Biochemical Pharmacology, Vol. 40, No. 6, pp. 1219–1225, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

CHARACTERIZATION OF [^{3}H]ESTRADIOL-17β-(β-D-GLUCURONIDE) BINDING SITES IN BASOLATERAL AND CANALICULAR LIVER PLASMA MEMBRANES

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(*Received* 10 *November* 1989; *accepted* 16 *February* 1990)

Abstract—The specific binding of [^{3}H]estradiol-17β-(β-D-glucuronide) ([^{3}H]$E_2$17G) was examined in isolated basolateral (bLPM) and canalicular (cLPM) liver plasma membranes. Two distinct binding sites were identified in each membrane fraction by competition and saturation experiments. Binding parameters obtained from competition studies were: K_{d_1} = 26 nM, B_{max_1} = 0.26 pmol/mg protein; K_{d_2} = 2.6 μM, B_{max_2} = 27 pmol/mg protein for bLPM; and K_{d_1} = 81 nM, B_{max_1} = 0.61 pmol/mg protein; K_{d_2} = 6.7 μM, B_{max_2} = 79 pmol/mg protein for cLPM. Binding parameters obtained from saturation experiments were not significantly different. There was no Na^+ requirement for binding. Kinetic dissociation experiments showed that binding was reversible and revealed two components. The dissociation rate constants did not vary with the method of dilution of radioligand, i.e. by "infinite" volume, or excess unlabeled ligand, thus ruling out the possibility of cooperativity. The ability of a series of compounds to inhibit the binding of [^{3}H]$E_2$17G was also examined. In bLPM, taurocholate (TC), estrone sulfate (E_1SO_4) and bromosulfophthalein (BSP) were able to compete with both binding sites, whereas estriol-17β-(β-D-glucuronide) ($E_3$17G), estriol-16α-(β-D-glucuronide) ($E_3$16G), testosterone glucuronide (TG), estradiol-3-(β-D-glucuronide) ($E_2$3G), estriol-3-(β-D-glucuronide) ($E_3$3G), cholate and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) were able to inhibit binding to only the low-affinity site. In cLPM, only the cholestatic steroid D-ring glucuronides ($E_3$17G, $E_3$16G and TG) and TC were able to compete with both sites, whereas the non-cholestatic steroid A-ring glucuronides ($E_2$3G and $E_3$3G), BSP and DIDS competed for only the low-affinity site. Based on the observed substrate specificities, the low-affinity sites in bLPM and cLPM are postulated to represent multispecific organic anion carriers. The high-affinity site in cLPM may play a role in mediating steroid D-ring glucuronide-induced cholestasis.

The transport of organic anions from blood into bile involves at least two carrier-mediated processes: the hepatic uptake of compounds across the basolateral membrane and the hepatic secretion of compounds across the canalicular membrane. Recent studies suggest that these processes utilize different carriers. Studies with photolabile bile salt derivatives have indicated that two polypeptides with apparent molecular weights of 54,000 and 48,000 are involved in sinusoidal uptake of bile salts [1]. The latter polypeptide has been shown to be the Na^+-dependent bile acid carrier [2], whereas the 54 kD protein is thought to represent a multispecific anion carrier since it seems to be shared with other organic and inorganic anions [3]. A bile salt-binding polypeptide with an apparent molecular weight of 100,000 has been identified in canalicular but not basolateral rat liver plasma membranes [4] and has been shown to be the canalicular bile salt carrier [5].

Estradiol-17β-(β-D-glucuronide) ($E_2$17G) is an organic anion which is avidly taken up by the liver, is secreted in bile, and has been shown to induce cholestasis [6]. We have used [^{3}H]$E_2$17G as a model organic anion and presented evidence for two distinct [^{3}H]$E_2$17G binding sites in mixed liver plasma membranes [7]. The substrate specificity of these binding sites is consistent with their identification as organic anion carriers [8]. Recently, Meier *et al.* [9] developed a method for the preparation of both basolateral (bLPM) and canalicular (cLPM) liver plasma membranes from the same rat livers. This method has made possible the characterization of organic anion binding sites/carriers in each of these membrane fractions. The present studies, therefore, were designed to identify and characterize [^{3}H]$E_2$17G binding sites in bLPM and cLPM to determine if the properties and substrate specificities of these sites were similar or unique.

