FUTILE CYCLING OF ESTRONE SULFATE AND ESTRONE IN LIVER

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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**ABSTRACT**

Futile cycling of estrone sulfate (E₁S) and estrone (E₁) is a pharmacologically important biocycle that regulates endogenous estrogens. In order to understand the influence of the futile cycling on E₁S and E₁ hepatic clearances, vascular and tissue binding, zonal transport and metabolism, and excretion were investigated *in-vitro* and the variables were integrated stepwise to intact hepatocytes and then to perfused rat liver preparations. *In-vitro* studies identified that E₁S and E₁ were highly bound to bovine serum albumin and tissues. In addition, E₁ was bound and metabolized by erythrocytes. In zonal hepatocytes, acinar homogeneity was found for the transport of E₁S and E₁ and the desulfation of E₁S; these processes occur more rapidly than the sulfation of E₁, mediated predominantly by estrogen sulfotransferase in the cytosol of the perivenous over the periportal hepatocytes. When E₁S was incubated with intact zonal hepatocytes, higher concentrations of E₁S and E₁ were observed in periportal than in perivenous hepatocytes, and saturation was evident at the higher concentrations. When the *in-vitro* and hepatocyte data were fitted to a cellular model, the fitted results revealed that E₁ sulfation was indeed the slowest step in futile cycling. The greater metabolism of E₁ in PV hepatocytes, sulfation, glucuronidation, and hydroxylation led to lower E₁ and E₁S levels in PV hepatocytes. Moreover, the nonlinear uptake, tissue binding, and vesicular accumulation of E₁S resulted in different elimination half-lives for E₁S and E₁. Hence, hepatic clearances of E₁S and E₁ were investigated in the recirculating liver preparation with simultaneous delivery of tracer [²H]E₁S and [¹⁴C]E₁. The hepatic clearances of [²H]E₁S and [¹⁴C]E₁ were high despite tight vascular binding. Low concentrations of [²H]E₁ were
detected following the tracer $[^3H]E_tS$ dose because $[^3H]E_t$ was highly partitioned into the microsomes. Thus, a distributed-in-space model that embodied vascular and tissue binding, transport, subcompartmentalization of the cytosol and endoplasmic reticulum, and zonal metabolism was used to interpret the perfusion results. The model described the perfusion data well. However, in the absence of drug partitioning into the endoplasmic reticulum, parallel elimination profiles for $E_t$ and $E_tS$, characteristic of compounds undergoing futile cycling, were observed in the simulation study.
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ABBREVIATIONS

ABC       ATP-binding cassette
APS       Adenosine-5’-phosphosulfate
ARS       Arylsulfatase
ASTIV     Rat liver phenol (minoxidil) sulfotransferase
ATP       Adenosine triphosphate
BSA       Bovine serum albumin
BSEP      Bile salt export pump
BSP       Bromosulphophthalein
CNT       Concentrative nucleoside transporter
CP        Chondrodysplasia punctata
DHEAS     Dehydroepiandrosterone sulfate
E1        Estrone
E1G       Estrone glucuronide
E1S       Estrone sulfate
E2        Estradiol
ENT       Equilibrative nucleoside transporter
EST       Rat liver estrogen sulfotransferase
GLUT      Glucose transporter
GSH       Glutathione
HPLC      High performance liquid chromatography
IDS       Iduronate-2-sulfate sulfatase
LPP       Periportal lysate
LPV       Perivenous lysate
MCT       Monocarboxylate transporter
MDR       Multidrug resistance protein
MLD       Metachromatic leukodystrophy
MPP       1-Methyl-4-phenylpyridinium
MPS       Mucopolysaccharidosis
MRP       Multidrug resistance associated-protein
MSD       Multiple sulfatase deficiency
MTX       Methotrexate
NMN       N1-methylnicotinamide
NTCP      Na+-taurocholate cotransporter
Oat       Organic anion transporter (animal)
OAT       Organic anion transporter (human)
Oatp      Organic anion transporting polypeptide (animal)
OATP      Organic anion transporting polypeptide (human)
Oct       Organic cation transporter (animal)
OCT       Organic cation transporter (human)
PAH       P-aminohippurate
PAP       3'-phosphoadenosine-5'-phosphate
PAPS      3'-Phosphoadenosine-5'-phosphosulfate
Pgp       P-glycoprotein
PP        Periportal
<table>
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<tr>
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<tbody>
<tr>
<td>PV</td>
<td>Perivenous</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cells or erythrocytes</td>
</tr>
<tr>
<td>ROH</td>
<td>Hydroxyl-substrate R</td>
</tr>
<tr>
<td>ROS</td>
<td>Sulfoconjugate of substrate R</td>
</tr>
<tr>
<td>sPgp</td>
<td>Bile salt efflux protein</td>
</tr>
<tr>
<td>ST</td>
<td>Sulfotransferase</td>
</tr>
<tr>
<td>STa</td>
<td>Rat liver hydroxysteroid (bile acids) sulfotransferase</td>
</tr>
<tr>
<td>STS</td>
<td>Steroid sulfatase</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium</td>
</tr>
<tr>
<td>UDPGA</td>
<td>Uridine-5′-diphosphoglucuronic acid</td>
</tr>
<tr>
<td>XLI</td>
<td>X-linked ichthyosis</td>
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**TERMINOLOGY**

