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Binding of ^{3}H-Estradiol-17β-(β-D-glucuronide), a Cholestatic Organic anion, to Rat Liver Plasma Membranes. Evidence Consonant with Identification of Organic Anion Carriers

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SUMMARY

The binding of ^{3}H-estradiol-17β(β-D-glucuronide) (^{3}H-E_2 17G), a cholestatic organic anion, was examined in rat liver plasma membranes, and two saturable, specific binding sites were identified. The binding parameters are $Kd_1 = 3.9 \times 10^{-7}$ M, $Bmax_1 =$ 69 pmol/mg of protein; $Kd_2 = 4.90 \times 10^{-6}$ M, $Bmax_2 = 495$ pmol/mg of protein according to Scatchard analysis of equilibrium experiments. Kinetic dissociation experiments showed that ^{3}H-$E_2$17G binding was reversible and revealed two components. The dissociation rate constants did not vary with the method of dilution of radioligand, i.e., by "infinite" volume, or excess unlabeled ligand, ruling out the possibility of cooperativity. The rate of association of ^{3}H-$E_2$17G binding was very rapid, so that maximal binding was reached within 15 sec at 4°. Na^+ was not required for binding and binding was not decreased in the presence of high osmolarity buffer (125 mM sucrose), indicating that transport into vesicles was not involved. The ability of a series of compounds to inhibit the binding of ^{3}H-$E_2$17G was also examined. Taurocholate, cholate, taurodehydrocholate, and testosterone glucuronide were identified as ligands selective for the high affinity site (site 1). The A- and D-ring glucuronide conjugates of estradiol and estriol, bromosulfophthalein, dibromosulfophthalein, and the glucuronide conjugates of phenolphthalein, 4-methylumbelliferone, and menthol inhibited binding of ^{3}H-$E_2$17G to both sites. Morphine glucuronide, estradiol, and glucuronic acid did not inhibit binding to either site. The substrate specificities of binding to the low affinity site (site 2) are consistent with data characterizing the transport of these substrates in hepatocytes and supports the postulate that site 2 represents a non-bile acid organic anion carrier. Site 1 is postulated to represent a carrier shared by the bile acids and non-bile acid organic anions.

The estrogen D-ring glucuronides, natural metabolites of estradiol and estriol, cause a rapid, reversible, and dose-dependent inhibition of canalicular bile flow and bile acid secretory rate in the rat (1) and decrease hepatic excretory function in the monkey (2). In contrast, the estrogen A-ring glucuronides stimulate bile flow under similar experimental conditions, indicating a specific structure-activity relationship necessary for inducing cholestasis. The estrogen D-ring glucuronides exhibit parallel dose response curves with respect to their inhibition of bile flow, with $E_2$17G being the most potent of these cholestatic agents (3). These data led us to postulate that the estrogen D-ring glucuronides induce cholestasis by interacting at a specific site in the liver.

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$E_2$17G is an organic anion which is efficiently transported by the liver. Following its intravenous administration in the rat, 75–80% of a dose of ^{3}H-$E_2$17G was recovered in bile within 3 hr and identified as $E_2$17G or metabolites (1–3). The uptake of ^{3}H-$E_2$17G by isolated rat hepatocytes has been shown to be saturable and at least partially dependent on energy and Na^+, indicating that its uptake is an active carrier-mediated process (4). The uptake of ^{3}H-$E_2$17G is inhibited by several organic anion dyes, e.g., DBSP and BSP, as well as by other estrogen A- and D-ring glucuronides (5). These data strongly suggest that $E_2$17G shares an uptake mechanism, i.e., a carrier, with the organic anion dyes, agents whose hepatic transport has been extensively studied (6–8). Numerous studies both *in vitro* and *in vivo* (8–12) have suggested that organic anions are transported into hepatocytes by multiple carrier systems. Such a carrier-mediated uptake involves two processes: 1) binding of ligand to the carrier protein and 2) translocation of ligand across the membrane.

ABBREVIATIONS: $E_2$17G, estradiol-17β-(β-D-glucuronide); DBSP, dibromosulfophthalein; BSP, bromosulfophthalein; HPLC, high pressure liquid chromatography; MeOH, methanol; $E_3$17G, estriol-17β-(β-D-glucuronide); $E_3$16G, estriol-16α-(β-D-glucuronide); $E_2$3G, estradiol-3β-(β-D-glucuronide).

The objective of the present studies were to determine if a specific binding site(s) for ^{3}H-$E_2$17G could be identified in rat liver plasma membranes and to characterize the substrate specificities of these binding sites in order to determine whether these sites represent hepatic organic anion carriers or, alternatively, a site that mediates cholestasis. The present studies describe the binding of ^{3}H-$E_2$17G to two sites in rat liver plasma membranes. The ability of a series of ligands to compete with ^{3}H-$E_2$17G for binding was examined and compared to their ability to inhibit uptake of ^{3}H-$E_2$17G into hepatocytes.

Experimental Procedures

Materials. ^{3}H-$E_2$17G (50 Ci/mmol) and ^{3}H-H_2O were obtained from New England Nuclear (Boston, MA). Nonlabeled $E_2$17G was purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals were of reagent grade.

Animals. Female Sprague-Dawley rats weighing 175–225 g were purchased from Harlan Industries (Indianapolis, IN) and housed in hanging steel wire cages. They were given food (Purina Rat Chow) and water *ad libitum* and were maintained on a 12-hr light/dark cycle. Animals were acclimated at least 5 days before use.

Preparation of liver plasma membranes, subcellular fractions and enzyme assays. Rat liver plasma membranes were prepared using discontinuous sucrose density gradient centrifugation by a modification of the methods of Song *et al.* (13) and Boyer and Reno (14) according to Scharschmidt *et al.* (15). The final pellet of liver plasma membranes was resuspended in modified Hanks' buffer (137 mM NaCl, 5.2 mM KCl, 0.9 mM $MgSO_4$, 0.12 mM $CaCl_2$, 10 mM Na_2HPO_4, pH 7.4) and stored for no longer than 5 days in liquid N_2 until use in the binding assay. Protein concentration was determined by the Lowry assay (16). Nuclei were prepared by discontinuous sucrose density gradient ultracentrifugation of rat liver homogenate as described by Tata (17). Mitochondria and microsomes were prepared by differential centrifugation as described by Fleisher and Kervina (18).

