Cloning and Epresentation of Human Liver
UDP-glucuronosyltransferase in COS-1 Cells

3,4-CATECHOL ESTROGENS AND ESTRIOL AS PRIMARY SUBSTRATES*

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The broad physiological roles of estrogen in sexual development (1), and the menstrual cycle and reproduction (2, 3) in humans have been well characterized. Estrogenic effects are altered but not abolished by oxidative metabolism, whereas these activities are blocked by glucuronidation and sulfation (4). In the liver, primarily, and brain, pituitary, and other peripheral tissues to a lesser extent (5–7), oxygenation of the steroids by specific cytochrome P-450-dependent monooxygenases converts the compounds to 2,3-catechol estrogens (8), 3,4-catechol estrogens (9, 10), or 16α-hydroxylated forms (8). Although catechol estrogens are rapidly cleared when injected in vivo (11), these derivatives, formed in estrogen-responsive tissue, bind the estrogen receptor with high affinity (12, 13) and are, thus, thought to be involved in the neuroendocrine mechanisms of estrogen action. The 2,3-catechol isomers are antiestrogenic (12, 14, 15), while the 4-hydroxyestrogens are quite potent estrogens with central effects on gonadotropin release (16–18), sexual behavior (19, 20), and puberty (21, 22) in rat. The 16α-hydroxy derivative, estriol, is thought to exert antiestrogenic effects during pregnancy (23). Because of these differences in biological activity, researchers consider that the catechol estrogens represent two classes of compounds with, most likely, different pathways of regulation and clearance.

A primary route of detoxification of steroids and other endogenous lipophiles is via glucuronidation catalyzed by the endoplasmic reticulum-bound UDP-glucuronosyltransferase enzyme system (24). An undetermined number of isozymes catalyzes the transfer of glucuronic acid from UDP-glucuronic acid to any of a large number of lipophilic acceptor substrates to generate water-soluble glucuronides which have enhanced rates of excretion from cells. Complementary DNAs isolated from rat (25–29), mouse (30), and human (31, 32) have been reported which encode transferases that glucuronidate primary estrogens (estrone (30), 17β-estradiol (18)), androgens (testosterone (28, 30), androsterone (27)), or exogenous compounds (p-nitrophenol (29, 30, 32), 4-methylumbelliferone (27, 28, 30)).

Based on glucuronidating activity following expression in COS-1 cells, we have uncovered a human UDP-glucuronosyltransferase which specifically glucuronidates 3,4-catechol estrogens and estriol. The form is encoded in a cDNA clone, UDPGT2, which was isolated from a human liver Agt11 cDNA library using a mouse transferase cDNA, UDPGT1, as probe. The UDPGT2-encoded protein represents a new and unique transferase activity specific for active estrogen metabolites.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05428.

**EXPERIMENTAL PROCEDURES**

*Materials—UDP-glucuronic acid and all aglycones tested for substrate activity were from Sigma, Aldrich, and Fluka. [14C]UDP-
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glucuronic acid and [3H]methionine were from Du Pont-New England Nuclear, and [3P]deoxyctydine triphosphate was from Amersham. Restriction enzymes and other reagents used in molecular biology techniques were from New England Biolabs, Pharmacia LKB Biotechnology Inc., IRI Biochemicals, Bethesda Research Laboratories, or Boehringer Mannheim. The pSVL vector and the oligo-labeling kit were from Stratagene (La Jolla, CA). The Erase-A-Base kit was from Promega (Madison, WI), tunicamycin was from Boehringer Mannheim, and Lipofectin™ was from Bethesda Research Laboratories. Dulbecco’s modified Eagle’s medium, fetal calf serum, and antibiotics were from GIBCO BRL. The Bluescript plasmids were from Stratagene (La Jolla, CA). The Erase-A-Base kit was from Promega (Madison, WI), tunicamycin was from Boehringer Mannheim, and Lipofectin™ was from Bethesda Research Laboratories. Dulbecco’s modified Eagle’s medium, fetal calf serum, and antibiotics were from GIBCO BRL.