- $CL_{E_i}$: Excretion of $E_i$ to intracellular vesicle
- $CL_{E_i \text{ uptake}}$: Uptake clearance for $E_i$
- $CL_{E_i \rightarrow E_1}$: Intrinsic clearance for $E_i$ sulfation
- $CL_{E_i \rightarrow E_1 \rightarrow M}$: Intrinsic clearance for sulfation of $E_i$ (formation of all metabolites except $E_i S$)
- $CL_{E_i \rightarrow M}$: Intrinsic clearance for glucuronidation, hydroxylation, and oxidation of $E_i$
- $CL_{E_i \text{ ex}}$: Intrinsic clearance for excretion of $E_i S$ into cellular vesicle
- $CL_{E_i \text{ diff. rbc}}$: Hematocrit normalized bidirectional RBC transmembrane constant for $E_i$
- $CL_{E_i \text{ m}}$: Sinusoidal bidirectional transmembrane clearance for $E_i$
- $CL_{E_i \text{ S}}$: Sinusoidal bidirectional transmembrane clearance for $E_i S$
- $CL_{E_i \text{ G}}$: Sinusoidal bidirectional transmembrane clearance for $E_i G$
- $CL_{E_i \text{ in}}$: Endoplasmic reticulum influx clearance for $E_i$
- $CL_{E_i \text{ eff}}$: Endoplasmic reticulum efflux clearance for $E_i$
- $CL_{E_i \text{ bie}}$: Endoplasmic reticulum bidirectional transmembrane clearance for $E_i S$
- $CL_{E_i \text{ bile}}$: Endoplasmic reticulum bidirectional transmembrane clearance for $E_i G$
- $CL_{E_i \text{ rbc}}$: Biliary intrinsic clearance for $E_i S$
- $CL_{E_i \text{ G}}$: Biliary intrinsic clearance for $E_i G$
- $CL_{E_i \text{ M}}$: RBC metabolic clearance of $E_i$
- $CL_{E_i \rightarrow E_1 S}$: Sulfation intrinsic clearance of $E_i$
- $CL_{E_i \rightarrow E_1 G}$: Glucuronidation intrinsic clearance of $E_i$
- $CL_{E_i \rightarrow M}$: Other intrinsic clearance of $E_i$ (formation of all metabolites except $E_i S$ and $E_i G$)
- $CL_{E_i S \rightarrow E_1}$: Desulfation intrinsic clearance of $E_i S$
- $CL_{E_i S \rightarrow M}$: Other intrinsic clearance of $E_i S$ (formation of all metabolites except $E_i S$ and $E_i G$)
- $f_{\text{blood}\ E_i}$: Unbound fraction of $E_i$ in blood
- $f_{\text{blood} E_i S}$: Unbound fraction of $E_i S$ in blood
- $f_c E_i$: Unbound fraction of intracellular $E_i$
- $f_{\text{cyt} E_i}$: Cytosolic unbound fraction of $E_i$
- $f_{\text{cyt} E_i S}$: Cytosolic unbound fraction of $E_i S$
- $f_{\text{ec} E_i}$: Unbound fraction of extracellular $E_i$
- $f_p E_i$: Unbound fraction of $E_i$ in plasma containing 4% BSA
- $f_{p E_i}$: Unbound fraction of $E_i$ in plasma containing 4% BSA
- $f_{\text{rbc} E_i}$: Unbound fraction of $E_i$ in RBC
- $f_{\text{rbc} E_i}$: Unbound fraction of $E_i$ in RBC
- $k_{E_i S}$: Association constant for $E_i S$
- $k_{E_i}$: Dissociation constant for $E_i$
- $K_{E_i S}$: Michaelis-Menten constant for $E_i S$ uptake
- $K_{E_i S \rightarrow E_1}$: Michaelis-Menten constant for desulfation of $E_i S$
- $K_{E_i \rightarrow E_1}$: Michaelis-Menten constant for sulfation of $E_i$
- $K_{E_i \rightarrow M}$: Michaelis-Menten constant for metabolism of $E_i$ (for glucuronidation, hydroxylation and oxidation of $E_i$ excepting sulfation of $E_i$)
- $n E_i S$: Number of binding sites for $E_i S$
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<td>$P_{diff}^{E_1S}$</td>
<td>Bidirectional diffusion constant for $E_1S$</td>
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<tr>
<td>$P_{diff}^{E_1}$</td>
<td>Bidirectional diffusion constant for $E_1$</td>
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<tr>
<td>$Q_{bile}$</td>
<td>Bile flow rate</td>
</tr>
<tr>
<td>$Q_{blood}$</td>
<td>Perfusate flow rate</td>
</tr>
<tr>
<td>$V_{bile}$</td>
<td>Volume of biliary compartment</td>
</tr>
<tr>
<td>$V_c$</td>
<td>Cellular volume</td>
</tr>
<tr>
<td>$V_{c_3y}$</td>
<td>Cytosol volume</td>
</tr>
<tr>
<td>$V_{ee}$</td>
<td>Extracellular volume</td>
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<td>$V_{er}$</td>
<td>Endoplasmic reticulum volume</td>
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<td>$v_{E_1S\to E_1S_{in}}^{max}$</td>
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<td>$V_r$</td>
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CHAPTER 1

INTRODUCTION
1.1 The Liver

1.1.1 Anatomy

The liver is one of the largest internal organs in the body and its blood supply is approximately 25% of the cardiac output in humans (Bradley, 1949). The blood supply to the liver consists of the portal vein (75%), formed by the convergence of the splenic and superior mesenteric veins, and the hepatic artery (25%), that arises from the aorta. Within the liver, distributing branches of the portal vein and hepatic artery continue in parallel, and eventually the terminal portal venule and the arterial capillary converge at the sinusoid. The sinusoids empty into the terminal hepatic venules and eventually drain into the inferior vena cava. The hepatic lymphatics drain principally into the lymph nodes of the porta hepatis. The hepatic nerve plexus is innervated from the vagus and phrenic nerves (Rappaport et al., 1954).

The sinusoids, the principal vessels involved in transvascular exchange between the blood and parenchymal cells or hepatocytes, are capillaries lined by highly fenestrated endothelial cells, Ito cells, and Kupffer cells. The endothelial fenestrae are dynamic openings of 100 to 150 nm in diameter that allow free exchange of plasma and proteins between the blood and the space of Disse, the extracellular space between the endothelial cells and hepatocytes. The sinusoids are lined by a sheet of approximately twenty to thirty hepatocytes that form the acinus, the functional unit of the liver. Hepatocytes can be roughly compartmentalized into zones that describe their position along the cell plate in the acinus. Zone one, the most highly oxygenated zone, comprises those hepatocytes close to the portal and the arterial blood supply, whereas zone three, which is oxygen poor, encompasses those hepatocytes adjacent to the hepatic venules. The intermediate area is referred to as zone two (Fig 1-1; Rappaport et al., 1954).

The hepatocytes represent about 60% of the total cell population of the liver, but account for
80% of the volume of the liver (Moulé and Chauveau, 1963). The diameter of the hepatocyte is around 30 μm, and the liver cell has three specialized membrane surfaces: sinusoidal, lateral, and canalicular. The sinusoidal membrane surface faces the sinusoid and allows an exchange of chemicals between plasma and hepatocytes. The lateral membrane surfaces are the intercellular surfaces and contain gap junctions. The canalicular membrane surfaces of adjacent hepatocytes form the bile canaliculus, which empty into the hepatic bile duct. The tight junction complex between adjacent hepatocytes prevents the leakage of bile from the canaliculus. Apart from the hepatocytes, about 8, 12, and 16% of the total cell population of the liver are the Ito cells, Kupffer cells, and endothelial cells, respectively (Blouin, 1982).

**Figure 1-1. Liver Acinus**

![Liver Acinus Diagram](Taken from Oinonen and Lindros (1998).)
1.1.2 Importance as drug removal and first-pass organ

The liver is exposed to many exogenous substances from diet and orally administered drugs via the gastrointestinal tract. By virtue of its position in the circulation, the liver is vulnerable to damage from toxic xenobiotics, but liver damage is ameliorated by its high capacity to metabolize xenobiotics and endogenous compounds. The biotransformation of drugs involves the conversion of hydrophobic molecules into hydrophilic metabolites via a broad range of biochemical reactions, e.g. oxidation, reduction, hydrolysis, hydration, and conjugation; the resultant metabolites can be eliminated more easily through biliary and urinary excretion. The purpose of drug biotransformation is to reduce the exposure of the body to the drugs and hence decrease their potential toxicity.

1.2 Factors Governing Hepatic Drug Clearance

In order to understand hepatic drug clearance, it is necessary to consider the physiological determinants for drug absorption, distribution, metabolism, and excretion. For a drug that is already in the circulation, blood flow, vascular protein binding, and membrane barriers are factors that govern the uptake of the drug into the liver. Subsequently, tissue binding, metabolic enzymes, and excretion transporters will influence the fate of the drug in the liver.

1.2.1 Hepatic blood flow

Blood flow can affect the hepatic drug clearance, especially for drugs with efficient cellular elimination. Rowland et al. (1973) used the “well-stirred” hepatic clearance model, rapidly mixed liver model, to derive the relationship among hepatic clearance ($CL$), blood flow ($Q$), and intrinsic clearance ($CL_{int}$) as shown in Eq. 1
\[ CL = \frac{Q \cdot CL_{\text{int}}}{Q + CL_{\text{int}}} \]  

Eq. 1

For a drug with high intrinsic clearance \((CL_{\text{int}} \gg Q)\), the hepatic clearance approaches the hepatic blood flow rate because the rate of drug removal is limited by the rate of delivery from blood. On the other hand, a poorly cleared drug \((CL_{\text{int}} \ll Q)\) is characterized by a hepatic clearance almost equal to the intrinsic clearance. The hepatic clearance is relatively insensitive to changes in \(Q\) and is limited by the activity of the metabolic enzymes.