5-Mononucleotidase activity, a liver plasma membrane marker, and glucose-6-phosphatase activity, a microsomal marker, were determined by the analysis of inorganic phosphate production according to the method of Fisk and Subbarow (19). Succinate dehydrogenase activity, a mitochondrial marker, was determined by the method of Shephard and Hubscher (20). Na^+K^+ATPase and Mg^+ATPase activities were determined by a coupled enzyme assay in which the oxidation of NADH is monitored according to the method of Scharschmidt *et al.* (15).

Binding assay. The binding assay was performed in 400-μl centrifuge tubes in a final volume of 200 μl. Unlabeled $E_2$17G or other ligands in 50 μl of modified Hanks' buffer or buffer alone were added to the tubes followed by the addition of 50 μl of ^{3}H-$E_2$17G. The binding reaction was begun by the addition of 70–100 μg of protein in 100 μl of buffer to the centrifuge tube. Binding of the radioligand to the centrifuge tube was negligible. All binding experiments were performed at 4° unless otherwise noted.

At the termination of the binding reaction (1 min, except where noted), bound ligand was separated from free ligand by centrifugation for 2 min at 10,000 × *g* in a Beckman benchtop Microfuge. Bound ligand was precipitated in seconds and at no time was it exposed to ligand-free media. Supernatant was removed by aspiration with a Pasteur pipette drawn out to a fine tip to prevent disruption of the pellet. The tube was cut near the pellet and the membrane pellet dissolved in 0.5 ml of Soluene 350 tissue solubilizer (United Technologies Packard, Downer's Grove, IL) in a 7-ml mini-vial for 1 hr at 50° or overnight at 37°. The solubilizer was neutralized with 17 μl of glacial acetic acid, 4 ml of Scintiverse II (Fisher Scientific Co., Cincinnati, OH) were added, and radioactivity was counted by liquid scintillation spectrometry. Supernatant trapped in the pellet was corrected for in control samples by incubation of 100 μl of ^{3}H-H_2O with membranes. Trapped H_2O was 0.3–0.5% of the amount of ^{3}H-$E_2$17G added. Specific binding was defined as the total amount bound (^{3}H-$E_2$17G alone) minus the amount bound in the presence of 100 μM $E_2$17G. Specific binding was 60% of total binding at the concentration of ^{3}H-$E_2$17G (5 nM) used in displacement experiments. The data from the inhibition experiments are graphically represented as the log of the concentration of unlabeled $E_2$17G (or other ligand) on the abscissa and the percentage inhibition of ^{3}H-$E_2$17G bound on the ordinate where 100% inhibition is that inhibition achieved by 100 μM $E_2$17G.

The time course of the binding reaction was determined at 4° and 37°. ^{3}H-$E_2$17G ($2–3 \times 10^{-9}$ M) was incubated with membranes as described above in the presence and absence of 100 μM unlabeled $E_2$17G, and binding was assayed at 0.25, 0.50, 0.75, 1, 2, 5, 15, 30, 45, and 60 min. Experiments designed to characterize the linearity of ^{3}H-$E_2$17G binding with respect to protein showed that binding was linear from 0.02 to 0.2 mg of protein. All assays were carried out in the range of linearity (0.07–0.1 mg of protein). The effect of increased osmolarity on total and specific binding was determined by incubating $2–3 \times 10^{-9}$ M ^{3}H-$E_2$17G with and without 100 μM unlabeled $E_2$17G with membranes in the presence and absence of 125 mM sucrose. The binding parameters for ^{3}H-$E_2$17G were also determined in Na^+-free Tris buffer (0.1 M, pH 7.4) in order to determine whether Na^+ was required for binding.

Two types of dissociation experiments were performed to determine whether negatively cooperative interactions were involved between ligand and bindings site as described by DeMeyts *et al.* (21). The first experimental approach used excess unlabeled $E_2$17G dilution to cause dissociation of the radioligand. ^{3}H-$E_2$17G (0.5 ml of 4×10^{-8} M) was incubated with 1 mg of membrane protein (0.5 ml) to equilibrium (2 min) at 4°. Binding was terminated by centrifugation in a Sorvall centrifuge for 2 min at 10,000 × *g*. Supernatant was removed by aspiration and the pellet was resuspended in 1 ml of 400 μM $E_2$17G. Aliquots (100 μl) were removed at various times after dilution from 15 sec to 30 min and centrifuged in the Microfuge at 10,000 × *g* for 2 min. Bound ^{3}H-$E_2$17G was determined in the same manner as described above. The second type of dissociation experiment used volumetric "infinite" dilution in which the pellet was resuspended with 20-fold excess buffer. The rest of the experiment was performed as described for dilution with excess unlabeled $E_2$17G.

Analysis of ^{3}H-$E_2$17G. ^{3}H-$E_2$17G was purified to greater than 98% radiochemical purity by HPLC using two C-18 μBondapak columns (Waters Associates, Inc., Milford, MA) in series with an MeOH/H_2O gradient (26% MeOH/74% H_2O to 30% MeOH/70% H_2O) over 20 min at a flow rate of 1 ml/min. Authentic ^{3}H-$E_2$17G eluted at 16.5–24 min and a polar impurity eluted at 7–13 min. In order to determine whether any metabolism or degradation of ^{3}H-$E_2$17G occurred during the binding reaction, the pellet obtained from a typical assay was acidified with HCl and extracted with ethyl acetate, then, the extract was analyzed by HPLC.

Data analysis. The binding parameters, K (affinity constant), B_{max} (receptor capacity), and N (nonspecific binding) for both saturation and inhibition experiments were determined by LIGAND, a nonlinear least squares iterative curve-fitting computer program by Munson and Rodbard (22). Statistical analysis of whether the increase of the goodness of fit for a model with additional parameters is significantly more than expected on the basis of chance alone is based on the "extra sum of squares" principle. The F test is performed as follows:

$$F = \frac{(SS_1 - SS_2)/(df_1 - df_2)}{SS_2/df_2}$$

where SS_1 and SS_2 are the residual sum of squares for the less and the more complicated models and df_1 and df_2 are the associated degrees of freedom, respectively.