Nucleotide Sequence Determination of UDPGT-2—The human liver clone, UDPGT-2, which encodes a UDP-glucuronosyltransferase, was isolated from a human liver Agt11 DNA library (supplied by Dr. Anul Jauswal, New York University, NY) based on its hybridization to the mouse transferase clone (34) (30 labeled by the oligo-primed technique (39) using a kit (Pharmacia), [α-3P]deoxyctydine triphosphate, and the supplier’s protocol. The full-length insert was isolated and ligated into EcoRI-digested pSMK13™ following the partial digestion of the Agt11 cDNA clone with EcoRI. The ligation mixture was used to transform XL-1 Blue cells. From a single colony, a subclone was grown overnight in a 250-ml culture, and the plasmid was purified according to Mierendorf and Pfeffer (34). The plasmid was linearized with progressively overlapping restriction endonucleases, constructed by utilizing the Erase-A-Base kit and the supplier’s protocol. Clones having appropriately spaced deletions were purified. Direct plasmid dideoxy sequencing (38) was carried out using the Sequel-DS/KF sequencing kit (IBI), according to the manufacturer’s protocol, but with the following modifications: (i) the composition of the 10X sequencing buffer was 83 mM Tris, pH 7.5, 83 mM MgCl2, 83 mM dithiothreitol, and 16.7 mM EDTA, and (ii) ddTTP reaction mixtures from Mierendorf and Pfeffer (34) were substituted. The concentration of dideoxynucleotide in the A, C, G, and M mix was slightly modified (18.8 μM ddATP, 16.5 μM ddCTP, and 16.5 μM ddGTP, respectively). Both strands of the cDNA were completely sequenced.

Northern Blot Analysis of Human Liver mRNA with 32P-Labeled UDPGT-2—Human liver mRNA was isolated from normal biopsy material according to the guanidinium isothiocyanate method of Chirgwin et al. (36) and affinity-purified by chromatography through oligo(dT)-cellulose (Collaborative Research Inc., Lexington, MA) with an intermediate heat step. Poly(A)+ RNA (4 μg) was electrophoresed in a 1% agarose gel containing 2.2 mM formaldehyde and transferred to Zetabind® membrane (AMF-CUNO, Meriden, CT) using the manufacturer’s instructions. The Northern blot was hybridized to the oligo-primed 32P-labeled EcoRI fragment from the most 3’-end of UDPGT-2 according to Church and Gilbert (37). Northern Blot of UDPGT-2 in COS-1 Cells—The cDNA from the pSKM13™ subclone was digested with SmaI and EcoRV and then ligated into the SmaI-digested pSVL expression vector. After appropriate subclones were characterized for the correct or reversed orientation of the cDNA with respect to the promoter element in pSVL, plasmid preparations were purified by cesium chloride banding (38). The expression unit is designated pUDPGT-2. Mock-transfected cells were carried out with the cDNA inserted in the reverse direction with respect to the promoter element of pSVL. COS-1 cells (80% confluent) were plated at 106/10 × 15 mm dish in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and grown overnight. The purified plasmid was then combined with the carrier, Lipofectin™, and added to culture dishes and incubated according to the manufacturer’s instructions. At the end of the 5-h transfection period which was blocked by adding excess methionine for 5 min. After washing the culture with phosphate-buffered saline, the cells were solubilized with 1 ml of 0.2 M potassium phosphate, pH 7.5, 0.15 M KCl, 2% sodium cholate, and 0.5% Zwittergent. Insoluble material was pelleted by centrifugation, and the solubilized cellular content was incubated with either goat control IgG or anti-mouse transference IgG (40) for 1 h on ice. An excess of protein A-Sepharose was added to the complexes and allowed to mix for at least 2 h in the cold. The protein A-Sepharose immunocomplexes were then washed with the solubilizing buffer until all unincorporated radioactivity was removed. The supernatants were from medium containing [3H]methionine (mock-transfected) and control IgG (data not shown). The immunocomplexes were detached and run on a single 7.5% sodium dodecyl sulfate-polyacrylamide gel (41) and processed for autoradiography by using a BAS 800 Imaging System Mark II (Ambis, San Diego, CA). The minimum detectable amount of glucuronide product formed, under the assay conditions employed, was 1.7 pmol (signal/background ratio is 2, with a background of approximately 50 cpm). For the determination of the Km value of the transference using 4-hydroxyestrone, 900 μM [14C] UDP-glucuronic acid (1.25 μC) was added to each reaction and incubated 1 h at 37 °C. It was predetermined that the generation of product was linear for at least 90 min. Plates were exposed to x-ray film for 21 days to obtain prints of the results.