1.2.2 Vascular and tissue binding

Drug binding to vascular components, albumin and red blood cells, and cellular tissues is expected to reduce hepatic drug clearance. Erythrocyte binding and metabolism of drug is often neglected in pharmacokinetics. According to classical theory, the liver can only take up unbound drug. Thus, the uptake of a bound drug is highly dependent on the dissociation rate constant of the drug-albumin complex (Gillette, 1973b) and the release rate constant of red blood cells (Goresky et al., 1975). Although the theory still holds true for most drugs, the transport of certain drugs may be rapid despite being highly bound. This has been postulated to occur with facilitated dissociation of the drug-albumin complex by a specific albumin receptor on the cell surface or a non-specific interaction between drug-albumin complex and cell membrane, as described in the albumin-receptor theory (Weisiger, 1985). For a low extraction ratio drug, the hepatic drug clearance is heavily dependent on the unbound fraction of the drug. On the other hand, the hepatic drug clearance may or may not depend on the unbound fraction for a high extraction ratio drug.

Tissue binding can affect both the metabolism and efflux of a drug in hepatocytes by means of decreasing the cellular concentrations of the unbound drug, and consequently less unbound drug
is available for the metabolic enzymes and efflux transporters (Gillette, 1973a; Meijer et al., 1977). Ligandin, an intracellular protein also known as glutathione-S transferase B, has been identified to be responsible for binding many organic anions (Inoue et al., 1983). In addition, fatty acid-binding protein is found to bind both organic anions and uncharged sex steroids (Ockner et al., 1972).

1.2.3 Membrane barriers (influx and efflux)

The sinusoidal membrane is a barrier that hinders drugs entry into liver cells and affects hepatic drug clearance (Gillette and Pang, 1977) by means of limiting the drug supply to the metabolic enzymes. Briefly, the cell membrane is a phospholipid bilayer interspersed with proteins, with carbohydrates and cholesterol as minor constituents. The ability of a drug to pass through the membrane depends on its physiochemical properties such as molecular weight, shape, polarity, and lipophilicity. Cell entry processes are categorized as passive diffusion, facilitated diffusion, active transport, and receptor-mediated endocytosis. Passive diffusion is driven by a drug concentration gradient between plasma and the cells. Uncharged xenobiotics of molecular weight less than 100 Dalton are capable of passing through the membrane pores (<45 Å) and larger uncharged lipophilic molecules are normally capable of diffusing through the membrane. Facilitated diffusion involves a substrate-specific membrane protein that is both saturable and inhibitable; drug movement occurs down a concentration gradient and is energy-independent. On the other hand, active transport, an energy-dependent transport process involving a specific carrier that is saturable, inhibitable, and able to transport a specific drug against a concentration gradient. Finally, endocytosis, which involves the invagination of the membrane to entrap the drug, requires energy and may be induced by the chemicals present in the plasma. More transport carriers have recently been discovered in the liver, due to realization that transport carriers may influence hepatic drug clearance by affecting drug
distribution in the liver. Many influx- and efflux-transporters have been discovered on the sinusoidal and lateral membranes. Some of the sinusoidal influx-proteins for the transport of nucleosides, glucose, organic anions, and organic cations found to date are summarized in Table 1-1. In addition, a few multidrug resistance associated-protein (MRP) efflux-transporters present on the lateral and sinusoidal membranes are summarized in Table 1-2.

1.2.4 Metabolic enzymes and cosubstrates

The metabolism of drug to its metabolites plays an important role in the hepatic drug clearance. The biotransformation of drugs is normally divided into two phases as shown in Table 1-3. Phase I is the alteration of a molecule via the addition of a functional group that can be further conjugated in phase II (Williams, 1959). The majority of phase I processes is catalyzed by the mixed-function oxidase system which consists of the members of cytochrome P450 superfamily and NADPH-cytochrome P450 reductase. At present, there are 14 cytochrome P450 families which consist of 26 mammalian subfamilies (Nelson et al., 1996). Phase II reactions involve the addition of a specific cofactor to form glucuronide, glutathione, sulfate, amino acid, methyl, and acetyl conjugates. 3'-Phosphoadenosine-5'-phosphosulfate (PAPS), uridine-5'-diphosphoglucuronic acid (UDPGA), glutathione (GSH), and acetyl-coenzyme-A are essential cosubstrates for the sulfoconjugation, glucuronidation, glutathione, and acetyl conjugation processes, respectively.
Table 1-1. Summary of influx-transporters in the liver