MATERIALS AND METHODS

Materials. [^{3}H]$E_2$17G ([6,7-^{3}H]estradiol; 47.3 Ci/mmol, >99.1% purity) was custom synthesized by New England Nuclear (Boston, MA). The nonlabeled steroid glucuronides, estriol-17β-(β-D-glucuronide) ($E_3$17G), estriol-16α-(β-D-glucuronide) ($E_3$16G), testosterone glucuronide (TG), estradiol-3-(β-D-glucuronide) ($E_2$3G), and estriol-3-(β-D-glucuronide) ($E_3$3G), the bile acids taurocholate (TC) and cholate, and sucrose (grade I) were purchased from the Sigma Chemical Co. (St. Louis, MO). Bromosulfophthalein (BSP) was obtained from Hynson, Westcott & Dunning, Inc. (Baltimore, MD). All other chemicals were of reagent grade.

Animals. Male Sprague–Dawley rats weighing 225 ± 25 g were purchased from Harlan Sprague–

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Dawley Inc. (Indianapolis, IN), and were allowed to acclimate in bedded cages for at least 5 days prior to use. Food (Purina Rat Chow) and water were available *ad lib*. The animal rooms were maintained on a 12-hr automatically timed light/dark cycle in a HEPA Filtered Laminar Flow Unit from Hazelton Research Products Inc. (Denver, PA). Rats were killed by guillotine between 8:00 and 9:00 a.m.

Preparation of rat liver plasma membranes. bLPM and cLPM were prepared by a discontinuous sucrose density-gradient centrifugation according to the method of Meier *et al.* [9]. The membranes used for binding were suspended in modified Hanks' buffer consisting of 137 mM NaCl, 5.2 mM KCl, 0.9 mM $MgSO_4$, 0.12 mM $CaCl_2$ and 10 mM Na_2HPO_4 adjusted to pH 7.5, and quick frozen in liquid nitrogen for no longer than 30 days. Protein was determined by the method of Lowry *et al.* [10].

Enzyme analyses. Na^+,K^+ and Mg^{2+}-ATPase activities (liver plasma membrane markers) were measured with an enzymatic assay according to Scharschmidt *et al.* [11] or an inorganic phosphorus detection method [12]. 5'-Mononucleotidase activity, a marker for canalicular liver plasma membranes, was determined by the method of Lesko *et al.* [13]. NADPH–cytochrome *c* reductase activity was used to measure microsomal contamination [14]. Mitochondrial contamination was determined by measurement of succinate-tetrazolium reductase activity according to the method of Pennington [15].

Binding assays. bLPM and cLPM preparations were removed from liquid nitrogen, quick thawed at 37°, and diluted with modified Hanks' buffer to give a protein concentration of 0.5 to 1.2 mg/mL. The standard binding assay was carried out at 4° in a cold room. All assays were performed in triplicate or quadruplicate.

Competition experiments. Modified Hanks' buffer (50 μL) containing various concentrations of unlabeled $E_2$17G or other competing ligands was added to a 400-μL polyethylene centrifuge tube followed by the addition of 50 μL of [^{3}H]$E_2$17G (2–3 nM, 47.3 Ci/mmol). The binding reaction was initiated by the addition of 0.05 to 0.12 mg protein in 100 μL buffer bringing the total volume to 200 μL. The tube was incubated for 2 min (4°), and then centrifuged for 2 min at 10,000 *g* in a Beckman benchtop Microfuge B (Beckman Instruments Inc., St. Louis, MO) to separate bound ligand from free ligand. Bound ligand was precipitated in seconds, and at no time was it exposed to ligand-free medium. The supernatant fluid was removed by vacuum 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 was added to a scintillation vial, dissolved in Safety-Solve (Research Products Internaional Corp., Mount Prospect, IL), and then shaken overnight at room temperature in a Big Vortexer (Glas-Col Apparatus Co., Terre Haute, IN). Radioactivity was counted by liquid scintillation spectrometry (Tri-Carb 4000 Series, Packard Instrument Co., Downers Grove, IL). Efficiencies were determined by using [^{3}H]toluene as an internal standard.