The log dissociation rate curves were analyzed by the computer program NON-LIN (23) which used the Gauss-Newton least squares iterative method to fit the data. A monoexponential function (Bound = $Ae^{-\alpha t}$) and a bioexponential function (Bound = $Ae^{-\alpha t}$ and $Be^{-\beta t}$) were fit to the data, and visual inspection of the data and predicted curve, as well as R^2 were used to determine the best fit. R^2 is [weighted corrected sum of squared observations (WYS) − sum of weighted squared deviations (WS)/WYS].

Results

The plasma membrane preparation used in these studies is enriched in enzymes that have been shown histochemically to be located in the plasma membrane: 5′-mononucleotidase, Mg^{2+} ATPase, and Na^+K^+ATPase (Table 1). There is little mitochondrial (succinate dehydrogenase) or microsomal (glucose-6-phosphatase) contamination. This is a heterogenous plasma membrane preparation since markers of both the sinusoidal domain (Na^+K^+ATPase) and the canalicular domain (Mg^{2+}ATPase and 5′-mononucleotidase) are enriched.

No metabolism of the radioligand occurred during the binding experiment since 98.6–100% ($n = 2$) of the radioactivity extracted from the membrane pellet was identified by HPLC as ^{3}H-$E_2$17G. Association experiments in which 2–3 × 10^{-9} M ^{3}H-$E_2$17G was incubated in the presence and absence of 100 μM $E_2$17G indicated that equilibrium was reached within 15 sec at both 37° and 4°. Analysis of the time course of binding by two-way analysis of variance showed no significant difference between time points for both total and specific binding, but did show a significant difference ($p < 0.001$) between temperatures. Specific binding was 3–4 times greater at 4° than at 37° in the association experiment so that all subsequent studies were carried out at 4°. When competition experiments were performed at 37° and 4°, there was a significant increase in the receptor capacity at the high affinity site at 4° as determined by the group test (Table 2). Incubation of membranes at 4° in Tris-substituted Na^+ free buffer did not significantly affect the binding parameters as determined by the group t test. There was no significant decrease in specific or total binding when membranes were incubated in the presence of increased osmolarity, 125 mM sucrose, as determined by the paired t test. We can therefore conclude that transport into osmotically active vesicles was not involved. This interpretation is supported by the absence of a decrease in binding at 4° which would be expected if transport into vesicles was occurring.

The displacement of ^{3}H-$E_2$17G by increasing concentrations of unlabeled $E_2$71G (Fig. 1) indicates that binding is saturable. Concentrations of $E_2$17G greater than 100 μM (up to 200 μM) did not displace ^{3}H-$E_2$17G any further. Analysis of these data by LIGAND yielded two populations of binding sites for ^{3}H-$E_2$17G in liver plasma membrane (mean ± SE): site 1, Kd_1 = 3.90 × 10^{-7} M ± 1.11 × 10^{-7} M, $B\text{max}_1$ = 69 pmol/mg of protein ± 11.1; and site 2, Kd_2 = 4.91 × 10^{-6} M ± 0.93 × 10^{-6} M, $B\text{max}_2$ = 495 pmol/mg of protein ± 67.1.

The Scatchard plot of the saturation data (Fig. 2) is a hyperbola, upwardly concave, which suggests the presence of multiple binding sites. $E_2$17G is not soluble in buffer above 200 μM (final concentration), so that concentrations greater than 10^{-5} M ^{3}H-$E_2$17G could not be used in the saturation experiments. LIGAND analysis showed that the two-binding site model fit these data best with the following parameters: site 1, Kd_1 = 1.20 × 10^{-7} M, $B\text{max}_1$ = 11.1 pmol/mg of protein, site 2, Kd_2 = 1.11 × 10^{-5} M, $B\text{max}_2$ = 881 pmol/mg of protein.

Fig. 3A shows the linear plot of the dissociation rate experiments. Dilution of bound radioligand by either means (infinite volume or excess unlabeled $E_2$17G) displaced all specific binding, indicating that ^{3}H-$E_2$17G specific binding is fully reversible. The amount bound at 5 and 10 min did not differ in either experiment. The excess $E_2$17G dissociation experiment was

TABLE 1

Enzymatic characterization of rat liver plasma membranes

The activities of the various marker enzymes were determined in aliquots of rat liver homogenate and rat liver plasma membranes as described under Experimental Procedures. Enrichment represents the enrichment of each marker enzyme activity in plasma membranes as compared to homogenate. Each value represents the mean ± standard error of three determinations.

	Homogenate	LPM[a]	Enrichment
Succinate dehydrogenase (μmol Formazan/mg protein·hr)	2.27 ± 0.05	2.17 ± 0.65	0.96
5′-Mononucleotidase (μmol P_i/mg protein·hr)	1.15 ± 0.31	37.97 ± 1.56	33.02
Glucose-6-phosphatase (μmol P_i/mg protein·hr)	3.22 ± 0.17	1.03 ± 0.32	0.38
Mg^{2+} ATPase (μmol P_i/mg protein·hr)	2.68 ± 0.24	48.58 ± 4.02	18.13
Na^+K^+ATPase (μmol P_i/mg protein·hr)	0.761 ± 0.43	32.29 ± 2.52	42.43

[a] LPM, liver plasma membranes.

TABLE 2

Effect of temperature on binding parameters

Inhibition experiments were performed at 37° and 4° in which 5 × 10^{-9} M ^{3}H-$E_2$17G was incubated with 10^{-10}–10^{-5} M unlabeled $E_2$17G. The binding parameters were determined by LIGAND analysis. Each point represents the mean ± standard error of three to four determinations.

Temperature	Kd_1 (M)	Kd_2 (M)	R1[a]	R2
4°	3.9 × 10^{-7} ± 1.11	4.91 × 10^{-6} ± 0.93	68.9 ±11.1	495 ±67.1
37°	1.81 × 10^{-7} ± 0.76	2.01 × 10^{-5} ± 0.84	5.33[b] ±2.21	913 ±326

[a] $R = B_{max}$ in pmol/mg of protein.
[b] $p < 0.05$, significantly different from 4° value.