RESULTS

Isolation of the Human Liver Clone, UDPGT-2—The cDNA clone hybridized at high stringency to the mouse liver clone, UDPGT-2, upon screening a human liver Agt11 DNA library. The cDNA was subcloned and sequenced using a series of progressively overlapping deleted clones as described under “Experimental Procedures”. Fig. 1 shows that the insert contains 1869 base pairs with a open reading frame of approximately 50 cpm. The determination of the Km value of the transference using 4-hydroxyestrone, 900 μM [14C] UDP-glucuronic acid (1.25 μC) was added to each reaction and incubated 1 h at 37 °C. It was predetermined that the generation of product was linear for at least 90 min. Plates were exposed to x-ray film for 21 days to obtain prints of the results.

The abbreviation used is: CHAPS, 3-[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate.
FIG. 1. Nucleotide sequence data for complete coding UDPGTh-2 with the deduced amino acid sequence data. The nucleotide sequence was determined according to the Sanger method (35) using double-stranded plasmid DNA and drawn using the PC-DNA draw program developed by Marvin Shapiro (Division of Computer Research and Technology, National Institutes of Health). The signal peptide sequence (residues 5–12) and the membrane-spanning region (residues 493–509) are underlined. The arrow (67, 68, and 315) represent consensus sequences for potential asparagine-linked glycosylation sites.

FIG. 2. Northern blot hybridization analysis. Hybridization of the 32P-labeled EcoRI fragment (from the most 3′-end of UDPGTh-2) to poly(A) RNA (4 µg) isolated from human liver biopsy was carried out as detailed under "Experimental Procedures." RNA molecular weight markers (Bethesda Research Laboratories) were hybridized to oligo-primed 32P-labeled λ phage DNA.

Allelic variant of HUG25, UDPGTh-1.2

Fig. 2 shows by Northern blot analysis that a 2200-base mRNA hybridizes to a 32P-labeled EcoRI fragment from the most 3′-end of UDPGTh-2. Under the conditions used (37), the full length clone cross-hybridizes to a 2300-base mRNA. Immunocomplexes of [35S]methionine-labeled UDP-glucuronosyltransferase in Transfected COS-1 Cells—In order to determine whether transferase is synthesized following transfection of COS-1 cells with the expression unit, pUDPGTh-2, [35S]methionine-labeled and solubilized cells were immunocomplexed with goat control IgG or goat anti-mouse transferase IgG (40), as described under "Experimental Procedures". Transfected cells incubated 48, 72, and 96 h and then immunocomplexed with anti-transferase IgG show a prominent protein with a molecular mass $\approx 52,000$ (Fig. 3, lanes 7, 9, and 11, respectively), with increasing incubation time these cells show increasing amounts of a second protein with a molecular mass $\approx 49,000$. The appearance of the 49,000-dalton protein with a parallel decrease in the 52,000-dalton protein between 72 and 96 h represents, most likely, the natural turnover of the transferase in COS-1 cells. Control studies were carried out by using preimmune IgG on both 72-h transfected cells (Fig. 3, lane 6) and on mocked transfected cells (Fig. 3, lane 3) and by exposing anti-transferase IgG to mocked transfected cells (Fig. 3, lane 4). There was a trace of a ~51,000-dalton radioactive immunocomplexed protein with immune serum from the mock-transfected cells (Fig. 3, lane 4). This result which suggests a low level of endogenous transferase protein could only be seen with exposures of greater than 24 h. In addition, there appeared to be a low level of the 52,000-dalton protein with preimmune serum using transfected cells (Fig. 3, lane 6). The lack of a corresponding band with preimmune serum in mock-transfected cells even after long exposure (Fig. 3, lane 3) suggests nonspecific trapping of the highly radioactive labeled transferase in transfected cells may be the basis. Further, we considered the possibility that the expressed protein is glycosylated via the potential asparagine-linked glycosylation sites in the deduced amino acid sequence. The