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Homology in amino acid sequence</th>
<th>Substrate</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNT1&lt;sub&gt;h&lt;/sub&gt;</td>
<td>72% of CNT1</td>
<td>Pyrimidine nucleosides</td>
<td>Ritzel et al., 1997</td>
</tr>
<tr>
<td>CNT2&lt;sub&gt;h&lt;/sub&gt;</td>
<td>64% of Cnt1</td>
<td>Purine nucleosides</td>
<td>Wang et al., 1997</td>
</tr>
<tr>
<td>Cnt1&lt;sub&gt;r&lt;/sub&gt;</td>
<td>60% of Cnt1</td>
<td>Pyrimidine nucleosides</td>
<td>Huang et al., 1994</td>
</tr>
<tr>
<td>Cnt2&lt;sub&gt;r&lt;/sub&gt;</td>
<td>64% of Cnt1</td>
<td>Purine nucleosides</td>
<td>Che et al., 1995</td>
</tr>
<tr>
<td>ENT1&lt;sub&gt;h&lt;/sub&gt;</td>
<td>49% of ENT1</td>
<td>Purine and pyrimidine nucleosides</td>
<td>Griffiths et al., 1997a</td>
</tr>
<tr>
<td>GLUT1&lt;sub&gt;h&lt;/sub&gt;</td>
<td></td>
<td>Glucose</td>
<td>Kasahara and Hinkle, 1977</td>
</tr>
<tr>
<td>GLUT2&lt;sub&gt;h&lt;/sub&gt;</td>
<td>56% of GLUT1</td>
<td>Glucose</td>
<td>Thorens et al., 1988</td>
</tr>
<tr>
<td>GLUT3&lt;sub&gt;h&lt;/sub&gt;</td>
<td></td>
<td>Glucose</td>
<td>Kayano et al., 1988</td>
</tr>
<tr>
<td>GLUT7&lt;sub&gt;h&lt;/sub&gt;</td>
<td>68% of GLUT2</td>
<td>Glucose</td>
<td>Waddell et al., 1992</td>
</tr>
<tr>
<td>GLUT9&lt;sub&gt;h&lt;/sub&gt;</td>
<td>38% of GLUT1</td>
<td>Glucose</td>
<td>Phay et al., 2000</td>
</tr>
<tr>
<td>LST1&lt;sub&gt;h&lt;/sub&gt;</td>
<td></td>
<td>conjugated steroids, eicosanoids and thyroid hormones</td>
<td>Abe et al., 1999</td>
</tr>
<tr>
<td>Lst1&lt;sub&gt;r&lt;/sub&gt;</td>
<td>60% of LST1</td>
<td>prostaglandin and bile salts</td>
<td>Kakyo et al., 1999</td>
</tr>
<tr>
<td>MCT1&lt;sub&gt;ht&lt;/sub&gt;</td>
<td>60% of MCT1</td>
<td>lactate, pyruvate, and benzoate</td>
<td>Garcia et al., 1995</td>
</tr>
<tr>
<td>MCT2&lt;sub&gt;ht&lt;/sub&gt;</td>
<td></td>
<td>lactate and pyruvate</td>
<td>Garcia et al., 1994</td>
</tr>
<tr>
<td>NTCP&lt;sub&gt;h&lt;/sub&gt;</td>
<td></td>
<td>taurocholate, bile salts, bumetanide, and BSP</td>
<td>Hagenbuch and Meier, 1994</td>
</tr>
<tr>
<td>Ntcp&lt;sub&gt;r&lt;/sub&gt;</td>
<td>70% of NTCP</td>
<td>bile salts, E&lt;sub&gt;1&lt;/sub&gt;S, and BSP</td>
<td>Hagenbuch et al., 1991</td>
</tr>
<tr>
<td>Oat2&lt;sub&gt;r&lt;/sub&gt;</td>
<td></td>
<td>salicylate, acetylsalicylate, PGE&lt;sub&gt;2&lt;/sub&gt;, dicarboxylates and PAH</td>
<td>Sekine et al., 1998</td>
</tr>
<tr>
<td>Oat3&lt;sub&gt;r&lt;/sub&gt;</td>
<td></td>
<td>PAH, E&lt;sub&gt;1&lt;/sub&gt;S, cimetidine, and ochratoxin A</td>
<td>Kusuhara et al., 1999</td>
</tr>
<tr>
<td>OATP&lt;sub&gt;h&lt;/sub&gt;</td>
<td></td>
<td>organic anions and organic cations</td>
<td>Kullack-Ublick et al., 1995</td>
</tr>
<tr>
<td>Oatp1&lt;sub&gt;r&lt;/sub&gt;</td>
<td></td>
<td>conjugated steroids, eicosanoids, and lipophilic organic anions</td>
<td>Jacquemin et al., 1994</td>
</tr>
<tr>
<td>Oatp2&lt;sub&gt;r&lt;/sub&gt;</td>
<td>77% of Oatp1</td>
<td>bile salts, conjugated steroids, digoxin, and thyroid hormones</td>
<td>Noé et al., 1997</td>
</tr>
<tr>
<td>Oatp3&lt;sub&gt;r&lt;/sub&gt;</td>
<td></td>
<td>bile salts and thyroid hormones</td>
<td>Abe et al., 1998</td>
</tr>
<tr>
<td>Oatp4&lt;sub&gt;r&lt;/sub&gt;</td>
<td></td>
<td>conjugated steroids, eicosanoids, and lipophilic organic anions</td>
<td>Cattori et al., 2000</td>
</tr>
<tr>
<td>OCT1&lt;sub&gt;h&lt;/sub&gt;</td>
<td>80% of Oct1</td>
<td>MPP, TEA, choline, quinine, d-tubocurarine, pancuronium, and cyanine</td>
<td>Gorboulev et al., 1997; Zhang et al., 1997</td>
</tr>
<tr>
<td>Oct1&lt;sub&gt;r&lt;/sub&gt;</td>
<td></td>
<td>MPP, TEA, NMN, and small organic cations</td>
<td>Gründemann et al., 1994</td>
</tr>
<tr>
<td>OCTN1&lt;sub&gt;h&lt;/sub&gt;</td>
<td>76% of OCTN1</td>
<td>carnitine</td>
<td>Tamai et al., 1997</td>
</tr>
<tr>
<td>OCTN2&lt;sub&gt;h&lt;/sub&gt;</td>
<td></td>
<td>carnitine</td>
<td>Tamai et al., 1998</td>
</tr>
</tbody>
</table>

<sup>h</sup> human; <sup>ht</sup> hamster; <sup>r</sup> rat
Table 1-2. Summary of efflux-transporters in the liver

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Homology in amino acid sequence</th>
<th>Substrate</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRP1&lt;sup&gt;h,l&lt;/sup&gt;</td>
<td></td>
<td>GSH and its conjugates, bilirubin glucuronides, conjugated steroids, MTX, eicosanoids, and anionic conjugates</td>
<td>Cole et al., 1992</td>
</tr>
<tr>
<td>Mrp1&lt;sup&gt;m,l&lt;/sup&gt;</td>
<td>84% of MRP1</td>
<td>GSH and its conjugates, bilirubin glucuronides, conjugated steroids, MTX, eicosanoids, and anionic conjugates</td>
<td>Stride et al., 1996</td>
</tr>
<tr>
<td>MRP3&lt;sup&gt;h,s&lt;/sup&gt;</td>
<td>58% of MRP1</td>
<td>GSH conjugates, conjugated steroids, and MTX</td>
<td>Kuichi et al., 1998</td>
</tr>
<tr>
<td>Mrp3&lt;sup&gt;r,s&lt;/sup&gt;</td>
<td>70% of Mrp2</td>
<td>GSH conjugates, conjugated steroids, and MTX</td>
<td>Hirohashi et al., 1998</td>
</tr>
<tr>
<td>MRP6&lt;sup&gt;h,s&lt;/sup&gt;</td>
<td></td>
<td>Anthracycline</td>
<td>Kool et al., 1999</td>
</tr>
</tbody>
</table>

<sup>h</sup> human; <sup>m</sup> mouse; <sup>r</sup> rat
<sup>l</sup> Lateral; <sup>s</sup> Sinusoidal

1.2.5 Excretion

ATP-binding cassette proteins, carriers present on the canalicular membrane of the liver, are the secretory-transporters that require energy to excrete drugs and metabolites into bile. These excretion processes affect hepatic drug clearance by controlling the intracellular concentrations of a drug and its metabolites. A summary of the secretory-transporters including multidrug resistance associated-protein (MRP), multidrug resistance protein (MDR), and bile salt efflux protein (sPgp) is shown in Table 1-4.
Table 1-3. Summary of major phase I and II enzymes

<table>
<thead>
<tr>
<th>Metabolic phase</th>
<th>Type of reaction</th>
<th>Enzyme involved</th>
<th>References for gene and nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase I</td>
<td>Oxidation</td>
<td>Alcohol dehydrogenase</td>
<td>Duester et al., 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aldehyde dehydrogenase</td>
<td>Nebert et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flavin-containing monooxygenase</td>
<td>Lawton et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed-function oxidase system (cytochromes P450 and NADPH-cytochrome P450 reductase)</td>
<td>Nelson et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monoamine oxidase</td>
<td>Boomsma et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prostaglandin-synthase</td>
<td>O'Neill, 1994</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td></td>
<td>Esterase</td>
<td>Satoh and Hosokawa, 1998</td>
</tr>
<tr>
<td>Epoxide hydration</td>
<td></td>
<td>Epoxide hydrolase</td>
<td>Beetham et al., 1995</td>
</tr>
<tr>
<td>Reduction</td>
<td></td>
<td>Mixed-function oxidase system</td>
<td>Nelson et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peptidase</td>
<td>Hersh, 1986</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sulfatase</td>
<td>Parenti et al., 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucuronidase</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quinone reductase</td>
<td>Gaedigk et al., 1998</td>
</tr>
<tr>
<td>Phase II</td>
<td>Acetylation</td>
<td>N-acetyltransferase</td>
<td>Vatsis et al., 1995</td>
</tr>
<tr>
<td></td>
<td>Amino acid conjugation</td>
<td>Amino acid N-acetyltransferase</td>
<td>Hutt et al., 1990</td>
</tr>
<tr>
<td></td>
<td>Glucuronidation</td>
<td>UDP-glucuronosyltransferase</td>
<td>Burchell et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Glutathione conjugation</td>
<td>Glutathione S-transferase</td>
<td>Mannervik et al., 1992</td>
</tr>
<tr>
<td></td>
<td>Methylatation</td>
<td>Methyltransferase</td>
<td>Thompson et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Sulfation</td>
<td>Sulfotransferase</td>
<td>Nagata and Yamazoe, 2000</td>
</tr>
</tbody>
</table>