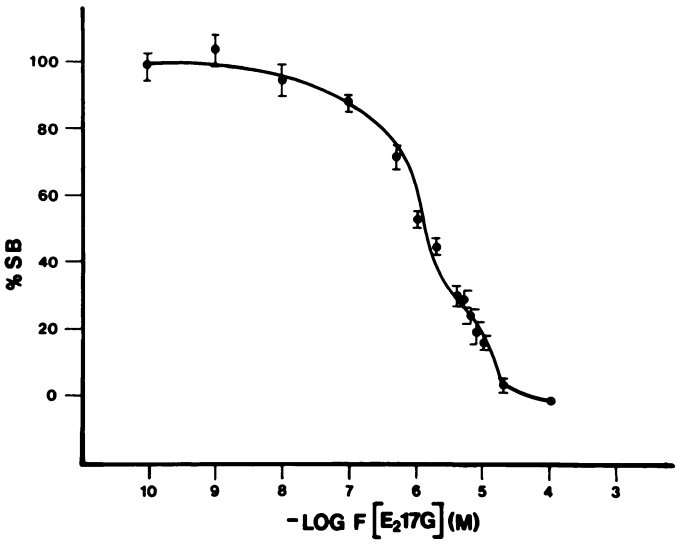

Fig. 1. Displacement of ^{3}H-$E_2$17G binding by increasing concentrations of unlabeled $E_2$17G. ^{3}H-$E_2$17G (5 × 10^{-9} M) was incubated in the presence of 10^{-10}–10^{-4} M unlabeled $E_2$17G. The percentage of specific bound (% *SB*) is displayed on the *ordinate* and 100% specific bound refers to binding when ^{3}H-$E_2$17G is incubated alone. Each *point* represents the mean ± standard error of 3–15 determinations each performed in quadruplicate. The binding parameters were determined by LIGAND analysis of the data.

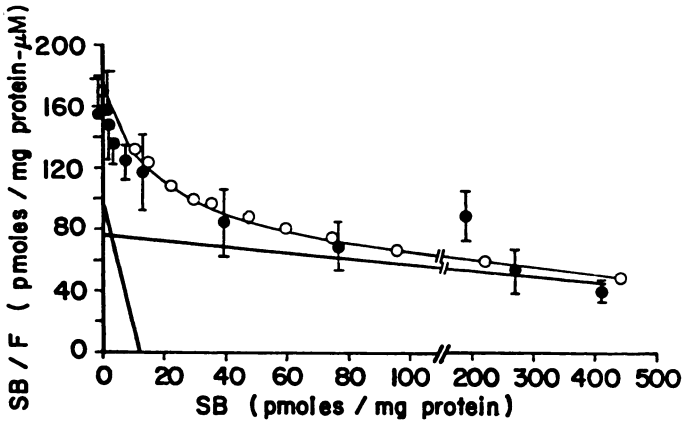

Fig. 2. Scatchard analysis of the saturation experiment. The data are represented graphically by specific bound/free (*SB/F*) versus specific bound. The binding parameters were determined by LIGAND analysis and the *solid lines* are those calculated by LIGAND with slopes = $1/Kd_1$ and $1/Kd_2$. ●, observed data points (mean ± standard error); ○, predicted values determined by performing geometric construction of the curved line (34).

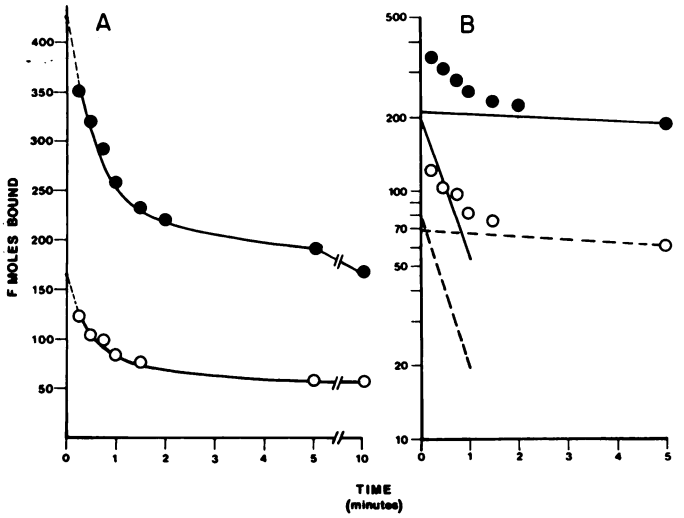

Fig. 3. Linear (A) and log (B) plots of dissociation rate experiments. The data are represented graphically by amount bound versus time after dissociation of bound ligand. ○ and ●, excess $E_2$17G dilution and infinite volume dilution, respectively. Each *point* represents the mean of two determinations performed in triplicate. The lines drawn are those calculated by Non-lin in which their slopes are equal to K_{-1} (site 1) and K_{-1} (site 2). R^2 = 0.984 and 0.996 for the biexponential fit of the data from excess $E_2$17G and infinite volume experiments, respectively.

carried out longer and the amount bound at 30 and 60 min was not different from that bound at 10 min (data not shown). The logarithmic conversion of these data is displayed in Fig. 3B. Both types of dissociation experiments exhibited biphasic log plots which denote heterogenous binding sites. Analysis by Non-lin showed that a biexponential rate equation fit these data best. The K_{-1} values (dissociation rate constants) as determined by Non-lin for the excess $E_2$17G experiment were 1.40 and 0.024 min^{-1} and for the infinite dilution experiment were 1.28 and 0.025 min^{-1}, respectively. The K_{-1} values do not differ between the two types of dilution used, indicating that no cooperative interactions are occurring at either site. Using the relation $K_{-1}/K_{+1} = K_d$, K_{+1} values for high and low affinity

TABLE 3

Binding of ^{3}H-$E_2$17G to liver subcellular fractions

Inhibition experiments were performed at 4° in which 5×10^{-9} M ^{3}H-$E_2$17G was incubated with 10^{-10}–10^{-5} M unlabeled $E_2$17G. The points are the parameters determined by LIGAND analysis when all experiments are fit together (n = 3–8).

Fraction	Kd_1 (10^{-7} M)	Kd_2 (10^{-5} M)	$Bmax_1$	$Bmax_2$
			pmol/mg of protein	
Homogenate	4.98	1.72	14.08	233.5
Nuclei	1.25	1.21	4.10	45.56
Mitochondria	3.09	1.41	14.27	296.7
Microsomes	7.80	5.05	111.5	5142
Plasma membrane	3.90	0.49	68.8	495.0

sites were calculated to be 6×10^4 M^{-1} sec^{-1} and $8 \times 10^1 M^{-1} sec^{-1}$, respectively. These rapid association rates are in agreement with the data generated by the time course of association experiments in which equilibrium was reached within 15 sec.