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2 J. K. Ritter and I. S. Owens, manuscript in preparation.
This report describes the cloning and expression of a novel steroidal UDP-glucuronosyltransferase, 3,4-catechol estrogen/estriol UDP-glucuronosyltransferase. The predicted amino acid sequence of this transferase isoform, specified by a 1590-base pair open reading frame in the cDNA clone, UDPGTt-2, shows strong identity with other cloned members of the steroid transferase family: UDPGTt-1 (66%), UDPGTt-2 (69%), UDPGTt-3 (66%), and UDPGTt-4 (65%). It is, therefore, not surprising that the encoded UDP-glucuronosyltransferase efficiently glucuronidates hydroxysteroids. Yet, this isoform clearly has a different acceptor substrate profile compared to the other cloned steroid transferases. The absence of detectable UDPGTt-2 encoded activity toward 3-hydroxy steroids, 17-hydroxy steroids, or either 1-naphthol, estrone, and testosterone which are metabolized by activity encoded in UDPGTt-4, UDPGTt-3/UDPGTt-2 (28), and UDPGTt-1 (30), respectively, indicates that this form is unique; it selectively glucuronidates two subsets of estrogen metabolites, 3,4-catechol isomers and estradiol.

Due to the difficulties associated with the purification of this labile and membrane-bound class of proteins (44, 45), very little in the literature exists regarding purification of human transferases. To our knowledge, there has been only one published purification study relevant to the activities encoded by UDPGTt-2. A human liver transferase was purified to apparent homogeneity by chromatofocusing and UDP-hexamethylenamine-Sepharose 4B affinity chromatography. It weakly catalyzed the glucuronidation of estradiol but not estrone, estradiol, testosterone, or androstenedione (46), whereas it was found to be 40-fold more active toward the planar phenols, p-nitrophenol and 4-methylumbelliferone, than estradiol. As shown in Table 1, we could detect no activity toward either of these substances (signal/background ratio = 1) in COS-1 cells expressing encoded UDPGTt-2 (as compared with estradiol activity: signal/background ratio > 180). These data strongly suggest a lack of identity between the purified and cloned (UDPGTt-2-mediated) estradiol transferase activities. However, it remains to be determined whether the mixed estradiol/phenol activity reflects a second estradiol transferase isoform or an impure transferase preparation containing the form encoded by UDPGTt-2.
FIG. 4. Substrate specificity of the UDPGT1-2-encoded UDP-glucuronosyltransferase expressed in COS-1 cells. A, pUDPGT1-2-transfected cells which were incubated for 48 h were assayed for the production of glucuronides (region of arrow) using 100 μM aglycone and 50 μM \(^{14}C\)UDP-glucuronic acid as described under "Experimental Procedures." Reactions were incubated for 16 h at 24 °C and then analyzed by TLC chromatography, scanning the plate as detailed under "Experimental Procedures" and exposed to x-ray film (20 days) for print. The aglycones and the radioactivity (in cpm) incorporated into glucuronides are recorded at the top of the chromatogram. B, substrate activities of aglycones expressed in picomoles of glucuronide formed/μg of protein. Counts shown in part A (arrow) were converted to picomoles of glucuronide formed based on counting efficiencies of the AMBIS system which ranged from 40% for 1,000 or less cpm to 27% for 10,000 cpm.