1.3 Functional Heterogeneity in Liver

Heterogeneity exists for transport proteins and metabolic enzymes among the hepatocytes located adjacent to the hepatic venules (perivenous) and the portal venules (periportal). Several implications arise from zonated expression of transporters and metabolic enzymes in the liver. Firstly, the disposition of drugs and their metabolites can be affected by the heterogeneity of transport carrier and metabolic enzyme (Dawson et al., 1985; Xu et al., 1990). This aspect is
important because drugs and metabolites in circulation cause pharmacological and toxicological effects. Secondly, many hepatotoxins cause localized injury in the liver acinus due to heterogeneous chemical activation and detoxification in the acinus. Some examples include carbon tetrachloride, which causes perivenous necrosis (Zhao and O’Brien, 1996), and allyl alcohol, which produces periportal injury (Pentilla, 1988).

Table 1-4. Summary of canalicular secretory-transporters in the liver

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Homology in amino acid sequence</th>
<th>Substrate</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSEP\textsuperscript{h} \textsuperscript{(sPgp)} Bsep\textsuperscript{r}</td>
<td>88% of Bsep</td>
<td>bile salts</td>
<td>Strautrieks et al., 1998 Gerloff et al., 1998</td>
</tr>
<tr>
<td>MDR1\textsuperscript{h} \textsuperscript{(Pgp)} Mdr1a\textsuperscript{m} Mdr1b\textsuperscript{f} (Pgpl) (Pgp2)</td>
<td>78% of Mdr1b</td>
<td>lipophilic compounds and organic cations</td>
<td>Chen et al., 1986 Hsu et al., 1990</td>
</tr>
<tr>
<td>MDR3\textsuperscript{h} \textsuperscript{(MDR2)} Mdr2\textsuperscript{r}</td>
<td>75% of MDR1</td>
<td>phospholipids and phosphatidylcholine</td>
<td>Van der Bliek et al., 1988 Brown et al., 1993</td>
</tr>
<tr>
<td>MRP2\textsuperscript{h} \textsuperscript{(cMOAT)}</td>
<td>46% of MRP1</td>
<td>GSH and its conjugates, bilirubin glucuronides, conjugated steroids, MTX, eicosanoids, and anionic conjugates</td>
<td>Kartenbeck et al., 1996</td>
</tr>
<tr>
<td>Mrp2\textsuperscript{r} \textsuperscript{(cmoat)}</td>
<td>48% of MRP1</td>
<td>GSH and its conjugates, bilirubin glucuronides, conjugated steroids, MTX, eicosanoids, and anionic conjugates</td>
<td>Paulusma et al., 1996</td>
</tr>
</tbody>
</table>

\textsuperscript{h} human; \textsuperscript{m} mouse; \textsuperscript{r} rat
1.3.1 Transport

Within the intact liver, drug disappearance is a result of drug uptake that occurs in a distributed-in-space fashion among hepatocytes located along the sinusoidal flow path. An enrichment of a specific transporter in a zone will influence the local drug concentration along the liver acinus, and subsequently this uneven drug concentration among the zonal hepatocytes will affect the intrinsic clearance of the drug. At present, few transporters have demonstrated zonal localization: GLUT1, GLUT2, MRP3, Oatp2, and Oct1 (Table 1-5). Interestingly, none of the canalicular transporters is heterogeneously localized within the liver acinus (Table 1-5).

Table 1-5. Summary of zonated transporters found to date

<table>
<thead>
<tr>
<th>Location</th>
<th>Transporter</th>
<th>Acinar localization</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sinusoidal</td>
<td>cysteine</td>
<td>Perivenous</td>
<td>Saiki et al., 1992</td>
</tr>
<tr>
<td></td>
<td>glutamate</td>
<td>Perivenous</td>
<td>Burger et al., 1989</td>
</tr>
<tr>
<td></td>
<td>GLUT1</td>
<td>Perivenous</td>
<td>Tal et al., 1990</td>
</tr>
<tr>
<td></td>
<td>GLUT2</td>
<td>Periportal</td>
<td>Ogawa et al., 1996</td>
</tr>
<tr>
<td></td>
<td>lactate</td>
<td>Even</td>
<td>Staricoff et al., 1995</td>
</tr>
<tr>
<td></td>
<td>α-ketoglutarate</td>
<td>Perivenous</td>
<td>Moseley et al., 1992</td>
</tr>
<tr>
<td></td>
<td>MRP3</td>
<td>Periportal</td>
<td>Kool et al., 1999</td>
</tr>
<tr>
<td></td>
<td>NTCP</td>
<td>Even</td>
<td>Stieger et al., 1994</td>
</tr>
<tr>
<td></td>
<td>Oatp1</td>
<td>Even</td>
<td>Abu-Zahra et al., 2000</td>
</tr>
<tr>
<td></td>
<td>Oatp2</td>
<td>Perivenous</td>
<td>Reichel et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Oct1</td>
<td>Even</td>
<td>Tirona et al., 2000</td>
</tr>
<tr>
<td></td>
<td>Bsep</td>
<td>Even</td>
<td>Meyer-Wentrup et al., 1998</td>
</tr>
<tr>
<td>Canalicul</td>
<td>Mdr (C219)</td>
<td>Even</td>
<td>Gerloff et al., 1998</td>
</tr>
<tr>
<td></td>
<td>MRP2</td>
<td>Even</td>
<td>Tan and Pang (unpublished)</td>
</tr>
<tr>
<td></td>
<td>Mrp2</td>
<td>Even</td>
<td>Kool et al., 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tirona et al., 1999</td>
</tr>
</tbody>
</table>
1.3.2 Metabolic enzymes and cosubstrates

Zonation of metabolic enzymes in the liver acinus plays an important role in regulating metabolite formation. Gluconeogenesis, cholesterol synthesis, ureagenesis, and bile production are predominantly found in the periportal zone. In contrast, glycolysis, lipogenesis, and most of the xenobiotic biotransformation processes are mainly localized in the perivenous zone (Table 1-6). These regional metabolic activities often result from the different expression of key enzymes of a given process. For example the concerted effects of zonal glucose uptake and regional expression of glucokinase and pyruvate kinase lead to the perivenous localization of glycolysis (Jungermann and Kietzmann, 1997). For the most part, the constitutive expression of many drug metabolising enzymes occurs in the perivenous zone (Table 1-6). The expression of most of the cytochrome P450 isoenzymes (CYP1A, CYP2B, CYP2E, CYP3A, and CYP4A), glutathione S-transferases, N-acetyltransferase (NAT2), and UDP-glucuronosyltransferase isoenzymes are mainly localized in the perivenous region (Jungermann, 1995; Oinonen and Lindros, 1998). For the sulfotransferase isoenzymes, hydroxysteroid sulfotransferase has been shown to be more dominant in the periportal region, whereas estrogen sulfotransferases are predominantly localized in the perivenous region (Homma et al., 1997). For the acinar distribution of cofactors, Smith et al. (1979) found that GSH is predominantly concentrated in the periportal region, but others (Tirona et al., 1999) found that GSH is evenly distributed in the liver acinus. However, the zonal distribution of other cofactors such as PAPS, UDPGA, and acetyl-coenzyme-A has yet to be investigated.

