fraction of hormone. The combined supernatants from initial sedimentation and successive washings of hydroxyapatite represent the free, or unbound, fraction of oestradiol.

The bound fraction was extracted with 5 vol. of methanol, and the precipitate which formed was washed twice with 5 ml of methanol (cf. Beers & Rosner, 1977). The methanol extracts were combined, evaporated under a stream of nitrogen, and the residue was taken up in 10 ml of ether and washed three times with 5 ml of water. The ether was evaporated under a stream of nitrogen, and the residue was analysed by t.l.c. with chloroform/acetic

acid (10:1, v/v) as described by Bishara & Jakovljevic (1969).

The free fraction was extracted twice with an equal volume of ether (cf. Beers & Rosner, 1977). The ether extracts were combined, evaporated and chromatographed as above.

Analysis of enzyme activities in plasma membrane and other subcellular fractions

Activity of 5'-nucleotidase (EC 3.1.3.5) in the several cell fractions was determined by the method of Touster *et al.* (1970). Analyses for alkaline phosphatase (EC 3.1.3.1) activity were as described by Pietras (1978). Activity of ouabain-sensitive $(Na^+,K^+$-activated)-ATPase [adenosinetriphosphatase $(Na^+,K^+$-activated; EC 3.6.1.3)] was determined by the method of Ismail-Beigi & Edelman (1971). Succinate dehydrogenase (EC 1.3.99.1) activity was determined by the method of Pennington (1961) and that of glucose 6-phosphatase (EC 3.1.3.9) by the method of Hübscher & West (1965). Acid phosphatase (EC 3.1.3.2) activity was analysed as described by Szego *et al.* (1971). For each enzyme activity, linearity of the reaction with time as well as sample protein was verified experimentally. In those experiments with media supplemented with EDTA, an appropriate excess of $CaCl_2$ was added to counteract any potential inhibitory effect of EDTA on enzyme activities (cf. Evans, 1978).

Chemical composition of plasma membranes

Plasma membranes collected at sucrose-gradient interfaces were diluted with Tris/HCl buffer (pH 7.4) and pelleted by centrifugation. To remove adsorbed or occluded material (cf. Emmelot & Bos, 1966; Kremmer *et al.*, 1976) pellets containing approx. 3 mg of protein were resuspended in 20 ml of 0.15 M-NaCl/5 mM-Tris/HCl (pH 7.4) and incubated for 1 h at 4°C, with repeated resuspensions at 10-min intervals with a Vortex stirrer. The membranes were then pelleted by centrifugation and resuspended in highly purified water (specific resistance $> 18\,M\Omega cm^{-1}$) at an approximate protein concentration of 1.5 mg/ml. Portions of this suspension were analysed chemically for protein, cholesterol, phospholipid, sialic acid, DNA and RNA. Lipids were extracted from washed plasma membranes with chloroform by the method of Bligh & Dyer (1959) as modified by Touster *et al.* (1970); portions were analysed for total cholesterol by the procedure of McDougal & Farmer (1957) and for total organic phosphate by the method of Galliard *et al.* (1965). N-Acetylneuraminic acid was determined by the procedure of Warren (1959) after hydrolysis of membranes in 0.1 M-H_2SO_4 at 80°C for 1 h. DNA and RNA were extracted by the

method of Maggio *et al.* (1963) and analysed as described by Schneider (1957).

Determination of wheat germ agglutinin binding in cell fractions

Binding of [^{3}H]wheat germ agglutinin (4.37 Ci/mmol, New England Nuclear) to liver cells was carried out by methods similar to those of Chang *et al.* (1975). Approx. 10^8 isolated cells were incubated at 22°C with 0.2 μCi of [^{3}H]wheat germ agglutinin (39 ng/ml) for 5 min in Ringer solution with 1 mM-sodium pyruvate. The free radioactivity in the medium was removed by sedimenting the cells at 50 g (5 min, 4°C) and washing twice with ice-cold homogenizing medium. Plasma-membrane and other cellular fractions were then prepared as described above. Over 95% of [^{3}H]wheat germ agglutinin binding to hepatocytes was blocked in the presence of a 100-fold molar excess of unlabelled wheat germ agglutinin (A grade; Calbiochem, San Diego, CA, U.S.A.) or by prior incubation of the lectin with 0.1 M-N-acetyl-D-glucosamine (A grade; Calbiochem).

Resolution of oestrogen-binding components by density-gradient centrifugation

Sets of three linear sucrose-density gradients (5 ml) were formed in $\frac{1}{2}$-inch × 2-inch polyallomer tubes with the use of a six-syringe Beckman density gradient former (Beckman Instruments, Mountain View, CA, U.S.A.). The low- and high-density solutions were, respectively, 5 and 20% (w/v) sucrose, each containing 10 mM-Tris/HCl (pH 7.4), 1.5 mM-EDTA, 0.01% (v/v) Triton X-100 and either 0.01 M-KCl (low salt) or 0.4 M-KCl (high salt). The following proteins were used as standards in the sedimentation experiments. Equine heart cytochrome c (Calbiochem) was assumed to have a sedimentation value of 1.9 S (Smigel & Fleischer, 1977). The value taken for ovalbumin (Calbiochem), 3.6 S, was an average of two reported determinations (Svedberg & Pedersen, 1940; Kegeles & Gutter, 1951). The sedimentation value used for albumin (Pentex; Miles) was 4.5 S (Eisenfeld *et al.*, 1976) and that used for bovine immunoglobulins (Calbiochem) was 7.0 S (Edsall, 1953).

Mixtures of protein standards (i.e. cytochrome c and albumin or ovalbumin and immunoglobulins), 0.2 ml of a solution of each protein (10 mg/ml) in high- or low-salt medium (see above), as appropriate, or 0.2 ml of Triton-solubilized membranes were layered on top of each gradient. The set of six tubes consisted of three high-salt and three low-salt sucrose gradients, with two pairs of solubilized membrane samples and one pair of protein standards in each experiment. The tubes were centrifuged at 50000 rev./min (233000 g at r_{av}; $\int_0^t \omega^2 dt = 1.75 \times 10^{12} rad^2/s$) for 18 h in a Spinco SW 50.1

rotor in a Beckman L5-75 centrifuge. The temperature was set at 4°C. After deceleration at 20% of the maximum rate, the bottoms of the tubes were pierced and 55% (w/w) sucrose was injected by means of a precision-driven syringe (Isco model D density gradient fractionator, Instrument Specialities Co., Lincoln, NE, U.S.A.). Fractions of 10 drops (approx. 40 drops/ml) were collected from the top, with an Isco model 1200 fraction collector. Alternate fractions from one tube with protein standards or membrane material were taken for determination of the refractive index with a Bausch and Lomb Abbé 3L refractometer. The remaining fractions were analysed for protein, and those containing solubilized plasma membranes were also analysed for specific [^{3}H]oestradiol binding.

To determine specific hormone binding to macromolecular components in the several solubilized membrane fractions, 0.1 ml samples were diluted to 1 ml with 50 mM-Tris/HCl (pH 7.2)/1.5 mM-EDTA/0.5 μM- leupeptin and either 0.01 or 0.4 M-KCl, as appropriate. Incubation for 1.5 h was begun with the addition of 2 nM-[^{3}H]oestradiol-17β at 4°C. A 200-fold molar excess of unlabelled oestradiol-17β was present with [^{3}H]oestradiol in paired samples for determination of displaceable binding. Separation of free oestrogen from steroid bound to macromolecular components was achieved by use of the hydroxyapatite-adsorption method as described above (cf. Pavlik & Coulson, 1976).