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 unlabeled $E_2$17G. Specific binding was 40% of total binding at the concentration of [^{3}H]$E_2$17G (2–3 nM) used in the competition experiments.

Saturation experiments. Membranes were incubated with 50 μL of various concentrations of [^{3}H]$E_2$17G (10^{-10} M–10^{-4} M) in the presence and absence of 100 μM unlabeled $E_2$17G. Binding assays were carried out as described above.

Characterization of the binding. The time course of the binding reaction was determined at 4° and 37°. [^{3}H]$E_2$17G (2–3 nM) was incubated with membranes, as described above, in the presence and absence of 100 μM unlabeled $E_2$17G, and binding was terminated at 0.25, 1, 2, 5, 30 and 60 min. Experiments designed to characterize the linearity of [^{3}H]$E_2$17G binding with respect to protein showed that specific binding was linear from 0.025 to 0.13 mg protein (0.125 to 0.625 mg protein/mL incubation medium). All assays were carried out in the range of linearity (0.05 to 0.12 mg protein).

The effect of pH on specific binding was determined by preparing [^{3}H]$E_2$17G (2–3 nM), 100 μM unlabeled $E_2$17G and membranes in buffer in the pH range of 5.5 to 9.5. The binding assays were then performed as described above. The binding experiments were also carried out in Na^+-free modified Hanks' buffer (where lithium replaced sodium) in order to determine whether Na^+ was required for binding.

Dissociation experiments. Two types of dissociation experiments were performed as described by De Meyts *et al.* [16], in order to determine whether negative cooperative interactions were involved between ligand and binding sites. The first experimental approach used infinite volume (100-fold) buffer dilution, whereas the second experimental approach used excess (100 μM) unlabeled $E_2$17G dilution to cause dissociation of the radioligand. In the case of negative cooperativity, the dissociation rate will increase in the presence of an excess of unlabeled $E_2$17G, since this increases the concentration of occupied receptors. [^{3}H]$E_2$17G (2.4 mL of 4×10^{-8} M) was incubated with the membrane suspension (2.4 mL of 2 mg protein/mL) to equilibrium (2 min) at 4°. Binding was terminated by centrifugation in a Sorvall centrifuge for 2 min at 10,000 *g*. The supernatant fluid was removed by aspiration, and the pellet was resuspended in 4.8 mL of ice-cold buffer. Under these conditions, a small portion of receptors is occupied and the free ligand concentration in the medium at this time is effectively zero. Aliquots (100 μL) were distributed to two sets of tubes, each in duplicate. One set contained 10 mL of buffer (for infinite volume dilution) and another set contained 5 mL of 100 μM $E_2$17G in buffer (for excess $E_2$17G dilution). At timed intervals, the entire contents of each tube were filtered through Millipore filters (0.45 μm). The filters containing the bound [^{3}H]$E_2$17G were assayed for radioactivity.