1.4 Sulfoconjugation

Sulfoconjugation is one of the major phase II conjugation reactions in the metabolism of endogenous compounds and xenobiotics. In addition, PAPS is required as an active sulfate donor in the sulfoconjugation as shown in Fig. 1-2.
Figure 1-2. Sulfoconjugations:
(A) Sulfation of 4-nitrophenol by phenol sulfotransferase (ST1);
(B) Sulfation of minoxidil by phenol sulfotransferase (ST1);
(C) Sulfation of dehydroepiandrosterone by hydroxysteroid sulfotransferase (ST2);
(D) Sulfamation of 2-naphthylamine by amine sulfotransferase (ST3)
1.4.1 Sulfate homeostasis

Inorganic sulfate is essential for PAPS synthesis. The liver contains a high level of sulfate (about 1 μmol/g or 1 mM; Kim et al., 1995). The sulfate pools are replenished by absorption of sulfate (Castranova et al., 1979), degradation of sulfate-containing macromolecules, the activity of sulfatases, and last but not least, the sulfoxidation of sulfur-containing amino-acids, cysteine and methionine, yielding inorganic sulfate (Kim et al., 1995). Glazenburg et al. (1983) found that the infusion of sodium sulfate, cysteine, and methionine increase serum and liver sulfate concentrations, with sodium sulfate being the most efficacious, then cysteine and lastly methionine. The hepatic clearance of inorganic sulfate is by sulfate utilization in the liver and efflux into plasma and bile (Basswig et al., 1994).

Table 1-6. Acinar gradients of major metabolic pathways in liver

<table>
<thead>
<tr>
<th>Periportal region</th>
<th>Perivenous region</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General metabolism</strong></td>
<td><strong>Drug metabolism</strong></td>
</tr>
<tr>
<td>albumin formation</td>
<td>GSH peroxidation</td>
</tr>
<tr>
<td>bile acid production</td>
<td>Hydroxysteroid</td>
</tr>
<tr>
<td>ureagenesis</td>
<td>sulfoconjugation</td>
</tr>
<tr>
<td>cholesterol synthesis</td>
<td></td>
</tr>
<tr>
<td>fatty acid oxidation</td>
<td></td>
</tr>
<tr>
<td>gluconeogenesis</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Jungermann (1995) and Oinonen and Lindros (1998).

The availability of PAPS for sulfation in vivo depends on its synthesis, transportation (Mandon et al., 1994), utilization, and degradation. PAPS is synthesized via a two-step synthesis reaction. The first reaction, which is catalysed by ATP sulfurylase, results in the formation of APS
from ATP and inorganic sulfate. The subsequent reaction catalysed by APS kinase results in the formation of PAPS from APS and a second molecule of ATP (Fig 1-3). The hepatic PAPS concentration is around 70 nmol/g liver (Brzeznicka et al., 1987), but the rate of sulfoconjugation in the liver can sustained to be as high as 100 nmol/min/g liver (Pang et al., 1981) due to the rapid biosynthesis of PAPS in the liver.

**Figure 1-3. PAPS synthesis pathways**

\[
\begin{align*}
\text{ATP} + \text{SO}_4^{2-} & \xrightarrow{\text{ATP sulfurylase}} \text{APS} + \text{PPi} \\
\text{APS} + \text{ATP} & \xrightarrow{\text{APS kinase}} \text{PAPS} + \text{ADP} \\
\text{PAPS} + \text{ROH} & \xrightarrow{\text{Sulfotransferase}} \text{PAP} + \text{ROS}
\end{align*}
\]

ATP, adenosine triphosphate; APS, Adenosine-5’-phosphosulfate; PAPS, 3’-phosphoadenosine-5’-phosphosulfate; ROH, hydroxyl-substrate R; ROS, sulfoconjugate of substrate R; PAP, 3’-phosphoadenosine-5’-phosphate.

### 1.4.2 Sulfotransferases

There are two classes of sulfotransferases: the cytosolic sulfotransferases that catalyze the sulfoconjugation of xenobiotics and small endogenous compounds, and the membrane-bound sulfotransferases localized in the Golgi apparatus that catalyze the sulfoconjugation of glucosaminoglycans and tyrosines in proteins. A large number of the cytosolic sulfotransferases found in mammals has been characterized at the mRNA level, and these sulfotransferases are divided into several gene families based on the homology of their amino acids sequences. Although “SULT” has been suggested as the abbreviation for the cytosolic sulfotransferases and their genes, it has not
been universally agreed upon. Recently, Nagata and Yamazoe (2000) summarized and divided the gene family of cytosolic sulfotransferases based on their amino acid sequences (Table 1-7). Each gene family has less than 40% similarity with the others. Within a gene family, a subfamily exhibits 40-65% similarity to other subfamilies. ST1, ST2, and ST3 are the phenol, hydroxysteroid, and amine sulfotransferases, respectively.