Table 3 shows the binding parameters for ^{3}H-$E_2$17G (as determined by inhibition experiments) in various liver subcellular fractions. Binding is most enriched in plasma membranes and microsomes. The binding seen in nuclei and mitochondria is probably due to contamination from other organelles since 5′-mononucleotidase activity and glucose-6-phosphatase activity were present in mitochondria at 50 and 100% of their concentration in homogenate, respectively.

The next group of experiments was designed to determine the specificity of the ^{3}H-$E_2$17G binding sites in rat liver plasma membranes. The inhibition of ^{3}H-$E_2$17G binding by a series of ligands with differing structures and functions with respect to hepatic transport was examined.

The first group of inhibitors examined were the cholestatic D-ring estrogen glucuronides, $E_3$17G and $E_3$16G, as well as the choleretic A-ring estrogen glucuronide, $E_2$3G. All of these estrogen glucuronides were nonselective inhibitors of ^{3}H-$E_2$17G binding in rat liver plasma membranes (Fig. 4). Due to their limited solubility, it was not possible to test higher concentrations of these glucuronides to determine if they were capable of inhibiting 100% of ^{3}H-$E_2$17G binding. When these data were analyzed by LIGAND, these inhibitors fit significantly better to a two-site than a one-site model. The criterion for a significantly better fit is a p value ≤ 0.1 as calculated by the F test (described under Experimental Procedures). The K_d values for all inhibitors are shown in Table 4. The estrogen D-ring glucuronides ($E_2$17G, $E_3$17G, $E_3$16G) all had very similar K_d values at the high affinity site (site 1), ranging from 3.9–6.06 × 10^{-7} M, whereas K_d values at the low affinity site (site 2) ranged from 4.9 × 10^{-6} M for $E_2$17G to 2.8 × 10^{-4} for $E_3$16G. The estrogen A-ring glucuronide, $E_2$3G, had a 5.3-fold higher K_d than $E_2$17G at site 1 and a 20.4-fold higher K_d at site 2.

The organic anion dyes, BSP and DBSP, also bound to both sites (Fig. 5) with K_d values 4.5- and 1.8-fold higher at site 1 and 2.5- and 6.6-fold higher than $E_2$17G at site 2, respectively.

Phenolpthalein glucuronide, 4-methylumbelliferyl glucuronide, and menthol glucuronide also bound to both sites 1 and 2 (Fig. 6). Their affinities at site 1 ranged from 1.9 × 10^{-7} M for 4-methylumbelliferyl glucuronide to 1.1 × 10^{-5} M for menthol glucuronide (Table 4). K_d values at site 2 were 65- to 306-fold higher than that for $E_2$17G.

The bile acids exhibited inhibition curves that were strikingly different from those of the glucuronides or organic anion dyes (Fig. 7). The bile acids were able to inhibit only about 60% of

^{3}H-$E_2$17G binding regardless of whether a micelle-forming (taurocholate), non-micelle-forming (taurodehydrocholate), or unconjugated (cholate) bile acid was the competing ligand. LIGAND fit these data best to a one-site model. The affinity at site 1 for these bile acids was nearly identical to that for $E_2$17G (Table 4), and the calculated binding capacities were 67.2 ± 14.9, 150.0 ± 10.4, and 43.2 ± 16.0 pmol/mg of protein for taurocholate, cholate, and taurodehydrocholate, respectively. These binding capacities are similar to the binding capacity of site 1 for $E_2$17G (69 pmol/mg of protein), suggesting that the bile acids are selective ligands for site 1.

Testosterone glucuronide and ouabain also inhibited only 60% of ^{3}H-$E_2$17G specific binding and are thus similar to the bile acids (Fig. 8). The K_d values of testosterone glucuronide and ouabain for site 1 were 4- and 27-fold higher than that for $E_2$17G, whereas the binding capacities were 135.0 ± 38.8 and 3053 ± 2050 pmol/mg of protein, respectively. These data suggest that testosterone glucuronide and, possibly, ouabain bind selectively to site 1. The high B_{max} and the large variability in this measure for ouabain do not permit a definite assignment of its binding site.

Morphine glucuronide and estradiol at concentrations of 2×10^{-4} M and 1×10^{-4} M, respectively, did not inhibit ^{3}H-$E_2$17G binding. Because of its low water solubility, estradiol binding was done in the presence of 12.5% ethanol. Although ethanol itself decreased ^{3}H-$E_2$17G binding by 40%, estradiol had no further inhibitory effect. Glucuronic acid (2×10^{-3} M) had no effect on ^{3}H-$E_2$17G binding but, at a concentration of 1×10^{-2} M, did inhibit binding by 30%.

From these studies, three classes of ligand became evident, those which were selective for site 1 (the bile acids, testosterone glucuronide, and ouabain), the glucuronides and organic anion dyes which bound to both sites, and those agents which did not inhibit ^{3}H-$E_2$17G binding (estradiol, morphine glucuronide, and glucuronic acid).

TABLE 4

K_d values of inhibiting ligands

K_d values for inhibiting ligands were predicted by LIGAND analysis of data shown in Figs. 4–8. The inhibition experiments were performed as described under Experimental Procedures. Each value represents the mean ± standard error of three to six determinations, each performed in quadruplicate.