Data analyses. The binding parameters, K (affinity constant), B_{max} (receptor capacity), and N (nonspecific binding) for both saturation and competition experiments were determined by LIGAND, a nonlinear least squares iterative curve-fitting computer program by Munson and Rodbard [17]. Statistical

Table 1. Enzymatic characterization of basolateral (bLPM) and canalicular (cLPM) rat liver plasma membranes*

Enzyme assay	Homogenate	bLPM (RE)†	cLPM (RE)†
Na^+,K^+ATPase (μmol P_i/mg protein/hr)	0.65 ± 0.04	13.2 ± 1.4 (20.9 ± 2.4)	2.7 ± 1.0 (4.6 ± 1.8)
Mg^{2+}-ATPase (μmol P_i/mg protein/hr)	1.44 ± 0.03	20.7 ± 1.4 (14.5 ± 0.9)	68.5 ± 8.7 (45.3 ± 5.8)
5'-Mononucleotidase (μmol P_i/mg protein/hr)	4.0 ± 0.25	38.3 ± 2.0 (9.7 ± 1.1)	137 ± 12 (35.1 ± 5.6)
NADPH–cytochrome *c* reductase (μmol cyt *c* red/mg protein/hr)	2.4 ± 0.29	1.5 ± 0.2 (0.61 ± 0.04)	6.2 ± 0.74 (2.7 ± 0.4)
Succinate dehydrogenase (μmol formazan/mg protein/hr)	3.6 ± 0.14	5.8 ± 0.9 (1.6 ± 0.19)	0.74 ± 0.19 (0.21 ± 0.06)
Protein yield (mg/g liver)	198 ± 15	0.22 ± 0.03	0.07 ± 0.01

* Each value is the mean ± SE of 3–6 determinations.

† RE represents the relative enrichment of each marker enzyme activity in liver membranes as compared to homogenate.

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 dissociation rates were analyzed by the computer program KINETIC by McPherson which uses a weighted non-linear curve-fitting technique [18]. A mono-exponential model and a biexponential model were fit to the data, and the best fit was determined by using the F-test as described above. Student's *t*-test was used to determine whether binding parameters from compeition experiments and saturation experiments were significantly different.

RESULTS

The specific activities for various marker enzymes and their enrichment compared with liver homogenate are summarized in Table 1. cLPM contained a small amount of Na^+,K^+-ATPase activity, whereas bLPM were 20.9-fold enriched in this enzyme activity. Mg^{2+}-ATPase and 5'-mononucleotidase activities, canalicular markers, were 45.3- and 35.1-fold enriched in cLPM and 14.5- and 9.7-fold enriched in bLPM respectively. There was little mitochondrial (succinate dehydrogenase) or microsomal (NADPH–cytochrome *c* reductase) contamination. These data are comparable to the results reported by Meier *et al.* [9].

Characterization of [^{3}H]$E_2$17G binding sites showed similar results for bLPM and cLPM: (1) Binding reactions reached equilibrium within 1 min and were stable for at least 60 min at both 4° and 37°. There was no significant difference in specific binding at 4° and 37°. Therefore, the binding assays were performed at 4° to obviate interference by potential transport processes. (2) Binding studies at various pH values indicated that specific binding was decreased at pH values above 7.5 and increased (cLPM) or did not change (bLPM) at pH values less than 7.5. The non-specific binding was also increased by up to 33% at pH values below 7.5, presumably due to the increased concentration of the nonionic species. However, none of these changes were statistically significant, so that all subsequent binding studies were carried out at the physiologically relevant pH of 7.5. (3) Specific binding in Na^+-free modified Hanks' buffer was not significantly different from specific binding in modified Hanks' buffer, indicating that there is no Na^+ requirement for binding.

Saturation experiments were carried out by incubating increasing concentrations of [^{3}H]$E_2$17G from 10^{-10} M to 10^{-4} M with bLPM and cLPM in the presence and absence of 100 μM unlabeled $E_2$17G. The Scatchard plots of these saturation data gave upwardly concave curves, as shown in Fig. 1A and Fig. 1B for bLPM and cLPM respectively. The binding of 2 nM [^{3}H]$E_2$17G to bLPM and cLPM was also carried out in the presence of unlabeled $E_2$17G at concentrations ranging from 10^{-10} M to 10^{-4} M; results from these experiments are shown in Tables 3 and 4 (see below). LIGAND analysis of the data from competition experiments and saturation experiments showed that the two-site model fit these data best for both bLPM and cLPM ($P < 0.01$). The two methods gave very similar binding parameters that were not significantly different.