Results

Analyses of [^{3}H]oestradiol 17β binding and metabolism in parenchymal cells isolated from livers of ovariectomized rats

The presence in liver of enzymes which convert oestradiol-17β into other metabolites (e.g. oestrone) and water-soluble derivatives (Jensen & Jacobson, 1962) complicates identification of hepatic components which bind the hormone. However, equilibrium-binding studies with [^{3}H]oestradiol show that hepatic metabolism of oestrogen can be minimized by conducting experiments at 4°C for 1–2 h (Eisenfeld et al., 1976; Beers & Rosner, 1977). Moreover, previous investigations of uterine 17β-hydroxysteroid: NADP$^+$ 17-oxidoreductase (EC 1.1.1.64), the enzyme responsible for conversion of oestradiol into oestrone in liver as well as uterus, demonstrate that activity of this enzyme is markedly depressed by ovariectomy (Macartney & Thomas, 1969). These results suggest that under the conditions chosen for the conduct of the present experiments, the steroid-metabolizing capacity of liver was substantially attenuated.

The results of experiments on the binding and metabolism of 1 nM-[^{3}H]oestradiol by hepatocytes isolated from livers of ovariectomized rats and

incubated at 4°C for 1.5 h are shown in Table 1. It is apparent that, under the present experimental conditions, hepatocytes metabolize only minimal amounts of [^{3}H]oestradiol. Parenchymal cells (10^8

Table 1. *Metabolism of [^{3}H]oestradiol-17β at 4°C by parenchymal cells isolated from livers of ovariectomized rats*

Parenchymal cells were incubated *in vitro* with 1 nM [^{3}H]oestradiol at 4°C for 1.5 h. After controlled homogenization (see the text), cell-bound and free hormone were separated and extracted with the organic solvents indicated in the Materials and methods section into water- and ether-soluble fractions. The latter fractions were analysed by t.l.c. as described in the text. For the fraction analysed after t.l.c. with chloroform/acetic acid only that portion of the radioactivity corresponding to authentic oestradiol-standard is given. Results are given as means ± s.e.m. (n).

Fraction	^{3}H Disintegrations per min (% of total radioactivity)	
	Bound	Free
Water-soluble	1 ± 0 (3)	2 ± 1 (3)
Ether-soluble		
Analysed directly	98 ± 2 (3)	96 ± 2 (3)
Analysed after chromatography with chloroform/acetic acid	95 ± 2 (3)	92 ± 2 (3)

Table 2. *Summary of the various homogenization conditions used to determine distribution of enzyme activities and binding of oestradiol-17β in liver cell fractions*

Hepatocytes were suspended at 4°C in 9 vol. of the indicated homogenization media, each supplemented with 0.5 μM-leupeptin. Subsequent preparation of cellular fractions was carried out with the corresponding homogenization medium. However, in protocols 4 and 5, the media used after homogenization contained no additional CaCl$_2$. Mean total number of manual strokes required to disrupt approx. 98% of cells, as monitored by phase-contrast microscopy is given.

Protocol	Homogenization medium	Homogenizer (no. of strokes)
1	5 mM-Tris/HCl (pH 7.4) /1.5 mM-EDTA	Glass–glass (20)
2	5 mM-Tris/HCl (pH 7.4) /1.5 mM-EDTA	Teflon–glass (31)
3	5 mM-Tris/HCl (pH 7.4) /1 mM-EDTA /0.25 M-sucrose	Teflon–glass (36)
4	5 mM-Tris/HCl (pH 7.4) /0.5 mM-CaCl$_2$ /0.25 M-sucrose	Glass–glass (28)
5	5 mM-Tris/HCl (pH 7.4) /0.5 mM-CaCl$_2$ /0.25 M-sucrose	Teflon–glass (40)

per experiment) bind about 6.2% (e.g., 4.14×10^5 d.p.m.) of the tritiated hormone at this concentration. On the basis of an estimated recovery of 1.25×10^8 parenchymal cells per g of rat liver (cf. Munthe-Kaas et al., 1976), calculations from these results indicate that hormone binding by hepatocytes corresponds to 1.6 fmol/mg of liver. The latter value compares closely with the estimate of 1.0 fmol of oestradiol bound per mg of whole liver in independent studies, by using liver from intact female rats (Eisenfeld et al., 1976).

Effects of homogenization methods on yield of enzyme activities and oestradiol-17β binding in major fractions of liver parenchymal cells

The effects of different homogenization conditions on yield of specific oestrogen binding in major subfractions of hepatocytes were investigated initially. Cells isolated from a single pool were disrupted and fractionated in parallel by each of the five different protocols specified in Table 2. After homogenization, the general scheme for isolation of crude nuclear, mitochondria/lysosome, microsome-rich and cytosol fractions (cf. Fig. 1) was used with the medium specified for each protocol (see Table 2).

The results of these experiments are presented in Table 3. The distribution of protein among the major cell fractions prepared from the homogenates of protocols 1–5 shows little diversity. In contrast, distribution of the activities of the three plasma-membrane marker enzymes exhibits marked variation which is most apparent in cytosol fractions, with cytosolic activity for each of these activities in protocol $1 > 2 > 3 > 4 > 5$. Since the occurrence and relative specific activities of 5'-nucleotidase, alkaline phosphatase and (Na$^+$,K$^+$-activated)-ATPase in crude nuclear and microsomal fractions, where the bulk of plasma membrane cosediments (Berman et al., 1969), follows a reverse order among these five protocols, it appears that the membrane marker enzymes are redistributed from particulate to soluble

Table 3. *Effect of various conditions of liver cell disruption on distribution of protein, enzymes and specific binding of [³H]oestradiol-17β among the resultant cell fractions*

Homogenization and preparation of crude nuclear (N), mitochondria/lysosome (M + L), microsomal (P) and cytosol (S) fractions were conducted at 4°C, under conditions as specified in Table 2. Results represent the average of determinations made with two independent preparations that agreed to within 5%. Specific binding of 2 nM-[³H]oestradiol 17β is given; relative specific activity or binding of fractions is with respect to that of the homogenate.

Determination	Homogenization protocol	Homogenate (units)	Subcellular fraction N	M + L	P	S	Recovery (%)	Subcellular fraction N	M + L	P	S
			(% of homogenate)					(relative specific activity)			
Protein (mg/10⁸ cells)	1	149.5	25.3	25.7	17.0	29.1	97.1				
	2	144.5	23.7	26.7	20.4	27.4	98.2				
	3	160.3	24.6	26.8	25.0	20.8	99.2				
	4	155.7	24.1	28.0	18.4	25.1	95.6				
	5	162.7	26.4	27.1	23.3	23.4	100.2				
Alkaline phosphatase (nmol min⁻¹ mg⁻¹ of protein)	1	1.5	28.4	20.2	18.4	25.1	92.1	1.12	0.79	1.08	0.86
	2	1.4	28.6	18.2	24.7	26.2	97.7	1.21	0.68	1.21	0.96
	3	1.6	30.8	20.2	31.8	11.0	93.8	1.15	0.75	1.27	0.53
	4	1.7	35.4	23.2	29.5	7.2	93.3	1.39	0.83	1.61	0.29
	5	1.8	41.7	19.2	33.5	5.3	99.7	1.58	0.71	1.44	0.23
5'-Nucleotidase (nmol min⁻¹ mg⁻¹ of protein)	1	17.2	32.6	20.9	21.9	19.9	95.3	1.29	0.81	1.29	0.69
	2	15.7	32.1	25.4	26.0	16.2	99.7	1.36	0.95	1.27	0.59
	3	16.1	35.1	19.9	33.6	11.3	99.9	1.43	0.74	1.34	0.55
	4	16.8	34.5	17.8	29.1	12.7	94.1	1.43	0.64	1.58	0.51
	5	16.3	43.4	13.6	33.9	4.9	95.8	1.66	0.50	1.45	0.21
(Na⁴,K⁺-activated)-ATPase (nmol min⁻¹ mg⁻¹ of protein)	1	9.9	27.3	15.5	19.4	37.3	99.5	1.08	0.61	1.14	1.28
	2	11.2	31.9	13.6	27.3	23.7	96.5	1.35	0.51	1.34	0.87
	3	12.5	34.3	16.5	29.7	11.8	92.3	1.39	0.62	1.18	0.57
	4	13.0	36.3	18.6	27.6	10.8	93.3	1.51	0.66	1.50	0.43
	5	12.8	40.1	17.8	32.4	4.6	94.9	1.52	0.66	1.39	0.19
Oestradiol-17β binding (d.p.m./mg of protein)	1	3530	6.0	12.0	28.2	49.9	96.1	0.24	0.47	1.66	1.72
	2	3991	11.2	11.1	26.6	46.7	95.6	0.47	0.42	1.30	1.70
	3	4252	20.1	21.7	22.7	34.3	98.8	0.81	0.81	0.90	1.65
	4	3950	26.9	28.1	22.2	21.9	99.1	1.12	1.00	1.21	0.87
	5	4010	31.0	37.3	29.2	3.1	100.6	1.17	1.38	1.25	0.13

fractions in protocol 1 ⪢ 5 (Table 3). Likewise, specific oestrogen binding, that occurs predominantly in cytosol fractions when the latter are prepared by protocols 1–3, is minimal in cytosols obtained by protocols 4 and 5. The bulk of oestradiol binding is associated with particulate fractions in the latter methods (Table 3). In consideration of the more characteristic distribution of 5′-nucleotidase, alkaline phosphatase and (Na⁺,K⁺-activated)-ATPase activities in particulate fractions of the homogenate after use of protocol 5, the latter method was used in the succeeding experiments.