1.4.3 Role of sulfoconjugation in inactivation, detoxification, and toxification

Sulfoconjugation plays an important role in 1) the detoxification of xenobiotics, 2) the functional alteration of endogenous compounds, and 3) the bioactivation of xenobiotics. The major physiological role of sulfoconjugation is the detoxification of xenobiotics by increasing their elimination rates via formation of their hydrophilic sulfoconjugates. The biological role of sulfating an endogenous hormone is to alter the physiological role of the hormone. For example, sulfoconjugation of estrone inactivates the hormone. Although estrone sulfate is water soluble, it serves as a storage form of estrogen in the circulation due to its high protein binding characteristic (Rosenthal et al., 1972). Another pharmacological role of sulfoconjugation is to bioactivate prodrugs. For example, the sulfoconjugate of minoxidil, which is susceptible to further reaction that leads to an electrophilic intermediate, can react with the target protein to produce an active therapeutic agent for antihypertension (DuCharme et al., 1973) and hair growth (Buhl et al., 1990). On the other hand, bioactivation of xenobiotics via sulfoconjugation can also produce carcinogens. For example, sulfation of N-hydroxyl-2-acetylaminofluorine results in a carcinogen which forms adducts with proteins and nucleic acids (Wu and Straub, 1976).
<table>
<thead>
<tr>
<th>Family</th>
<th>Subfamily</th>
<th>Name</th>
<th>Species</th>
<th>Correspondence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST1</td>
<td>1A</td>
<td>1A1</td>
<td>rat</td>
<td>PST-1, Mx-STb, ASTIV, and Tyrosine-ester ST</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1A2</td>
<td>human</td>
<td>SULT1A2, HAST4V, HAST4, and STP2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1A3</td>
<td>human</td>
<td>SULT1A1, HAST1, Hpsta, TS-PST1, HAST2, P-PST, STP, and H-PST</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1A4</td>
<td>mouse</td>
<td>mSTp1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1A5</td>
<td>human</td>
<td>SULT1A3, hTLpST, HAST3, TL PST, hEST, STM, and HAST5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1A6</td>
<td>bovine</td>
<td>PST</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1A7</td>
<td>dog</td>
<td>dPST-1</td>
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<tr>
<td></td>
<td></td>
<td>1A8</td>
<td>rabbit</td>
<td>monPST-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1A9</td>
<td>monkey</td>
<td>dopa/tyrosine SULT</td>
</tr>
<tr>
<td></td>
<td>1B</td>
<td>1B1</td>
<td>rat</td>
<td>hST1B2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1B2</td>
<td>human</td>
<td>mouse SULT1B1</td>
</tr>
<tr>
<td></td>
<td>1C</td>
<td>1C1</td>
<td>rat</td>
<td>HAST-1 and N-hydroxy-2-acetylaminofluorene ST</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1C2</td>
<td>human</td>
<td>human SULT1C1</td>
</tr>
<tr>
<td></td>
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<td>1C3</td>
<td>human</td>
<td>human SULT1C2</td>
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<tr>
<td></td>
<td></td>
<td>1C4</td>
<td>mouse</td>
<td>mouse P-ST</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1C5</td>
<td>rabbit</td>
<td>rabbit stomach SULT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1C6</td>
<td>rat</td>
<td>sultK1</td>
</tr>
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<td></td>
<td></td>
<td>1C7</td>
<td>rat</td>
<td>sultK2</td>
</tr>
<tr>
<td></td>
<td>1D</td>
<td>1D1</td>
<td>mouse</td>
<td>SULT-N</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1D2</td>
<td>rat</td>
<td>tyrosine-ester SULT</td>
</tr>
<tr>
<td></td>
<td>1E</td>
<td>1E1</td>
<td>bovine</td>
<td>OST</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1E2</td>
<td>rat</td>
<td>EST-1, Ste 1, and EST-3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1E3</td>
<td>guinea pig</td>
<td>gpEST</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1E4</td>
<td>human</td>
<td>hEST and hEST-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1E5</td>
<td>mouse</td>
<td>mouse testis-specific estrogen SULT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1E6</td>
<td>rat</td>
<td>EST-2, Ste2, and EST-6</td>
</tr>
<tr>
<td>ST2</td>
<td>2A</td>
<td>2A1</td>
<td>rat</td>
<td>ST-20, ST-21a, and ST-21b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2A2</td>
<td>rat</td>
<td>ST-40 and ST-41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2A3</td>
<td>human</td>
<td>DHEA-SULT, hSTa, DHEA-ST8, and HST-hfa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2A4</td>
<td>mouse</td>
<td>mSTa1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2A5</td>
<td>rat</td>
<td>ST-60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2A6</td>
<td>guinea pig</td>
<td>gpHST1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2A7</td>
<td>guinea pig</td>
<td>gp HST2 and Preg-ST</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2A8</td>
<td>rabbit</td>
<td>AST-RB2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2A9</td>
<td>mouse</td>
<td>mSTa2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2A10</td>
<td>monkey</td>
<td>monHST-1</td>
</tr>
<tr>
<td></td>
<td>2B</td>
<td>2B1</td>
<td>human</td>
<td>SULT2B1a and SULT2B1b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2B2</td>
<td>mouse</td>
<td>mouse SULT2B1</td>
</tr>
<tr>
<td>ST3</td>
<td>3A</td>
<td>3A1</td>
<td>rabbit</td>
<td>AST-RB1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3A2</td>
<td>mouse</td>
<td>SULT-X2</td>
</tr>
</tbody>
</table>

Adapted from Nagata and Yamazoe (2000)
1.5 Desulfation

Desulfation also plays an important role in the metabolism of hormone and drug sulfates. The enzyme responsible for cleaving the sulfate moiety from a drug sulfate is sulfatase, which is a membrane-bound enzyme that has the highest activity in the liver.

1.5.1 Sulfatases

There are eleven sulfatase enzymes found in humans and nine of their corresponding genes have been identified as shown in Table 1-8. Most of the sulfatases are located in lysosomes, since they can best perform in acidic environments. ARSC, a microsomal arylsulfatase, best performs in neutral environments and is responsible for desulfation of steroids. Recently, three non-lysosomal sulfatases - ARSD, ARSE, and ARSF - were found in the same chromosome as ARSC, but they are unable to hydrolyze steroid sulfates (Franco et al., 1995). However, they are active in desulfating 4-methylumbelliferyl sulfate (4MUS). All members of the sulfatase family cleave the sulfate ester bonds of a wide variety of substrates ranging from the complex glycosaminoglycan to 4MUS. Schmidt et al. (1995) have discovered the cause of the multiple sulfatase deficiency disease (MSD); the sulfatase in the MSD patients are inactive due to the lack of an essential post-translational modification of the active side cysteine-19 residue to 2-amino-3-oxopropionic acid.

1.5.2 Biological role of desulfation

Desulfation plays an important role in the activation of sulfate conjugates of endogenous compounds. During the second half of pregnancy, the human fetal adrenal gland secretes large amount of dehydroepiandrosterone sulfate (DHEAS), which is desulfated by ARSC and metabolised by P450 to produce estrogen (Kuss, 1994). In addition, hydrolysis of cholesterol sulfate by ARSC is reported to be important in skin cell formation and differentiation because a skin disease,
ichthyosis, was observed in patients with ARSC deficiency (Ballabio et al., 1995). Moreover, there are many diseases such as chondrodysplasia punctata, metachromatic leukodystrophy; mucopolysaccharidosis, and X-linked ichthyosis that are caused by sulfatase deficiencies.

Table 1-8. Summary of sulfatases in humans

<table>
<thead>
<tr>
<th>Name</th>
<th>Subcellular localization</th>
<th>Natural substrate</th>
<th>Chromosomal location</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARSA</td>
<td>Lysosomes</td>
<td>Cerebroside sulfate</td>
<td>22q13</td>
<td>MLD and MSD</td>
</tr>
<tr>
<td>ARSB</td>
<td>Lysosomes</td>
<td>Dermatan sulfate</td>
<td>5q13-14</td>
<td>MPS and MSD</td>
</tr>
<tr>
<td>ARSC</td>
<td>Microsomes</td>
<td>Sulfated steroids</td>
<td>Xp22.3</td>
<td>XLI and MSD</td>
</tr>
<tr>
<td>ARSD</td>
<td>Endoplasmic reticulum</td>
<td>Unknown</td>
<td>Xp22.3</td>
<td>Not established</td>
</tr>
<tr>
<td>ARSE</td>
<td>Golgi Apparatus</td>
<td>Unknown</td>
<td>Xp22.3</td>
<td>CP and MSD</td>
</tr>
<tr>
<td>ARSF</td>
<td>Endoplasmic reticulum</td>
<td>Unknown</td>
<td>Xp22.3</td>
<td>Not established</td>
</tr>
<tr>
<td>IDS</td>
<td>Lysosomes</td>
<td>Dermatan sulfate and heparan sulfate</td>
<td>Xq27-28</td>
<td>MPS and MSD</td>
</tr>
<tr>
<td>Galactose 6-sulfatase</td>
<td>Lysosomes</td>
<td>Keratan sulfate and Chondroitin 6-sulfate</td>
<td>16q24</td>
<td>MPS and MSD</td>
</tr>
<tr>
<td>Glucosamine 6-sulfatase</td>
<td>Lysosomes</td>
<td>Keratan sulfate and heparan sulfate</td>
<td>12q14</td>
<td>MPS and MSD</td>
</tr>
<tr>
<td>Glucuronate 2-sulfatase</td>
<td>Lysosomes</td>
<td>Heparan sulfate</td>
<td>Unknown</td>
<td>Not established</td>
</tr>
<tr>
<td>Glucosamine 3-sulfatase</td>
<td>Lysosomes</td>
<td>Heparan sulfate</td>
<td>Unknown</td>
<td>Not established</td>
</tr>
</tbody>
</table>

Adapted from Parenti et al. (1997). ARS, arylsulfatase; CP, chondrodysplasia punctata; MLD, metachromatic leukodystrophy; MPS, mucopolysaccharidosis; MSD, multiple sulfatase deficiency; XLI, X-linked ichthyosis.