The results from dissociation rate experiments in cLPM are shown in Fig. 2. Comparable data were obtained for bLPM. Dilution of bound radioligand by either infinite volume or in the presence of an excess of unlabeled $E_2$17G displaced all specific binding, indicating that [^{3}H]$E_2$17G specific binding is fully reversible. Analyses of these data by KINETIC indicated that a biexponential rate model fit best

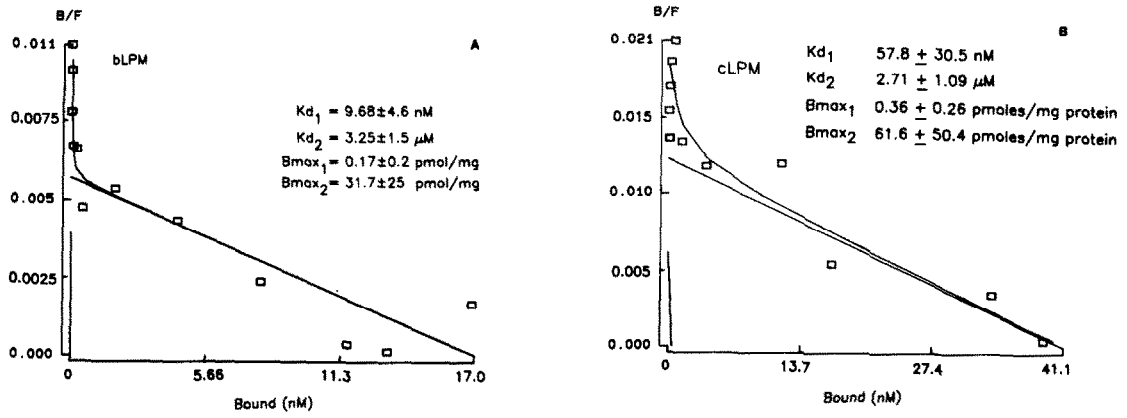

Fig. 1. Scatchard analysis of saturation experiments in bLPM (A) and cLPM (B). Data are represented graphically by specific bound/free (B/F) versus specific bound. Binding parameters (mean ± SE) were determined by individual LIGAND analysis of the data (from 3 to 4 experiments). The straight lines are those calculated by LIGAND with slopes = $1/K_{d_1}$ and $1/K_{d_2}$. The curved lines represent the best fit of the data to the two sites. Each point is the mean of 3–4 determinations, each performed in at least triplicate.