Cellular distribution of protein, enzymes and binding sites for wheat germ agglutinin and oestradiol-17β in major fractions of hepatocytes disrupted by mild procedures

Table 4 presents results of more extensive analyses of the cellular distribution and overall recoveries of protein, oestrogen- and wheat germ agglutinin-binding sites, and several marker enzymes in the four main subcellular fractions prepared by the present methods. 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 principally in microsome-rich and crude nuclear fractions. The latter fraction also accounts for $95 \pm 3\%$ ($n = 4$) of the DNA in the initial homogenate. Moreover,

binding sites for oestradiol as well as wheat germ agglutinin occur predominantly in particulate fractions. Additional results from two experiments show that specific binding of 2nM-[³H]oestradiol by crude nuclear, mitochondria/lysosome, microsomal and cytosol fractions is reduced to 29–35% of paired controls by competition with a 200-fold molar excess of unlabelled diethylstilbestrol. In contrast, binding was uninfluenced by an equivalent level of either testosterone or cortisol (not shown).

Characterization of partially purified membranes and associated cellular fractions

The crude nuclear sediment is known to be contaminated with plasmalemmal as well as microsomal membrane components (cf. Neville, 1976). Accordingly, further fractionation of crude nuclei was undertaken by isopycnic centrifugation through a discontinuous sucrose-density gradient. The resultant bands at $\rho < 1.13$ (Fraction 1), $\rho = 1.13–1.16$ (Fraction 2), $\rho = 1.16–1.18$ (Fraction 3) and $\rho = 1.18–1.20$ (Fraction 4) and the semipurified nuclear sediment (Fraction 5) were collected, washed by centrifugation as outlined, and analysed for ligand binding and enzyme activities (Table 5). Partially purified nuclei exhibit essentially no specific binding sites for oestradiol or wheat germ agglutinin and little of the predominantly extranuclear enzyme activities shown in Table 5. Fraction 4 represents a mixture of particulate material with no enrichment of plasma-membrane marker enzymes or binding sites for oestrogen or wheat germ agglutinin. The

Table 4. *Distribution of protein, enzymes and specific* [³H]*oestradiol-17β binding sites in major subfractions of hepatocytes*

Levels of protein (mg/10⁸ cells), enzyme activity (nmol min⁻¹ mg⁻¹ of protein) and hormone- and wheat germ agglutinin-binding (d.p.m./mg of protein) in the homogenate and corresponding subcellular fractions are given as means ± S.E.M., as appropriate, in two to eight independent experiments. In two experiments to determine the extent of [³H]oestradiol metabolism by the cell homogenate, and by crude nuclear (N), mitochondria/lysosome (M + L), microsomal (P) and cytosol (S) fractions under the present conditions, more than 95% of the radioactivity extractable from each fraction was identified by t.l.c. as unmodified oestradiol-17β.

Determination (n)	Initial homogenate (units)	Subcellular fraction				Recovery (%)
		N	M + L	P	S	
			(% of homogenate)			
Protein (8)	138 ± 10	25.5 ± 1.4	30.0 ± 1.5	26.0 ± 0.8	16.3 ± 1.0	97.8 ± 0.8
Succinate dehydrogenase (4)	32.2 ± 4.0	6.7 ± 1.2	88.6 ± 4.4	2.0 ± 1.2	0.2 ± 0.2	97.5 ± 2.8
Glucose 6-phosphatase (4)	21.3 ± 2.9	6.2 ± 1.4	18.0 ± 2.7	69.0 ± 4.0	6.3 ± 1.4	99.5 ± 3.3
Acid phosphatase (5)	4.6 ± 0.9	14.2 ± 1.2	74.1 ± 3.6	10.6 ± 3.0	2.1 ± 0.5	100.9 ± 0.8
Alkaline phosphatase (6)	2.3 ± 0.7	38.8 ± 2.0	17.3 ± 3.5	46.8 ± 2.3	0.3 ± 0.2	103.2 ± 1.0
5′-Nucleotidase (5)	18.6 ± 1.7	28.8 ± 2.2	15.6 ± 2.5	50.2 ± 2.1	3.1 ± 0.7	97.6 ± 1.0
Wheat germ agglutinin binding (2)	22867	36.4	18.1	39.9	1.1	95.5
Oestradiol 17β binding (8)	3619 ± 780	29.7 ± 2.7	36.7 ± 5.0	30.5 ± 4.3	3.1 ± 0.6	99.9 ± 1.7

Table 5. *Distribution of protein, enzymes and specific [³H]oestradiol-17β-binding sites in hepatocyte plasma membranes and their subfractions*

Fractions 1–4 and the semipurified nuclear sediment (Fraction 5) were obtained by discontinuous sucrose-density-gradient centrifugation of the washed crude nuclear fraction (see Fig. 1 and Table 4). Plasma-membrane subfractions, P2, 2B and 3B, were prepared by further centrifugation of the microsomal fraction, Fraction 2 and Fraction 3 respectively, as described in the text. Results are from two to four independent experiments and are given as means ± S.E.M., as appropriate. Relative specific activity or binding of fractions is with respect to that of the homogenate (see Table 4). N.D., Not determined.

Determination	Yield, gradient I (% of homogenate) Fraction					Yield, gradient II (% of homogenate) Fraction			Relative specific activity Fraction		
	1	2	3	4	5	2B	3B	P2	2B	3B	P2
Protein (mg/10⁹ cells)	1.5 ±0.5	2.9 ±0.9	5.1 ±1.1	7.3 ±0.7	10.5 ±1.5	0.5 ±0.1	4.0 ±1.6	0.2	—	—	—
Succinate dehydrogenase (nmol min⁻¹ mg⁻¹ of protein)	0.2 ±0.2	0.5 ±0.3	1.3 ±0.8	4.9 ±0.7	0.2 ±0.1	0.0 ±0.0	0.1 ±0.0	0.0	0.02 ±0.00	0.02 ±0.01	0.01
Glucose 6-phosphatase (nmol min⁻¹ mg⁻¹ of protein)	0.6 ±0.2	1.4 ±0.5	1.6 ±0.7	1.3 ±0.3	1.6 ±0.5	0.1 ±0.0	1.2 ±0.6	0.1	0.49 ±0.08	0.63 ±0.27	0.38
Acid phosphatase (nmol min⁻¹ mg⁻¹ of protein)	1.9 ±0.8	3.7 ±0.2	3.8 ±0.5	3.4 ±0.7	1.6 ±1.0	1.5 ±0.2	3.0 ±0.6	N.D.	5.04 ±1.90	2.45 ±1.64	N.D.
Alkaline phosphatase (nmol min⁻¹ mg⁻¹ of protein)	5.3 ±2.8	16.1 ±1.1	9.3 ±1.1	2.8 ±1.1	1.6 ±0.3	9.8 ±2.6	5.2 ±1.9	5.1	28.40 ±5.73	4.50 ±1.22	25.14
5′-Nucleotidase (nmol min⁻¹ mg⁻¹ of protein)	4.2 ±1.1	13.6 ±1.1	7.9 ±0.8	2.1 ±0.4	1.2 ±0.5	10.0 ±1.0	4.2 ±1.4	5.5	31.90 ±5.15	2.75 ±1.19	27.74
Wheat germ agglutinin binding (d.p.m. ³H/mg of protein)	0.3	25.1	8.8	1.2	0.8	22.8	8.7	13.9	45.95	1.73	55.60
Oestradiol-17β binding (d.p.m. ³H/mg of protein)	0.3 ±0.1	27.7 ±1.0	3.4 ±0.4	0.0 ±0.0	0.0 ±0.0	25.1 ±0.7	3.4 ±0.2	15.0	50.96 ±4.66	1.16 ±0.20	62.70