Ligand	No. of sites	K_d values (M)	
$E_2$17G	2	$3.90 \cdot 10^{-7}$ ±1.11	$4.90 \cdot 10^{-6}$ ±0.93
$E_2$3G	2	$2.05 \cdot 10^{-6}$ ±0.26	$1.00 \cdot 10^{-4}$ ±0.87
$E_3$16G	2	$6.06 \cdot 10^{-7}$ ±2.90	$2.80 \cdot 10^{-4}$ ±1.78
$E_3$17G	2	$4.40 \cdot 10^{-7}$ ±2.19	$1.17 \cdot 10^{-5}$ ±0.87
BSP	2	$1.80 \cdot 10^{-6}$ ±1.73	$1.23 \cdot 10^{-5}$ ±0.21
DBSP	2	$7.02 \cdot 10^{-7}$ ±1.36	$3.23 \cdot 10^{-5}$ ±0.57
Phenolphthalein glucuronide	2	$2.59 \cdot 10^{-6}$ ±0.54	$1.50 \cdot 10^{-3}$ ±0.59
4-Methyl Umbelliferyl glucuronide	2	$1.86 \cdot 10^{-7}$ ±0.81	$3.20 \cdot 10^{-4}$ ±2.41
Menthol glucuronide	2	$1.10 \cdot 10^{-5}$ ±0.93	$6.33 \cdot 10^{-4}$ ±5.74
Taurocholate	1	$4.71 \cdot 10^{-7}$ ±0.79	
Cholate	1	$7.47 \cdot 10^{-7}$ ±3.56	
Taurodehydrocholate	1	$3.16 \cdot 10^{-7}$ ±1.43	
Ouabain	1	$1.05 \cdot 10^{-5}$ ±0.61	
Testosterone glucuronide	1	$1.49 \cdot 10^{-6}$ ±0.65	

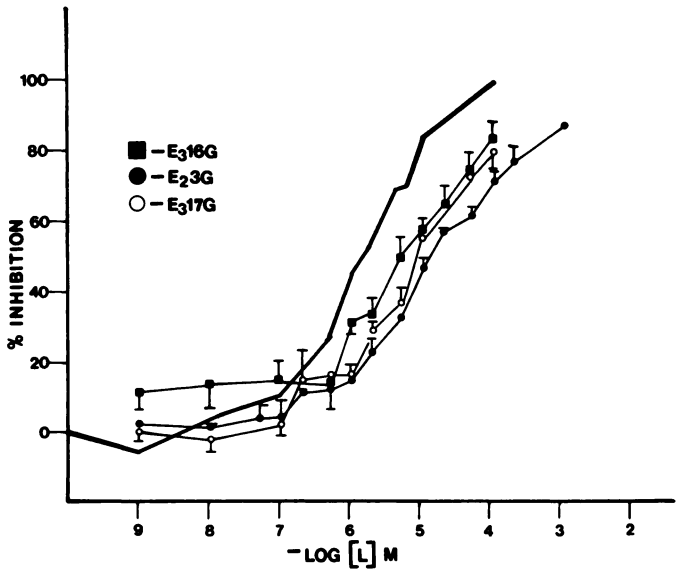

Fig. 4. Inhibition of ^{3}H-$E_2$17G binding by estrogen glucuronides. Unlabeled estrogen glucuronides (10^{-9}–10^{-3} M) were incubated with $5 \cdot 10^{-9}$ M ^{3}H-$E_2$17G and the binding experiment was performed as described under Experimental Procedures. The *ordinate* is the percentage inhibition of the maximal displacement achieved by 100 μM $E_2$17G, and the *abscissa* is the negative logarithm of concentration of unlabeled ligand employed. Each *point* represents the mean ± standard error of three to six determinations each performed in quadruplicate. The *bold line* represents the inhibition curve for $E_2$17G for comparison.

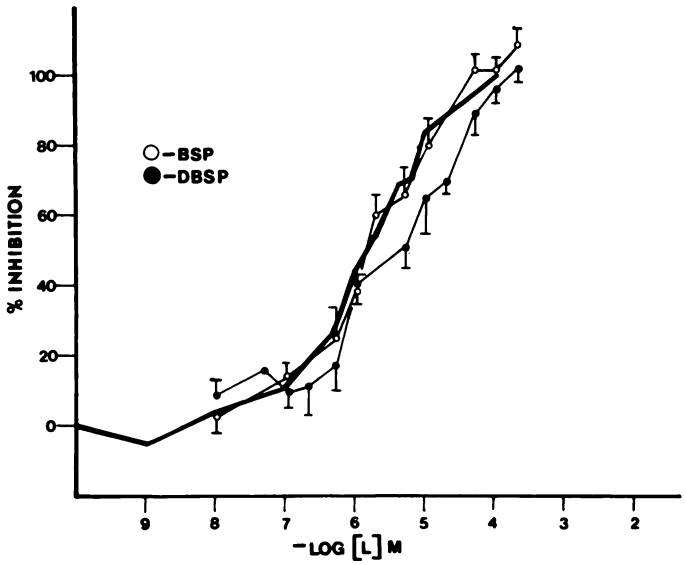

Fig. 5. Inhibition of ^{3}H-$E_2$17G binding by organic anion dyes, BSP, and DBSP. Unlabeled BSP and DBSP (10^{-9}–10^{-4} M) were incubated with $5 \cdot 10^{-9}$ M ^{3}H-$E_2$17G and the binding experiment was performed as described under Experimental Procedures. See the legend for Figure 4.

Discussion

The present data clearly show that ^{3}H-$E_2$17G binds specifically to liver plasma membranes. Binding was shown to be

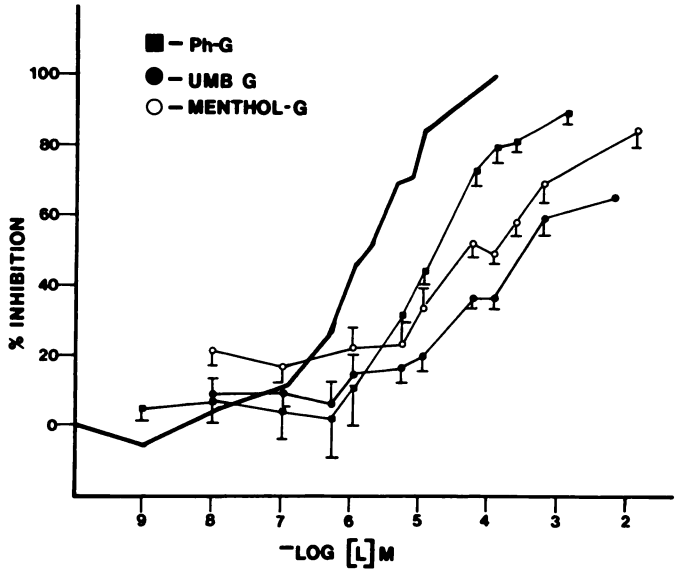

Fig. 6. Inhibition of ^{3}H-$E_2$17G binding by nonsteroidal organic anions, phenolphthalein glucuronide (*Ph-G*), 4-methylumbelliferyl glucuronide (*UMB G*), and menthol glucuronide (*Menthol-G*). Unlabeled inhibitors (10^{-9}–10^{-2} M) were incubated in the presence of $5 \cdot 10^{-9}$ M ^{3}H-$E_2$17G and the binding experiment was performed as described under Experimental Procedures. See the legend for Figure 4.