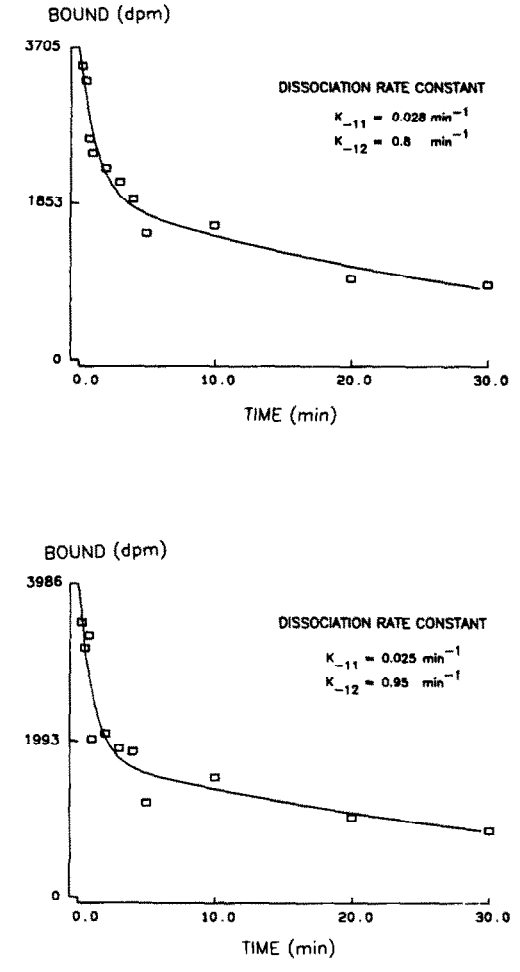

Fig. 2. Dissociation of [^{3}H]$E_2$17G binding in cLPM by infinite volume of buffer (upper panel) or excess unlabeled $E_2$17G (lower panel). Each point is the mean of a single experiment performed in duplicate.

Table 2. Dissociation rate constants of $E_2$17G in bLPM and cLPM

	bLPM (N = 2)		cLPM (N = 1)	
	Infinite volume	Excess $E_2$17G	Infinite volume	Excess $E_2$17G
High-affinity site	0.024	0.025	0.028	0.025
Low-affinity site	1.25	1.31	0.80	0.95

Dissociation rate constants are expressed in units of (min^{-1}). Each number is the mean of 1–2 experiments each performed in duplicate.

($P < 0.01$) and gave the dissociation rate constants as shown in Table 2. These data are consistent with identification of two binding sites. The rate constants do not differ between the types of dilutions used, indicating that no cooperative interactions are occurring at either site.

To determine the substrate specificity of [^{3}H]$E_2$17G binding sites, the inhibition of [^{3}H]$E_2$17G binding by a series of ligands with differing structural features was examined. The ligands examined included steroid glucuronides, bile acids (TC and cholate) and other organic anions such as estrone sulfate, BSP and the classic anion transport inhibitor, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS). Some of these ligands were tested in bLPM only due to the limited availability of cLPM. The results of these experiments are shown in Tables 3 and 4 for bLPM and cLPM respectively.

Three classes of ligands were obtained in bLPM: (1) a compound which did not inhibit [^{3}H]$E_2$17G binding (morphine glucuronide), (2) those compounds which bound only to the low-affinity site (TG, $E_3$17G, $E_3$16G, $E_3$3G, $E_2$3G, DIDS and cholate) and (3) those which bound to both sites (TC, estrone sulfate and BSP). The substrate specificities differed in some important regards in cLPM. Only TC and the cholestatic steroid glucuronides (TG,

Table 3. Binding parameters for $E_2$17G and other ligands in bLPM*

Ligand	Site(s)	K_{d_1} (nM)	B_{max_1} (pmol/mg protein)	K_{d_2} (μM)	B_{max_2} (pmol/mg protein)	P†
$E_2$17G	2	26 ± 11	0.26 ± 0.1	2.6 ± 0.8	27 ± 5	0.002‡
TG	1			6.8 ± 2.4	37 ± 7	NF§
$E_3$17G	1			23 ± 7	64 ± 19	0.7
$E_3$16G	1			13 ± 13	23 ± 3	0.6
$E_2$3G	1			303 ± 70	84 ± 20	0.6
$E_3$3G	1			85 ± 80	30 ± 16	0.14
BSP	2	23 ± 15	0.45 ± 0.4	10 ± 2	74 ± 40	0.002‡
TC	2	370 ± 320	0.33 ± 0.3	230 ± 76	34 ± 16	0.016‡
C‖	1			3.6 ± 1.8	36.3 ± 1	0.153
E_1SO_4	2	7.1 ± 5.5	0.18 ± 0.003	35 ± 31	33 ± 10	0.003‡
DIDS	1			8.8 ± 1.5	19 ± 6	NF
MG‖	0					

* Each value is the mean ± SE of 3–5 experiments each determined in triplicate or quadruplicate.
† P values were obtained from the F-test which compared the goodness of fit between the one-site model and the two-site model.
‡ Indicates a statistically better fit by the two-site model.
§ NF indicates that the data could not be fit to the two-site model.
‖ C = cholate; and MG = morphine glucuronide.