highest specific activiites of 5′-nucleotidase and alkaline phosphatase, valid plasma-membrane marker enzymes in liver (cf. Solyom & Trams, 1972; Evans, 1978), occur predominantly in Fractions 2 and 3, and to a lesser extent in Fraction 1 (Table 5). No enrichment of enzymes characteristic of microsomal or mitochondrial/lysosome sediments is found in these fractions. Thus Fractions 1–3 appear to be derived from plasma membranes of hepatocytes. Fraction 2 exhibits substantial concentration of specific binding sites for oestradiol as well as for wheat germ agglutinin, with Fraction 2 ≫ Fraction 3 ≫ Fraction 1.

Characterization of purified plasma membrane subfractions

As further revealed in Table 5, additional purification of Fractions 2 and 3 and microsomal fractions was achieved by their flotation through sucrose density gradient II (see Fig. 1). Material accumulated at $\rho = 1.13$–1.16 (Fractions 2B and P2) and at $\rho = 1.16$–1.18 (Fraction 3B) in the respective tubes was collected, washed at the centrifuge and analysed for specific oestrogen- and wheat germ agglutinin-binding and enzyme activities. The distributions of the plasma-membrane marker enzymes, 5′-nucleotidase and alkaline phos-

phatase, correlate very closely with each other in Fractions 2B, P2 and 3B respectively. Enrichment of these activities is greatest in Fraction 2B > Fraction P2 ≫ Fraction 3B. Likewise, binding sites for the plasma-membrane marker, wheat germ agglutinin (Chang et al., 1975), occur in Fractions 2B and P2, as well as in Fraction 3B, but are markedly enhanced, to 56- and 46-times that of the homogenate, only in Fractions P2 and 2B respectively (Table 5).

Contamination of plasma-membrane fractions by other intracellular marker enzymes appears to be very low. Thus comparison of relative specific activities of glucose 6-phosphatase between Fraction 2B (Table 5) and microsomal fractions (Table 4) reveals a ratio of 0.107, indicating a microsomal contamination of 10.7%. Microsomal contamination of 8.3 and 13.8% is found for Fractions P2 and 3B respectively. Similar calculations for succinate dehydrogenase activity yield a ratio of 0.008, or 0.8% contamination of Fraction 2B plasma membranes with mitochondrial elements. The latter value compares well with a mitochondrial contamination of 0.8 and 0.4% in membrane fractions 3B and P2 respectively.

The enzymic analyses confirm a predominantly plasma-membrane origin of Fractions P2, 2B and

3B. The results of analyses for cholesterol, phospholipid, sialic acid, DNA and RNA are presented in Table 6. Subfractions 2B and P2 showed relatively higher cholesterol to protein or phospholipid to protein ratios than fraction 3B. The molar ratios of total cholesterol to phospholipid were within the range of values reported by others (Touster et al., 1970; Thinès-Sempoux, 1973; Kremmer et al., 1976; Evans, 1978). Fraction 2B contained the highest such ratio and Fraction P2 the lowest. The values for sialic acid, which may be

bound to either protein or lipid, were higher in Fractions 2B and P2 than in Fraction 3B. The DNA and RNA contents of the several membrane subfractions were low, with Fraction 3B showing the greatest contamination by nucleotides.

Specific binding of [³H]oestradiol-17β to purified plasma-membrane subfractions: evidence of intrinsic nature

Specific binding sites for oestradiol occur in each of the purified plasma-membrane subfractions prepared from hepatocytes (Table 5). Of the three fractions, Fractions 2B and P2, composed of membrane components with densities of 1.13–1.16, are more selectively enriched in this property than is Fraction 3B, to 51–63 times that of the homogenate. Since specific binding of [³H]oestradiol in Fraction 2B accounted for approx. 25% of total cellular binding, the nature of this activity was further analysed.

Because occlusion of soluble oestrogen-binding proteins in plasma-membrane vesicles and/or non-specific adsorption to membrane surfaces could contribute to the hormone binding detected in Fraction 2B, a series of control experiments was instituted to investigate these potential artifacts (cf. Emmelot & Bos, 1966; Evans, 1978). The results in Table 7 show that oestradiol binding to plasma membranes was not appreciably reduced by extraction of the latter with buffered physiological saline, hypotonic buffer (i.e., 5 mM-Tris/HCl/1.5 mM-EDTA) or high-salt buffer (5 mM-Tris/HCl/1.5 mM-

Table 6. *Chemical analyses of plasma-membrane fractions of liver parenchymal cells*

The given plasma-membrane fractions were prepared and analysed as described in the Materials and methods section. The results represent the averages of analyses of two separate membrane preparations that agreed to within 5%. N.D., Not detectable.

	Plasma-membrane fraction		
Determination (units)	P2	2B	3B
Total cholesterol (μmol/mg of protein)	0.75	0.84	0.73
Phospholipid phosphorus (μmol of P/mg of protein)	0.97	0.98	0.94
Cholesterol/phospholipid	0.77	0.86	0.78
Sialic acid (nmol/mg of protein)	50.9	55.7	40.3
DNA (μg/mg of protein)	N.D.	N.D.	1.1
RNA (μg/mg of protein)	22.3	21.9	37.6

Table 7. *Effect of diverse extraction conditions on retention of bound [³H]oestradiol-17β in hepatocyte plasma-membrane subfraction 2B*

Samples of membrane (approx. 250 μg of protein) were incubated with 2 nM-[³H]oestradiol-17β for 1.5 h at 4°C, sedimented, and washed by centrifugation with 5 mM-Tris/HCl (pH 7.4) and 0.5 μM-leupeptin 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 [³H]oestradiol is given as a percentage of control samples extracted for 1 h with 5 mM-Tris/HCl (pH 7.4) and 0.5 μM-leupeptin in 0.25 M-sucrose; control values for specific binding were 158 ± 17 d.p.m./μg of protein. All extraction media included 0.5 μM-leupeptin Results are given as means ± s.e.m. (n), as appropriate.

Extraction conditions	[³H]Oestradiol-17β specifically bound (% of control)	Membrane protein (% of control)	[³H]Oestradiol-17β specifically bound/μg of membrane protein (% of control)
5 mM-Tris/HCl/0.15 M-NaCl	95.7 ± 0.6 (3)	96.2 ± 1.1 (3)	99.5 ± 0.6 (3)
5 mM-Tris/HCl/1.5 mM-EDTA			
(a) Without sonication	95.5 (2)	95.4 (2)	100.1 (2)
(b) With sonication (20 s)	85.2 (2)	86.6 (2)	98.4 (2)
5 mM-Tris/HCl/1.5 mM-EDTA/0.4 M-KCl	94.3 (2)	94.8 (2)	99.5 (2)
5 mM-Tris/HCl/0.25 M-sucrose/:			
(a) 0.01% (v/v) Triton X-100	89.4 (2)	89.6 (2)	99.8 (2)
(b) 0.05% (v/v) Triton X-100	75.0 (2)	85.1 (2)	88.1 (2)
(c) 0.1% (v/v) Triton X-100	51.0 ± 5.4 (3)*	68.4 ± 1.0 (3)*	74.7 ± 8.6 (3)*

* Value significantly different from that of control at $P < 0.05$.