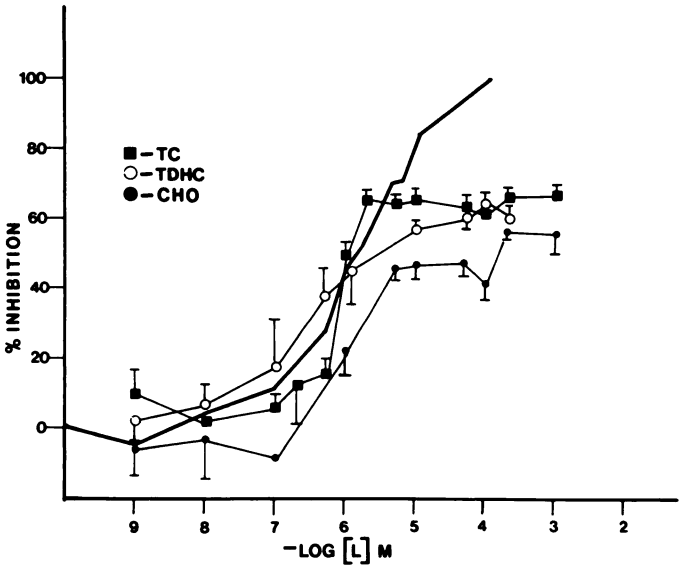

Fig. 7. Inhibition of ^{3}H-$E_2$17G binding by bile acids. Unlabeled taurocholate (*TC*), cholate (*CHO*), and taurodehydrocholate (*TDHC*) (10^{-9}–10^{-3} M) were incubated in the presence of $5 \cdot 10^{-9}$ M $E_2$17G and the binding experiment was performed as described in Experimental Procedures. See the legend for Figure 4.

saturable, reversible, and independent of Na^+. Binding to plasma membranes did not represent transport into vesicles since binding was not decreased in the presence of increased osmolarity or at 4°. The time course of ^{3}H-$E_2$17G binding and the association constants (6×10^4 M^{-1} sec^{-1} at site 1 and 8.2×10 $M^{-1}sec^{-1}$ at site 2) calculated from the equilibrium and dissociation constants both indicated that the association rate is rapid.

Several lines of evidence indicated that ^{3}H-$E_2$17G binds to two distinct sites in the membrane preparation. The evidence for multiplicity is derived from equilibrium and kinetic studies and inhibition studies identifying selective ligands. Both displacement experiments and saturation experiments fit significantly better to the two-site model when analyzed by LIGAND. The hyperbolic-concave upward Scatchard analysis of the saturation data (Fig. 2) is indicative of heterogeneity of binding sites. Curvilinear Scatchard plots can have other causes, such as ligand heterogeneity, incorrect definition of nonspecific binding, ligand-ligand interactions, two-step reactions with ternary complex formation, and negative cooperativity (24). HPLC analysis of bound ^{3}H-$E_2$17G indicated a single moiety, thus ruling out ligand heterogeneity as a cause of the basis for nonlinearity. Since excess unlabeled $E_2$17G was used to determine nonspecific binding and not a ligand different from the radioligand, nonspecific binding is probably defined correctly. The inhibition experiments identified ligands selective for the high affinity site, which argues against ternary complex formation and ligand-ligand interaction.

Additional approaches were taken in order to determine whether negative cooperativity was responsible for the curvilinear Scatchard plot. The dissociation rate of ^{3}H-$E_2$17G was determined by infinite dilution with buffer and by dilution with excess unlabeled $E_2$17G. These kinetic experiments revealed biphasic log dissociation plots, which can also be indicative of multiple sites. Boeynaems and Dumont (25) have described the dissociation kinetics for two classes of binding sites as follows:

$$[RL] = [R_1L]_0 \exp(-k_{-1}\, t) + [R_2L]_0 \exp(-k_{-2}\, t)$$

where $[RL]$ = total bound, $[R_1L]_0$ and $[R_2L]_0$ = amount bound to site 1 and site 2 at zero time respectively, and k_{-1} and k_{-2} are the kinetic dissociation constants of each site. Non-lin fit the data best to this biexponential rate equation, which also supports the hypothesis that $E_2$17G binds to two sites. Biphasic dissociation plots can also reflect ligand heterogeneity, ligand-induced site-site interactions, or a ligand-induced conformational change in the receptor (26). Since the dissociation rate constants were not influenced by the method of displacement

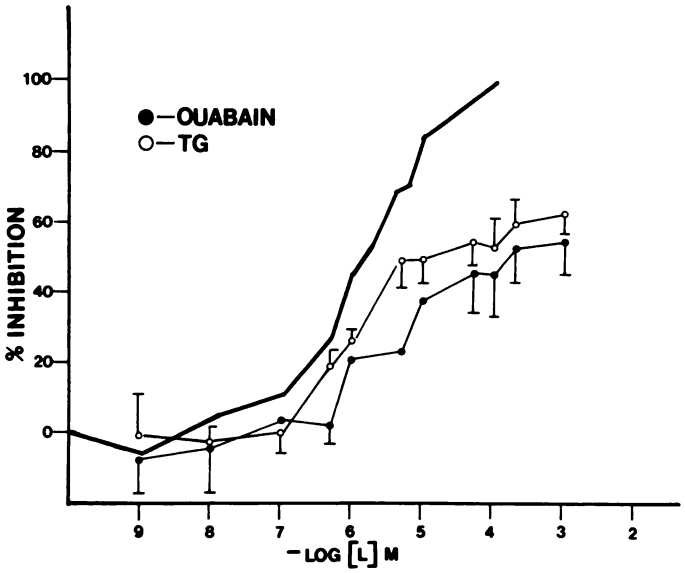

Fig. 8. Inhibition of ^{3}H-$E_2$17G binding by ouabain and testosterone glucuronide (*TG*). Unlabeled inhibitors (10^{-9}–10^{-3} M) were incubated in the presence of $5 \cdot 10^{-9}$ M ^{3}H-$E_2$17G and the binding experiment was performed as described under Experimental Procedures. See the legend for Figure 4.

of bound $E_2$17G, cooperative interactions are ruled out. Ligand-induced conformational changes in the receptor are also unlikely since this would yield a linear Scatchard plot (26). As discussed above, ligand heterogeneity can also be discounted.