$E_3$17G and $E_3$16G) bound to both sites. The non-cholestatic steroid glucuronides ($E_3$3G and $E_2$3G), DIDS and BSP bound to the low-affinity site only. Morphine glucuronide, as in bLPM, did not bind to either site.

DISCUSSION

In the present study, we characterized two [^{3}H]$E_2$17G specific binding sites in both bLPM and cLPM which showed no Na^+ requirement for binding and were optimal at pH 7.5. Several lines of evidence indicate a multiplicity of [^{3}H]$E_2$17G binding sites in both bLPM and cLPM. Both the competition and saturation experiments gave essentially the same binding parameters, and fit significantly better to the two-site model when analyzed by LIGAND. Scatchard transformation of the specific binding for these data gave hyperbolic, concave upward curves, suggesting two better known possibilities: (1) the presence of multiple classes of binding sites that have different but fixed affinities or (2) the existence of site–site interactions of the type defined as negative cooperativity [16]. Two types of dissociation experiments, one by infinite dilution and a second by excess unlabeled $E_2$17G, were carried out to determine whether negative cooperativity was responsible for the curvilinear Scatchard plot. The results from these kinetic experiments gave dissociation rate constants that were not influenced by the method of displacement of bound $E_2$17G (Table 2), indicating that there were not any cooperative interactions. Furthermore, these data fit best to the biexponential model, thus providing the second line of evidence for multiple sites.

The third line of evidence for two binding sites came from the identification of ligands that selectively bound to only one of the [^{3}H]$E_2$17G binding sites. In bLPM, TC, estrone sulfate and BSP were able to compete with $E_2$17G binding to both sites, whereas the other steroid glucuronides, DIDS and cholate were able to inhibit binding to only one site. In cLPM, different substrate specificities were observed. TC and only the cholestatic steroid D-ring glucuronides were able to compete with both sites, whereas BSP, DIDS and the steroid A-ring glucuronides were able to compete for binding at only one site. The calculated binding parameters of those ligands binding to a single site were more similar to the binding parameters of [^{3}H]$E_2$17G at the low-affinity site, suggesting that these compounds were selective inhibitors at the low-affinity site.

In cLPM, the $E_2$17G high-affinity binding site was shared by taurocholate and all the steroid D-ring glucuronides studied (TG, $E_3$17G and $E_3$16G), but not by the steroid A-ring glucuronides ($E_2$3G or $E_3$3G), BSP or DIDS. This structure–activity relationship parallels that seen for cholestasis [19], suggesting that this binding site represents a site mediating the cholestasis induced by steroid D-ring glucuronides. It does not seem likely that this site represents the bile acid carrier, however, since the anion transporter DIDS did not bind to this site. Meier *et al.* [20] demonstrated that DIDS inhibits electrogenic carrier mediated efflux of taurocholate from cLPM. There may be accessory sites on the carrier to which the cholestatic glucuronides bind and block access of taurocholate to the "active" site. Transport studies in cLPM vesicles will be needed to further delineate the functional significance of this high-affinity site.

The high-affinity site in bLPM clearly differed from that in cLPM; it was shared by TC, BSP and estrone sulfate, but not the other steroid glucuronides or DIDS. This site also appears not to represent the "active" site of an anion carrier, since it is not shared by DIDS. The K_d of $E_2$17G for this site (26 nM) is also much lower than the K_m of 4.5 μM shown for transport of $E_2$17G into isolated hepatocytes [8].