EDTA/0.4 M-KCl); after such extraction, the level of specific oestradiol binding relative to membrane protein was essentially unchanged or even slightly enhanced due to selective removal of protein (i.e., 4–6%; Table 7). However, extraction of fraction 2B with hypotonic buffer followed by sonication (two bursts of 10s duration at 4°C with a 60s cooling interval) elicited a more significant reduction in hormone binding as well as protein content (Table 7). The addition of 0.1% (v/v) Triton X-100, to the membrane suspension under essentially isotonic conditions was most effective in removing a substantial portion of membrane-bound radioactivity and protein (Table 7).

To test further for inadvertent adsorption of [^{3}H]oestradiol to plasma-membrane subfraction 2B, mixing experiments were undertaken. Cytosol prepared by protocol 1 (see Table 2) was labelled by incubation with 2 nM-[^{3}H]oestradiol-17β. Unbound hormone was removed by the dextran-coated charcoal procedure and samples (approx. 200 μg of protein) of the cytosol so labelled (6250 d.p.m./mg of protein) were mixed with samples of plasma-membrane fraction 2B (approx. 500 μg of protein), previously unexposed to oestrogen. After 1.5 h incubation at 4°C with Vortex stirring at 10-min intervals, Fraction 2B was again sedimented, resuspended in 5 mM-Tris/HCl (pH 7.4)/0.25 M-sucrose, and resedimented as described. Analysis of ^{3}H in the pellets and supernatants revealed that only 10.8 $\pm$ 1.1% ($n = 3$) of the initial cytosolic radioactivity became associated with the final membrane sediment under these conditions (not shown). In addition, extraction of the latter with 5 mM-Tris/HCl/1.5 mM-EDTA/0.4 M-KCl reduced the apparent association of cytosolic oestradiol–receptor with plasma membranes to only 2.2 $\pm$ 1.0%.

Specific binding of 2 nM-oestradiol-17β to plasma-membrane fraction 2B at 4°C is blocked completely by prior exposure of membranes (100 μg of protein) for 1 h to bovine pancreatic trypsin (250 μg $\simeq$ 2250 Bz-Arg-OEt units) at 22°C. Heat denaturation at 60°C also destroys the oestrogen-binding activity of this membrane subfraction ($n = 3$; not shown).

Saturation analysis of [^{3}H]oestradiol-17β binding to a plasma-membrane subfraction

Association of [^{3}H]oestradiol with plasma-membrane fraction 2B was analysed in equilibrium-binding experiments (Fig. 2). Samples of Fraction 2B were exposed to a series of [^{3}H]oestradiol concentrations ranging from 0.15 to 4.5 nM. As shown in Fig. 2, specific binding of the hormone by plasma membranes is saturable.

The oestradiol-binding properties of plasma membranes were analysed further to obtain both the concentration of specific binding sites for oestra-
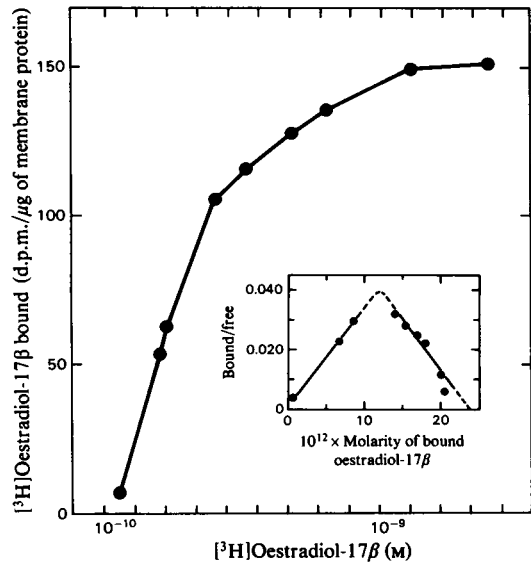

Fig. 2. *Binding of [^{3}H]oestradiol-17β by a plasma-membrane subfraction prepared from isolated hepatocytes of ovariectomized rats*

Plasma membranes (Fraction 2B) were incubated in Tris-buffered solution with 0.25 M-sucrose and 0.5 μM-leupeptin at approx. 0.12 mg of membrane protein per 2 ml, for 1.5 h at 4°C, with the concentrations of [^{3}H]oestradiol-17β shown on the abscissa. Specific binding of hormone by plasma membrane was determined by the method of Williams & Gorski (1973). Each point represents a mean value of duplicate determinations (that agreed to within 5%) from one representative experiment; a total of three independent equilibrium-binding experiments were done. Radioactivity extracted from membrane fractions exposed to 0.3 nM- or 1.9 nM-oestradiol-17β was identified as essentially unmetabolized oestradiol by t.l.c. with chloroform/acetic acid as described in the Materials and methods section. A Scatchard plot of specific [^{3}H]oestradiol binding by Fraction 2B membranes is shown as an inset.

diol-17β and the equilibrium constant. Fig. 2 shows a graphical analysis of the data by the method of Scatchard (1949). The biphasic nature of the plot at very low molarities of oestrogen is apparently due to co-operative binding effects and will be considered below. Mathematical resolution of the binding data at higher molarities of oestradiol (i.e., 0.46–1.9 nM) by the method of least-squares indicates that the association constant for the binding process is 3.6 nM. In three independent experiments the mean association constant of plasma-membrane Fraction 2B was estimated to be 3.5 $\pm$ 0.2 (S.E.M.) nM. With the assumption that the association follows the law of mass action, total binding sites for oestradiol at

saturation correspond to 526 ± 10 fmol/mg of membrane protein ($n = 3$).

In two experiments, samples of membrane fraction 3B were exposed to a series of [^{3}H]oestradiol concentrations ranging from 0.16 to 1.7 nM. Specific binding of the hormone by Fraction 3B membranes was also saturable. Likewise, a Scatchard plot of the binding showed a positive slope at low molarities of hormone (0.16–0.30 nM) and a negative slope at concentrations ranging from 0.34 to 1.7 nM. The mean association constant for the binding process is estimated to be 1.7 nM, and total binding sites for oestradiol-17β at saturation correspond to 9.4 fmol/mg of membrane protein. Thus the hormone-binding capacity of Fraction 2B membranes, although saturable, exceeds that of Fraction 3B by 56-fold.

All Scatchard plots obtained for oestradiol/membrane fraction 2B interactions showed a positive slope at low ligand concentrations similar to that presented in Fig. 2. This phenomenon is apparently not attributable to failure to attain equilibrium at the lower concentrations of oestradiol, since the extent of specific binding did not vary significantly with time of incubation from 1 to 10 h. Another experimental artifact which could produce such a plot is degradation of unbound receptor during incubation at low ligand concentrations (Rodbard, 1973; Chamness & McGuire, 1975). To test for this possibility, we incubated membranes with a saturating concentration of [^{3}H]oestradiol (i.e., 2 nM) after

initial incubation at a lower concentration of ligand (i.e., 0.15 nM). Under these conditions the extent of specific oestrogen binding by membranes was essentially equal to that found by incubating with the saturating level of hormone throughout ($n = 2$; not shown). Such an hyperbolic plot is typical of positive co-operativity in the binding of a ligand to its high-affinity receptor (cf. Changeux & Rubin, 1968). Evidence for the co-operative nature of the oestradiol-binding component of plasma-membrane fraction 2B is obtained from a Hill plot (Brown & Hill, 1922; Ellis & Ringold, 1971) of the binding data used in Fig. 2. A Hill coefficient of 1.8 was obtained from the data presented in Fig. 3. In such graphical analysis of the Fraction 2B binding activities of three independent preparations, the mean Hill coefficient was 1.6 ± 0.1 ($r = 0.93 \pm 0.02$).