In summary, the evidence that ^{3}H-$E_2$17G binds to two sites in liver plasma membranes is as follows. 1) The displacement of ^{3}H-$E_2$17G binding by increasing concentrations of unlabeled $E_2$17G is fit best by LIGAND to the two-site model. 2) The Scatchard transformation of the specific binding saturation curve is hyperbolic, concave upwards, and LIGAND fit these data best to a two-site model. 3) Dissociation rate experiments reveal biphasic log plots. 4) The bile acids, ouabain and testosterone glucuronide, selectively bind to site 1.

The binding sites were localized primarily in the plasma membrane and in the microsomal fractions of the liver. Inhibition data, as discussed later, strongly suggest that the binding sites in plasma membrane represent organic anion carriers. Carriers would be expected to be present only in those subcellular fractions involved in transport, a function not usually associated with the 100,000 × *g* "microsomal" fraction. Vesicular transport of organic anions such as the bile acids across the hepatocyte and into the canaliculus has been suggested by Jones *et al.* (27) and Boyer *et al.* (28). These studies reported that, after infusion of choleretic amounts of taurocholate or taurodehydrocholate, an increase in Golgi membranes and vesicles in the pericanalicular region of the hepatocyte was seen. Simion *et al.* (29) recently showed that taurocholate was transported by microsomes and that this transport was inhibited by BSP, an organic anion. Preliminary studies indicate that taurocholate competes with $E_2$17G for binding to microsomes (30). The microsomal binding sites could therefore be involved in the postulated vesicular transport of organic anions across the hepatocyte. Alternatively, $E_2$17G could be binding to enzymes involved in the metabolism of estradiol, i.e., cytochrome P-450 or UDP-glucuronyltranferase, or to serum proteins synthesized in the endoplasmic reticulum.

The substrate specificities demonstrated by the inhibition studies argue against either of these binding sites representing a site that mediates cholestasis. Since only the steroid D-ring glucuronides are cholestatic, whereas the A-ring glucuronides are choleretic, a site mediating cholestasis should be specific for the D-ring glucuronides. However, $E_2$3G bound to both sites as did BSP and DBSP, organic anions not associated with cholestatic activity.

The substrate specificities support the postulate that both binding sites represent organic anion carriers. The kinetics for uptake of ^{3}H-$E_2$17G in isolated hepatocytes over a wide concentration range (0.1–100 μM) have been examined as discussed in the accompanying paper (31). Two carriers were identified with the following kinetic parameters: Km_1 = 4.5 μM, $V\text{max}_1$ = 0.15 nmol/min/mg of protein, Km_2 = 149 μM, $V\text{max}_2$ = 0.64 nmol/min/mg of protein (31). Taurocholate and testosterone glucuronide were shown to inhibit the high affinity carrier selectively. BSP was a nonselective inhibitor; i.e., it inhibited $E_2$17G uptake by both carriers, whereas morphine glucuronide had no effect on ^{3}H-$E_2$17G uptake at either carrier. There is excellent concordance between the ability of this series of organic anions to inhibit binding of ^{3}H-$E_2$17G to either site and their ability to inhibit ^{3}H-$E_2$17G uptake at either carrier. Taken together, these data provide strong evidence in support of the hypothesis that the two binding sites identified in the present study represent organic anion carriers, with the high affinity site representing a carrier which is shared by the bile acids.

^{35}S-BSP (specific activity = 40–112 mCi/mmol) has been used previously to describe binding sites which might correspond to organic anion carriers. Wolkoff and Chung (32) reported a single binding site for ^{35}S-BSP in liver plasma membranes with $K_d = 3.7 \times 10^{-6}$ M and $B\text{max}$ = 6.3 nmol/mg of protein. Reichen *et al.* (33) reported three ^{35}S-BSP binding sites with K_d values of 1.8×10^{-8}, 5.9×10^{-6} and 6.7×10^{-4} M. The present studies are the first to use a high specific activity radioligand to characterize binding as well as to identify selective ligands to help elucidate the function of these organic anion binding sites.

The analyses of the binding characteristics of a large group of organic anions (see Table 4) have allowed us to hypothesize the substrate structural requirements for binding to ^{3}H-$E_2$17G binding sites and possibly for hepatic uptake as well. The binding characteristics to site 2 of the various structurally different compounds used in the inhibition studies suggest that an ionic bond, as well as accessory van der Waals bonds, is essential for binding of ^{3}H-$E_2$17G. The postulate that an ionic bond is required for the ^{3}H-$E_2$17G-binding site interaction is supported by the observation that all compounds that inhibit ^{3}H-$E_2$17G binding have a carboxyl, sulfate, or lactone ring group that is ionized at physiologic pH. The converse is not true, however, since not all anions were able to inhibit binding. Morphine glucuronide possesses the glucuronide carboxylic acid group but does not bind. However, morphine glucuronide also has a cationic group (N-17) which could provide sufficient repulsion to prevent its binding. Even though the binding energy involved in formation of an ionic bond is sufficient to account for the K_d at site 2 of 5 μM, glucuronic acid itself also did not inhibit binding. This indicates that accessory bonds must be essential for binding and may act to facilitate formation of the ionic bond. Such facilitation may occur by bringing the anionic group spatially closer to the cationic group on the binding site, or by inducing a tertiary or quarternary conformational change in the binding site which increases access of the anionic group to the cationic site.

In summary, we have shown that ^{3}H-$E_2$17G binds specifically to two independent sites in rat liver plasma membranes. Characterization of the substrate specificities of the binding sites and comparison with hepatocyte uptake data provide strong evidence supporting the hypothesis that site 2 represents an organic anion carrier that is not shared by the bile acids, whereas site 1 represents a separate, higher affinity transport system which is shared by the bile acids and other organic anions.

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