The low-affinity binding sites in cLPM and bLPM appear to be very similar since both sites shared the

Table 4. Binding parameters for $E_2$17G and other ligands in cLPM*

Ligand	Site(s)	K_{d_1} (nM)	B_{max_1} (pmol/mg protein)	K_{d_2} (μM)	B_{max_2} (pmol/mg protein)	P†
$E_2$17G	2	81 ± 56	0.61 ± 0.2	6.7 ± 1.4	79 ± 19	0.001‡
TG	2	22 ± 20	0.58 ± 0.2	75 ± 33	328 ± 95	0.009‡
$E_3$17G	2	52 ± 39	0.49 ± 0.1	14 ± 5	249 ± 124	0.032‡
$E_3$16G	2	7.7 ± 7.1	0.54 ± 0.3	1.9 ± 0.5	220 ± 198	0.074‡
$E_2$3G	1			206 ± 55	204 ± 44	NF§
$E_3$3G	1			164 ± 140	269 ± 136	0.31
TC	2	140 ± 80	0.21 ± 0.05	99 ± 43	88 ± 18	0.005‡
BSP	1			17 ± 13	474 ± 116	NF
DIDS	1			1.6 ± 0.7	46 ± 5	
MG‖	0					

* Each value is the mean ± SE of 3–5 experiments each determined in triplicate or quadruplicate.

† P values were obtained from the F-test which compared the goodness of fit between the one-site model and the two-site model.

‡ Indicates a statistically better fit by the two-site model.

§ Indicates that the data could not be fit to the two-site model.

‖ MG = morphine glucuronide.

same ligands with approximately the same order of potency. In both cLMP and bLPM, TC and the steroid A-ring glucuronides had the lowest affinity for these sites, while DIDS bound with high affinity in both cLMP and bLPM. Based on the substrate specificities of this site and the magnitude of the binding parameters, we postulate that this site represents a multispecific organic anion carrier. The K_d for binding at this site in bLPM (2.6 μM) is similar in magnitude to the K_m for uptake of 4.5 μM in isolated hepatocytes [8]. Morphine glucuronide did not bind to this site, and we have shown that it does not inhibit $E_2$17G uptake into hepatocytes [8]; morphine glucuronide is also not taken up by the liver [21, 22]. Morphine is a highly charged base (pK_a 9.8), and this could provide sufficient repulsion to prevent its binding even though it does possess the glucuronic acid moiety. Morphine glucuronide is excreted in bile [22], however, suggesting that it should bind in the canalicular membranes. There are several suggestions in the literature that morphine glucuronide is secreted by a different carrier than other substrates. Thus, Fuhrman-Lane and Fujimoto [23, 24] demonstrated that *trans*-stilbene oxide treatment increases the formation of morphine 3-glucuronide but inhibits its excretion in bile; *trans*-stilbene oxide had no effect, however, on the biliary excretion of BSP. We have shown that treatment of rats with estradiol increases the transport maximum (nmol/min/liver) for dibromosulfophthalein (DBSP) but decreases this value for morphine glucuronide in the isolated perfused rat liver [25]. These data are consistent with the hypothesis that morphine glucuronide is transported by a carrier distinct from that for BSP and DBSP.

The present data obtained in male rats agree in some respects with our work in female rats characterizing the binding of [^{3}H]$E_2$17G to a mixed liver plasma membrane preparation which is enriched in both basolateral and canalicular plasma membranes [7]. In these studies, we also found two specific binding sites for $E_2$17G with no evidence for cooperative interactions. Based on the similarity in the substrate specificies for binding and for transport in isolated hepatocytes [8], we concluded that these two binding sites represented organic anion carriers with only the high-affinity site shared with the bile acids. The results in the present studies differ in the magnitude of the binding parameters and in the substrate specificities of the binding sites. Such differences are to be expected since the mixed liver plasma membranes represent a composite of cLPM and bLPM. The present studies in which we have resolved the two domains offer the opportunity to determine if there are important differences in the substrate specificities in bLPM versus cLPM. Characterization of transport of $E_2$17G in bLPM and cLPM vesicles will also permit definitive assignment of the functional significance of these binding sites.

Acknowledgements—The authors thank Mr. Steve Teo for his help with the preparations of liver plasma membranes and Mrs. Colleen Haney and Trina Johnson for their help in preparation of the manuscript. This work was supported by USPHS Grant HD13250.

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