Ligand specificity of [^{3}H]oestradiol-17β binding to a plasma-membrane subfraction

The ligand specificity of [^{3}H]oestradiol binding to plasma-membrane fraction 2B was analysed by effectiveness of competition of a 200-fold molar excess of unlabelled steroids or diethylstilbestrol (Table 8). The extent of [^{3}H]oestradiol binding by Fraction 2B was essentially uninfluenced by such excess of oestradiol-17α, progesterone, testosterone or cortisol. In contrast, physiologically active
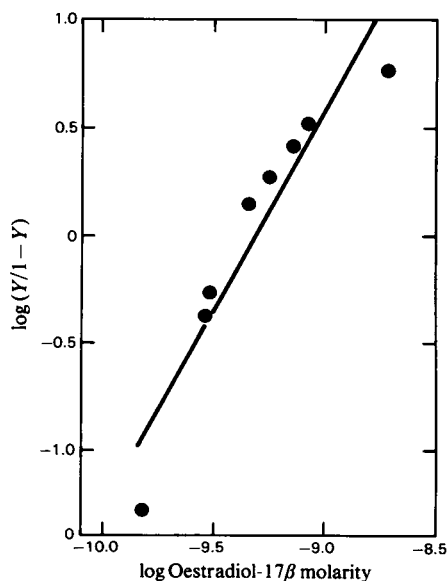

Fig. 3. *Hill plot (Brown & Hill, 1922) of* [^{3}H]*oestradiol-17β binding by plasma-membrane fraction 2B, based on the data of Fig. 2.*

Y represents the fraction of binding sites that are occupied by oestradiol (cf. Ellis & Ringold, 1971).

Table 8. *Ligand specificity of* [^{3}H]*oestradiol-17β binding to purified plasma-membrane subfraction 2B isolated from hepatocytes*

Specific binding of 2 nM-[^{3}H]oestradiol-17β was determined after incubation for 1.5 h at 4°C. Such binding was estimated by the method of Williams & Gorski (1973). Specific binding of [^{3}H]oestradiol in samples of the whole homogenate averaged 2.9 ± 0.5 d.p.m./μg of protein in three independent experiments. Results are given as means $\pm$ S.E.M. (n), as appropriate.

Competing compound	Specific [^{3}H]oestradiol-17β binding to purified plasma membranes	
	(d.p.m./μg of protein)	(% of paired control)
Vehicle control (0.02% ethanol)	128.7 ± 23.2 (3)	100 ± 0 (3)
Oestradiol-17β	0.0 ± 0.0 (3)*	0 ± 0(3)*
Oestradiol-17α	123.6 ± 22.1)3)	96 ± 1 (3)
Oestriol	74.8 (2)	65 (2)
Diethylstilbestrol	31.6 ± 7.3 (3)*	24 ± 3 (3)*
Progesterone	110.9 (2)	96 (2)
Testosterone	111.6 (2)	97 (2)
Cortisol	107.9 (2)	94 (2)

Competing compounds were 0.4 μM unless indicated otherwise.

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

oestrogens elicited either moderate (e.g., oestriol) or marked (e.g., oestradiol-17β,diethylstilbestrol) reduction of specific [^{3}H]oestradiol-binding to the purified plasma-membrane preparation (Table 8).

Resolution by sucrose-density-gradient centrifugation of Triton-solubilized membrane binding-sites for oestradiol-17β

A substantial amount of [^{3}H]oestradiol that was bound to plasma-membrane subfraction 2B is rendered soluble by Triton X-100 (see Table 7). Sedimentation in linear sucrose-density gradients was performed to determine whether the radioactive moiety so extracted was bound to macromolecular species.

In the present experiment, samples of Fraction 2B not exposed to oestrogen (approx. 7 mg of protein) were extracted in 1.4 ml of 10 mM-Tris/HCl (pH 7.4)/1.5 mM-EDTA/10 mM-KCl/0.1% (v/v) Tri-

ton X-100. After 90 min at 4°C with Vortex stirring at 10-min intervals, samples were centrifuged at 105000 g for 1 h at 4°C. The supernatant was collected and 0.2 ml of solubilized membrane material (approx. 0.3 mg of protein) was layered on top of each sucrose density gradient containing 0.01% (v/v) Triton X-100. Analysis of the distribution of phosphorus along a representative gradient (cf. Smigel & Fleischer, 1977) revealed that the bulk of the extracted membrane phospholipids did not enter the gradient.

The sedimentation of solubilized plasma-membrane components in 0.01 M-KCl/sucrose gradients is shown in Fig. 4. Sedimentation in 0.4 M-KCl/sucrose gradients is presented in Fig. 5. Sedimentation coefficients were estimated by the method of McEwen (1967) as well as by comparison with standards centrifuged in parallel gradients (cf. Martin & Ames, 1961). Values determined by either
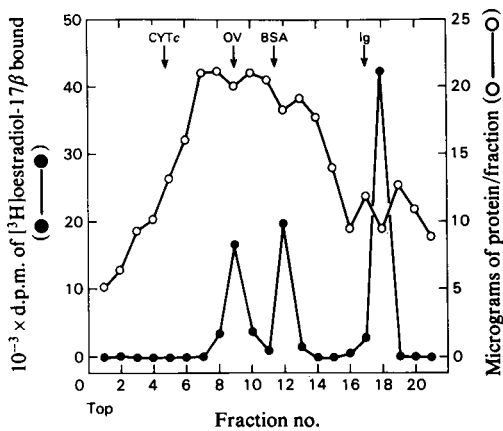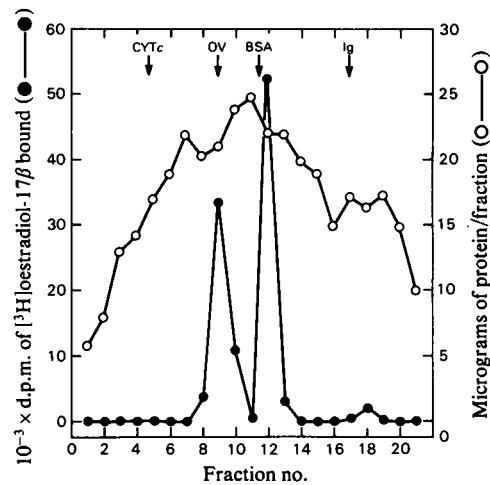

Fig. 4. *Sedimentation of Triton-solubilized [^{3}H]oestradiol-binding macromolecules from plasma membranes in 0.01 M-KCl/sucrose density gradients*

Linear 5–20% (w/v) sucrose gradients containing 0.01 M-KCl, 1.5 mM-EDTA, 10 mM-Tris/HCl (pH 7.4) and 0.01% (v/v) Triton X-100 were prepared as described in the Materials and methods section. Triton extracts of Fraction 2B membranes or mixed protein standards, including cytochrome *c* (CYT*c*), ovalbumin (OV), albumin (BSA) or immunoglobulins (Ig), were layered on these. Tubes were centrifuged and fractionated as described in the text. Portions were taken for determination of protein and specific [^{3}H]oestradiol binding (see the Materials and methods section). Peaks for protein standards are indicated by arrows. Peak positions for [^{3}H]oestradiol-binding macromolecules are at 3.6 S, 4.8 S and 7.5 S. In three independent experiments, binding components sedimented at predominantly 3.6 ± 0.2 S (756 ± 111 d.p.m. [^{3}H]/μg of protein), 4.7 ± 0.3 S (955 ± 66 d.p.m./μg) and 7.4 ± 0.1 S (5344 ± 474 d.p.m./μg).