TABLE I

<table>
<thead>
<tr>
<th>Compounds tested that showed no detectable conversion to glucuronide</th>
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<tr>
<td>Estrogens/derivatives</td>
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<tr>
<td>Estrone</td>
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<td>17β-Estradiol</td>
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<td>16α-OH-17β-Estradiol</td>
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<tr>
<td>2-Methoxyestradiol</td>
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<td>16α-OH Estrone</td>
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<td>16β-OH Estrone</td>
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<td>Diethylstilbestrol</td>
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<td>Neuromodulators</td>
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<tr>
<td>Dopamine</td>
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<tr>
<td>5-OH Tryptamine</td>
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<tr>
<td>6-OH Melatonin</td>
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<tr>
<td>Vitamins</td>
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<tr>
<td>Ergocalciferol</td>
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<tr>
<td>Vitamin E</td>
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<td>Vitamin K*</td>
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<td>Type 1</td>
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<tr>
<td>Harmol</td>
</tr>
<tr>
<td>α-Naphthol*</td>
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<tr>
<td>p-Nitrophenol</td>
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<td>4-Aminophenol</td>
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<tr>
<td>4-Methylumbelliferoneb</td>
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<tr>
<td>3-OH Benzo(a)pyrene</td>
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* Assayed in the presence of NADH/NADPH.
* Low activity in mock-transfected cells.

By using a highly sensitive radiometric assay to establish the aglycone specificity of the transferase, some 60 potential endogenous and exogenous substrates (Table I) were tested. There are three categories of chemical structures among metabolites of the primary estrogens and other steroids which support glucuronidation by this enzyme. The highest activity is for the 3,4-catecholic structure in 4-hydroxyestrone. A similar structure in 4-hydroxyestradiol ranks fourth in substrate activity. Steroids with dihydroxy substitutions at C-16 and C-17, but with a third hydroxy group in the A-ring, are excellent substrates. Estriol and 2-hydroxyestradiol rank second and third in rate of conversion. In contrast, two similar structures in 2-methoxyestradiol and 6α-hydroxyestradiol are among the weakest substrates, suggesting that the 2-methoxy and the 6-hydroxy substituents, respectively, interfere with substrate activity. The failure of 16α-hydroxytestosterone (C-
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16,17-dihydroxy) to generate glucuronides supports the claim that an additional hydroxyl group is needed in the A-ring for this type of substrate. Finally, the trihydroxy-substituted compounds, 5α-androstane 3α, 11β, 17β-triol, its isomer 5β-androstane 3α, 11β, 17β-triol, and 6α-hydroxyestriol (3-, 6-, and 17-trihydroxy), are also weak substrates. The poor substrate activity of these compounds suggests that the positions of the three hydroxyl groups around the molecules are less optimal than those in estriol.

We emphasize here that the 2,3-catecholic structures in 2-hydroxyestrone and 2-hydroxyestradiol show only a trace of glucuronidation (Fig. 4). Hence, the 3,4-catecholic structure is a strict requirement. Estradiol (C-3, C-17 dihydroxy, aromatic A-ring) and 16α-hydroxyestrone (C-3, C-16 dihydroxy, aromatic A-ring), which lack either a catechol or a 16,17-diol substitution, are not glucuronidated. The position of glucuronide linkage cannot be established for either category of substrates because two or more hydroxyl groups are present on all active substrates.

The biochemical significance of the strict specificity for a 3,4-catechol estrogen UDP-glucuronosyltransferase could relate to the physiological requirement for differential regulation of the 2,3- and the 3,4-catechol estrogens. The 2,3-catechol estrogens are primarily inactive metabolites (12, 47) while the 3,4-catechol estrogens have potent effects on gonadotropin release (16, 18). The very different metabolic profiles for the two types of catechol estrogens would be difficult to reconcile with one detoxifying pathway. Indeed, marked differences in the urinary profiles of the 2,3- and 3,4-catechol estrogen metabolites have been observed. Whereas methyl derivatives are the dominant forms of 2,3-catechol estrogens excreted in the urine (8), glucuronide conjugates are predominant in the case of the 3,4-catechol derivatives (48).