Fig. 5. *Sedimentation of Triton-solubilized [^{3}H]oestradiol-binding macromolecules from plasma membranes in 0.4 M-KCl/sucrose density gradients*

Linear 5–20% (w/v) sucrose gradients containing 0.4 M-KCl, 1.5 mM-EDTA, 10 mM-Tris/HCl (pH 7.4) and 0.01% (v/v) Triton X-100 were prepared as described in the Materials and methods section. Triton extracts of Fraction 2B membranes or mixed protein standards (see Fig. 4) were layered on these. Tubes were centrifuged and fractionated as described in the text. Portions were taken for determination of protein and specific [^{3}H]oestradiol binding. Peaks for protein standards are indicated by arrows. In the representative gradient shown, peak positions for [^{3}H]oestradiol-binding macromolecules are at 3.6 S and 4.8 S. In three independent experiments, binding components sedimented at 3.6 ± 0.0 S (1722 ± 197 d.p.m. [^{3}H]oestradiol/μg of protein) and 4.9 ± 0.1 S (2381 ± 164 d.p.m./μg).

method were essentially identical. The extent of specific [³H]oestradiol binding to macromolecular species was determined in each fraction by hydroxyapatite adsorption. Analyses of sedimentation and hormone-binding data from three independent experiments yielded the following results. In low-salt gradients (see Fig. 4) $55.4 \pm 5.0\%$ of total oestradiol-binding activity banded at $7.4 \pm 0.1\,S$, with minor bands at $3.6 \pm 0.2\,S$ (i.e., $18.3 \pm 0.8\%$) and $4.7 \pm 0.3\,S$ (i.e., $21.2 \pm 1.1\%$). Binding activity which sedimented at about $7.4\,S$ was enriched to 1561 ± 39 times that of the homogenate. In high-salt gradients (see Fig. 5) $52.7 \pm 3.1\%$ of total hormone binding occurred at $4.9 \pm 0.1\,S$ and $36.6 \pm 3.2\%$ at $3.6 \pm 0.0\,S$, with negligible binding activity in the $7.4\,S$ region. Oestradiol-binding components sedimenting at $4.9\,S$ and $3.6\,S$ showed relative specific activities of 702 ± 54 and 504 ± 42 respectively. Levels of oestrogen binding per mg of protein (cf. Figs. 4, 5) are based on determinations of protein in each gradient fraction prior to adsorption of hormone-binding macromolecules to hydroxyapatite. On the assumption that not all proteins in the fraction adsorb to hydroxyapatite, the final receptor enrichment probably exceeds the estimates given above. Thus preliminary experiments showed that $45 \pm 3\%$ $(n = 3)$ of protein in the $7.4\,S$ fraction which adsorbs to hydroxyapatite can be eluted

upon addition of 0.5 m-phosphate buffer (cf. Erdos *et al.*, 1971; Williams & Gorski, 1974). Accordingly, the final purification of receptor could be as high as 3469 times its concentration in the homogenate.

The ligand specificity of [³H]oestradiol binding in the gradient fractions sedimenting at approx. $7.4\,S$ in low-salt conditions and at about $3.6\,S$ and $4.9\,S$ in high-salt conditions was investigated in two independent experiments. A 200-fold molar excess of unlabelled diethylstilbestrol suppressed specific binding of $2\,\text{nM-[}^3\text{H]oestradiol-}17\beta$ by 3.6, 4.9 and $7.4\,S$ fractions to 32, 21 and 29% respectively, of that in paired controls. In contrast, the extent of specific hormone binding by these fractions was essentially uninfluenced by a corresponding molar excess of oestradiol-17α, testosterone or cortisol.

Cellular distribution of [³H[oestradiol-17β after exposure of liver cells to hormone at 4 or 22°C

Nuclear transfer of oestrogen–receptor complexes is known to be promoted by treatment of target cells with hormone at elevated temperatures (Jensen *et al.*, 1974; Eisenfeld *et al.*, 1976). Accordingly, the subcellular distribution of hormone binding sites was determined after treatment of approx. 10^8 cells with oestradiol-17β for 10 min at 22°C or for 1.5 h at 4°C. A low concentration of

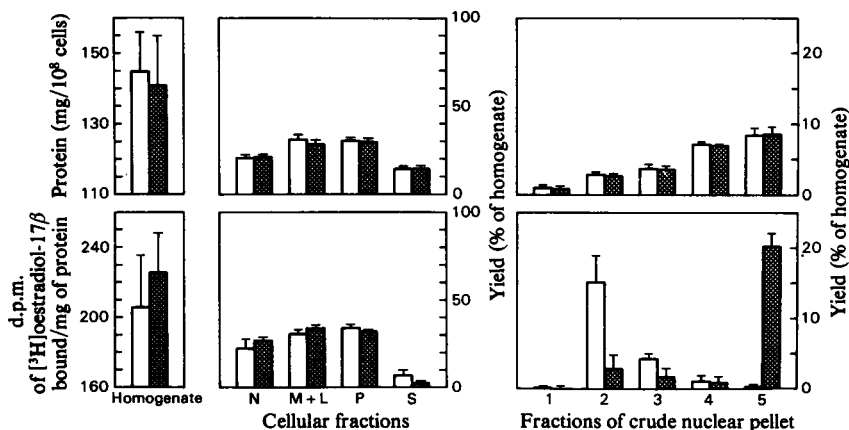Fig. 6. *Cellular distribution of [³H]oestradiol-17β after in vitro exposure of hepatocytes to hormone at 4 or 22°C*
Approx. 10^8 cells were incubated with 0.1 nM-[³H]oestradiol for 10 min at 22°C (shaded bars) or for 1.5 h at 4°C (open bars). Cells were then sedimented and washed by centrifugation, homogenized in the presence of a 200-fold molar excess of unlabelled oestradiol-17β, and fractionated into washed crude nuclear (N), mitochondria/lysosyme (M + L), microsome-rich particulate (P) and cytosol (S) components. Partially purified nuclei (Fraction 5), plasma membrane (Fractions 1, 2 and 3) and particulate (Fraction 4) fractions were then prepared by sucrose-density-gradient centrifugation of crude nuclei (cf. Fig. 1). Levels of protein and of oestradiol binding in the several fractions are presented as the mean percentage $\pm$ s.e.m. of that in the initial homogenates in three independent experiments. In each of these experiments, total radioactivity bound by homogenate preparations incubated for 1.5 h at 4°C or for 10 min at 22°C was extracted with organic solvents and analysed by t.l.c. (see the text). The results showed that more than 96 and 92% $(P > 0.40)$ of the radioactivity corresponded to authentic oestradiol-17β standard in experiments conducted at 4 and 22°C respectively. ·

oestrogen, 0.1 nM, was used in these experiments to minimize non-specific [^{3}H]oestradiol binding. After incubation with hormone, cells were washed twice by centrifugation at $50g$ for 2 min at 4°C. The sedimented cells were then suspended in 9 vol. of ice-cold homogenization medium supplemented with a 200-fold molar excess of unlabelled oestradiol-17β (i.e., final concentration 20 nM). The additional oestradiol served to reduce inadvertent intracellular binding of hormone during cell disruption (cf. Williams & Gorski, 1971; Jackson & Chalkley, 1974). Cells in both groups were then subjected to controlled homogenization and fractionation (protocol 5 of Table 2). The results of these experiments are shown in Fig. 6. Levels of protein per 10^8 cells and yields of protein in the indicated cellular fractions with respect to the homogenate were not significantly different for cells incubated with hormone at 4 or 22°C (all at $P > 0.80$). The extent of [^{3}H]oestradiol binding per mg of homogenate protein in experiments at 4°C was likewise not significantly different from that found for cells incubated at 22°C ($P > 0.40$). In crude nuclear and mitochondria/lysosome fractions, the concentration of bound [^{3}H]oestradiol at 22°C tended to increase over that at 4°C, but without statistical significance ($P > 0.20$), whereas that in supernatant fractions declined ($P < 0.001$) when compared with corresponding fractions from incubations at 4°C (Fig. 6).

Profound differences were observed as a function of temperature in the distribution of bound [^{3}H]-oestradiol among Fractions 2, 3 and 5 purified by sucrose step-gradient centrifugation of the washed crude nuclear sediment (cf. Fig. 1). Cells incubated with [^{3}H]oestradiol for only 10 min at 22°C exhibited substantial hormone retention in partially purified nuclei (i.e., Fraction 5; Fig. 6) whereas little binding occurred in nuclei from cells exposed to oestradiol for as long as 1.5 h at 4°C ($P > 0.001$). In contrast, [^{3}H]oestradiol bound to plasma-membrane fractions 2 and 3 was markedly higher in cells incubated at 4°C than in those maintained at 22°C ($P < 0.001$).

Discussion

The present work provides new evidence that under basal conditions a significant proportion (i.e., approx. 44%) of macromolecules with specificity for binding oestrogen is concentrated in plasma-membrane subfractions purified from hepatocytes of the ovariectomized rat. The sensitivity of the macromolecular oestradiol-binding component(s) in plasma membranes to trypsin and to heat denaturation, as well as capacity to discriminate between stereoisomers of oestradiol, suggest that the nature of the binding activity is, at least in part, protein. The apparent binding constant, i.e. 3.5 nM, for the

oestradiol–plasma-membrane association is high, as expected for a steroid–macromolecular interaction of high affinity (cf. King & Mainwaring, 1974).

The occurrence in target cells of oestradiol-binding activity in several particulate fractions, including mitochondria/lysosome (King et al., 1965; Noteboom & Gorski, 1965; Pietras & Szego, 1979b), lysosome (Hirsch & Szego, 1974), microsome (King et al., 1965; Noteboom & Gorski, 1965; Blyth et al., 1971; Little et al., 1972; Jackson & Chalkley, 1974; Pietras & Szego, 1979b) and plasma membrane (Pietras & Szego, 1977, 1979a,b; Hernández-Pérez et al., 1979; Nenci et al., 1980), has been reported previously (cf. also Sen et al., 1975). Nevertheless, the present findings are in apparent contrast to widely reported data that demonstrate occurrence of oestrogen binding predominantly in cytosol fractions of responsive tissues (cf. Gorski & Gannon, 1976), including liver (Eisenfeld et al., 1976). Moreover, the results of two further investigations have also suggested that binding of oestradiol by plasma membranes from liver (Blyth et al., 1973) or from uterus (Müller et al., 1979) is minimal. However, such discrepancy appears to be attributable, in part, to our application of homogenization and isolation procedures that differ significantly from those adopted early on and at present in general use (cf. Pietras & Szego, 1980; Szego & Pietras, 1980).

Plasma-membrane fractions prepared by the present methods are well preserved by both biochemical and biophysical criteria. Thus surface membrane and other major cell fractions exhibit satisfactory yield and high enrichment of appropriate marker enzymes. Levels of DNA, RNA and sialic acid in plasma membranes are in the range of values previously reported (Emmelot et al., 1964; Berman et al., 1969; Touster et al., 1970). The molar ratios of cholesterol to phospholipid in the various surface membrane fractions are close to the highest values found by others (Emmelot et al., 1964; Coleman, 1968; Touster et al., 1970; Thinès-Sempoux, 1973) and provide further basis for considering these preparations to be of high purity.

The marked enrichment of specific wheat germ agglutinin-binding sites in plasma membranes of hepatocytes has been found previously (Chang et al., 1975), but concentration of oestrogen receptors in hepatic plasmalemmal fractions has not been reported before, except in preliminary form (Pietras et al., 1978). Potential artifacts which might lead to this latter observation were investigated. However, the resultant data indicated that no more than a small fraction of the specific oestrogen binding by plasma membranes may be attributable to cytosol protein entrapment or adsorption artifacts during cell fractionation by the present methods.

Comparison of results obtained with protocols 1–5

shows that the extent of plasmalemmal marker enzyme activities occurring in soluble fractions (i.e., 5–37% of homogenate) parallels very closely the proportion of total cellular oestradiol binding that appears in cytosol. In the cases of both marker enzymes and oestrogen binding, elevation of activity in the cytosol fraction is accompanied by a corresponding decline in the other cell fractions. Since 5′-nucleotidase, alkaline phosphatase and $(Na^+,K^+$-activated)-ATPase activities are enriched normally only in particulate fractions and, indeed, predominantly in plasma membranes, the present findings are in accord with previous reports that use of hypotonic conditions (Berman et al., 1969; DePierre & Karnovsky, 1973) as well as excessive force to break cells (Plagemann, 1969; Thinès-Sempoux, 1973) elicit fragmentation of cell membranes and organelles with consequent redistribution of their components. Previous workers have also reported that the drastic homogenization commonly used to disrupt segments of another oestrogen target-organ, the uterus, results in very small fragments of plasma membrane, most of which are lost in postnuclear supernatant fractions at the first step of low-speed centrifugation (Kidwai et al., 1971).

In the present work, plasma membranes purified from isolated hepatocytes were distributed primarily among three subfractions, 2B and 3B (resulting from fractionation of crude nuclei) and P2 (derived from microsomal fractions). In physical and biochemical properties, these membranous subfractions correspond closely with those prepared from whole liver as well as from isolated hepatocytes (Kremmer et al., 1976; Wisher & Evans, 1977; Evans, 1978). The subfractions obtained by the Evans group were identified by biochemical, morphologic and surface-labelling techniques as derivatives of predominantly three functional areas of the hepatocyte surface, including blood-sinusoidal, lateral, and bile-canalicular areas (cf. Evans, 1978). It is consistent with such data that plasma-membrane subfraction 3B was probably derived from the lateral areas of the hepatocyte surface involved in interhepatocytic adhesion and communication. The membrane subfraction 2B probably represents a mixture of parenchymal cell surfaces bordering on blood sinusoids and bile canaliculi (cf. Evans, 1978). Similarly, plasma-membrane fraction P2 exhibits properties characteristic of the predominantly blood-sinusoidal surface region. It is reasonable to expect concentration of oestrogen-binding sites at the blood front (Tchernitchin et al., 1976) as well as at the canalicular interface (cf. Cantarow et al., 1942) of the hepatocyte surface, but verification of these assumptions must await further investigation.

The present results indicate that the oestro-gen-binding component of plasma-membrane subfraction 2B exhibits a moderate degree of positive co-operativity (i.e., Hill coefficient = 1.6) in interacting with hormone. Ellis & Ringold (1971), as well as other investigators (Erdos et al., 1971; Puca et al., 1971; Sanborn et al., 1971), have also provided evidence for co-operativity in the interaction of oestradiol with cytosolic receptor extracted from uterus, with Hill coefficients ranging from 1.1 to 1.6. However, Williams & Gorski (1974) were unable to demonstrate co-operativity of binding, although Rosner et al. (1979) found evidence for negative co-operativity of oestradiol binding to cytosol extracts from uterus.

Neither the potential significance nor the mechanism of the apparent positive co-operativity of the plasma-membrane binding component(s) is known. Most models for co-operative binding are based on interaction of multiple subunit structures that can exist in more than one conformation (Monod et al., 1965). The present work provides evidence that the hepatic plasma-membrane receptor for oestrogen may occur in multiple molecular forms, and could accommodate mechanisms of co-operative interactions. Oestradiol-binding components sedimenting at 3.5 S, 4.5 S and in the 6–10 S region of sucrose density gradients have previously been extracted in variable ratios from the microsomal fraction of pig-uterus homogenates (Little et al., 1972).

The present results provide evidence that a substantial portion of native binding sites for oestrogen in hepatocytes are intrinsic to the plasma membrane. However, the relation of such binding to biologic actions of the hormone in hepatocytes remains to be determined. In liver (Eisenfeld et al., 1976), as in other target tissues for oestradiol (Jensen et al., 1974; King & Mainwaring, 1974; Pavlik et al., 1979), interaction of hormone with its extranuclear receptor at physiological temperatures, but not at 4°C, seems to mediate transfer of the agonist to the nucleus, where the complex is believed to promote expression of the phenotypic effects (cf. Buller & O'Malley, 1976). In accord with these predictions, the present results demonstrate a profound increase in oestradiol binding in purified nuclei, coupled with a marked decline in hormone binding in plasma membranes, after exposure of intact hepatocytes to 0.1 nM oestradiol-17β at 22°C, as compared with paired cells treated at 4°C.

The occurrence of binding sites specific for oestradiol in multiple cellular-loci suggests that future research might well be directed toward investigation of a biosynthetic pathway (Szego & Pietras, 1980) or nuclear-translocation circuit (Jensen et al., 1974; King & Mainwaring, 1974; Szego, 1974, 1975, 1978; Szego & Pietras, 1980) involving microsomal, mitochondrial, lysosomal and plasma-lemmal, as well as cytosolic, elements. Data sug-

gestive of surface recognition of oestrogen and subsequent intracellular processing of either native or modified hormone–receptor complexes have been presented elsewhere (cf. Szego, 1974; Pietras & Szego, 1980; Szego & Pietras, 1980). Such data indicate that our understanding of the cellular mechanism of steroid hormone action requires further consideration of the role of cell membranes in both the recognition of and response to the agonist.

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