It is significant that the catechol estrogens are also metabolized by the catechol-0-methyltransferase system and are, thus, potent inhibitors of catecholamine catabolism (49). The catechol estrogens \( K_m \approx 10-20 \mu M \) have a 10-fold higher affinity for catechol-0-methyltransferase than the catecholestrogens \( K_m \approx 250-300 \mu M \) (50, 51). The finding in this study that 4-hydroxyestrone has an apparent \( K_m \approx 13 \mu M \) for UDP-glucuronosyltransferase is evidence that this enzyme can compete effectively with catechol-0-methyltransferase in the production of 3,4-catechol estrogen metabolites. This result may explain the predominance of the glucuronidated forms over the methylated forms of the 3,4-catechol estrogens in the urine (48). Glucuronidation of the 3,4-catechol isomers without a great dependence on catechol-0-methyltransferase for methylation may provide more precise regulation of the concentration of the active 3,4-catechol isomers.

A study on the interaction of catechol estrogens and their methyl ether derivatives showed that, in contrast to the rapid clearance of catechol estrogens from the blood when administered alone, long lasting elevations were induced when administered together with their methyl ether derivatives (52). We examined the effect of 2-methoxyestradiol on the transferase described in this study. Preliminary studies show that this compound competitively inhibits the glucuronidation of 4-hydroxyestrone. These results suggest that complicated interactions between products of the catechol-O-methyltransferase reaction and glucuronidation may, in fact, occur.

Since the production of the 3,4-catechol isomer, 4-hydroxyestradiol, is as high in the brain and pituitary as liver (7), we are interested in whether this 3,4-catechol estrogen UDP-glucuronosyltransferase plays any role in these tissues as a regulator of this catechol estrogen, or whether the liver is the only site of glucuronidation. Prior studies reporting concentrations of catechol estrogens in nonhepatic tissues have not discriminated between the parent compounds and their glucuronides.

In COS-1 cells, the clone expresses a transferase with \( M_r \approx 52,000 \) which is a glycoprotein as shown by a substantial shift in molecular mass when produced in cells treated with tunicamycin. The presence of three consensus sequences for asparagine-linked glycosylation moieties in the deduced amino acid sequence is consistent with the tunicamycin effect on this protein. The extent of glycosylation (number of sites occupied) is difficult to assess. If one assumes that the glycosyl structures incorporated at the level of the endoplasmic reticulum are always the core oligosaccharide (53), we can conclude that at least two or more moieties are inserted to account for the shift of \( M_r \approx 3,000-4,000 \). It is reported for an expressed cDNA transferase which has one consensus sequence for glycosylation that the shift in relative molecular mass is \( \approx 2,000 \) (54).

In summary, we have presented the cDNA and deduced amino acid sequence of a human UDP-glucuronosyltransferase cDNA, UDPGT-2, which upon expression in COS-1 cells, was found to encode a form highly selective for two important metabolites of estrogen, 3,4-catechol derivatives and estriol. Putative roles of these metabolites in target cells of endocrine tissue, either as estrogens (15) or anti-estrogens (55), indicate that this enzyme may be of major importance in their regulation and/or detoxification. In addition, the fact that this transferase glucuronidates a class of metabolites also catabolized by the catechol-O-methyltransferase system is evidence for the interaction of the drug-metabolizing pathway and the neuroendocrine system. The physiological role of this interaction remains to be established.

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REFERENCES

\(^3\) J. K. Kitter, Y. Y. Sheen, and I. S. Owens, manuscript in preparation.

FIG. 5. Lineweaver-Burk plot of 4-hydroxyestrone concentration versus reaction velocity using pUDPGTh-a-bans-methyl ether derivatives showed that, in contrast to the rapid
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