1.6 Model Substrates For Futile Cycling: Estrone Sulfate And Estrone In Liver

1.6.1 Futile cycling

The liver is the most important organ in drug metabolism. Hepatic drug clearance is
influenced by organ blood flow, protein and erythrocyte binding, transmembrane clearance, tissue binding, metabolic enzyme activity, and biliary excretion. Recent investigation adds futile cycling as an added variable that influences drug and metabolite clearances (Ratna et al., 1993). Futile cycling describes the interconversion of two moieties involving two different enzymatic reactions. Some examples include the futile cycling of estrone and estrone sulfate, catalysed by estrogen sulfotransferase and arylsulfatase C (Fig. 1-4), and the interconversion of 4-MU and 4-MUS, catalysed by phenol sulfotransferase and arylsulfatase C, D, E, and F (Ratna et al., 1993; Franco et al., 1995). The consequence of futile cycling is that both the apparent drug clearance and net metabolite formation are reduced. In addition, futile cycling also reduces the sequential metabolism of the formed metabolite.

1.6.2 Biological role of estrogens

Estrone (E₁) and estradiol (E₂) are the principal estrogens which are essential for sexual development and maintenance of the normal function of the female reproductive system. In addition, estrogens promote bone growth, prevent bone resorption, increase the levels of high-density lipoproteins, affect hepatic protein synthesis, and are involved in the feedback mechanism of gonadotrophs. Estrone undergoes reversible interconversion with E₂, which involves 17β-hydroxysteroid dehydrogenase. The estrogen hormones are produced mainly in the ovaries and placenta, but small amounts may be synthesised in testes and adrenals. The ovary is capable of converting androstenedione to E₁ by aromatase, and the formation of estrogens by ovarian follicles is regulated by the follicle stimulating hormone and luteinizing hormone. The plasma levels of E₁, E₂, and E₁S in humans are shown in Table 1-9. The major use of estrogens is in combination with progestins as oral contraceptives and hormone replacement therapy. Estrone sulfate is the predominant estrogen in the circulation after the oral administration of either E₁ or E₂ in humans. In addition, E₁S serves
as a storage form of estrogen in the circulation and is part of the proprietary drug in Premarin® and C.E.S®. Thus, E₁S plays an important role in regulating the levels of E₁ and E₂ in humans.

Table 1-9. Plasma levels of principal estrogens in humans

<table>
<thead>
<tr>
<th>Subject</th>
<th>Estradiol (nM)</th>
<th>Estrone (nM)</th>
<th>Estrone sulfate (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men</td>
<td>10-30</td>
<td>10-70</td>
<td>150-700</td>
</tr>
<tr>
<td>Women:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular phase</td>
<td>25-600</td>
<td>30-200</td>
<td>100-1,000</td>
</tr>
<tr>
<td>Luteal phase</td>
<td>100-300</td>
<td>70-100</td>
<td>200-2,000</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>10,000-40,000</td>
<td>2,000-30,000</td>
<td></td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>5-25</td>
<td>30-70</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Loriaux et al. (1971) and Henderson (2000).

1.6.3 Toxicology of estrogens

Estrogens are efficacious in hormone replacement therapy and as oral contraceptives. The major concern for the use of estrogens is the possibility of developing cancer. In 1936, Lacassagne found that estrogens induced breast, uterus, and bone tumors in various animal species. In a later report (Shapiro et al., 1985), an increased risk of endometrial carcinoma is associated with the use of conjugated estrogens in hormone replacement therapy. However, the risk of breast cancer is still uncertain for women using estrogens in both hormone replacement therapy and oral contraceptives (Bernstein et al., 1992). Some other deleterious effects of estrogen therapy include gastrointestinal upset, retention of sodium and water, increased risk of cholelithiasis, and other nonspecific adverse effects. Thus, the decision of using estrogens is highly dependent on the risk-to-benefit evaluation for each patient.
Figure 1-4. Metabolic scheme of estrone

- Estrone sulfotransferase
- Estrone glucuronidase
- Estrone UDP-glucuronosyltransferase
- Arylsulfatase C
- 17-beta-hydroxysteroid dehydrogenase

Other metabolites such as estriol (E3), catechol estrogens, glucuronide and sulfate conjugates of estrogen metabolites.
1.6.4 Pharmacokinetics of estrone and estrone sulfate

1.6.4.1 Vascular binding and transmembrane characteristics

Both $E_1$S and $E_1$, circulating in the blood are strongly bound to plasma protein. Estrone is 15-20\% bound to sex-hormone binding globulin and is 80-89\% bound to serum albumin (Jasonnie et al., 1988). Thus, only about 3\% of $E_1$ is unbound in the circulation (Jasonnie et al., 1988), and the percentage of unbound $E_1$S in the circulation is about 1.6\% (Rosenthal et al., 1972). In addition, it has been reported that when plasma proteins are saturated, erythrocytes can bind significant amounts of unconjugated estrogens (Challis et al., 1973). Hence, in the presence of drug-drug interaction or in the disease state, erythrocytes will play an important role in vascular binding and metabolism of $E_1$.

Most researchers believe that $E_1$ enters the target cells via simple diffusion, while others suggest that a cellular protein may facilitate diffusion into the cytoplasm (Rao et al., 1977). Indeed, $E_1$ is able to diffuse into target cells due to its high lipophilicity (log octanol-water partition coefficient of $E_1$ is 3.1; Howard and Meylan, 1997). The cellular uptake of $E_1$S is mediated by both diffusion and carrier proteins such as NTCP (Hagenbuch and Meier, 1994), the OATP family (Kullack-Ublick et al., 1995), and LST1 (Abe et al., 1999) for human.

1.6.4.2 Disposition in-vivo

After intravenous injection of $^{14}$C-estrone into women, a biphasic elimination profile was observed with half-lives of approximately 20 and 70 minutes. About 80\% of the radioactivity was found in the urine, predominantly as estrogen glucuronides during the following 120 hour period. The fecal excretion was about 7\% of the dose. However, patients with bile-fistulas excreted 47\% of the injected radioactivity in urine during the 120 hour period, and only 52\% of the dose was excreted...
in the bile during the first 12 hours. In normal patients, about 45% of the dose was reabsorbed in the gastrointestinal tract, demonstrating that large amounts of the estrogens participate in the enterohepatic circulation. These findings of Sandberg and Slaunwhite (1957) suggest that the liver plays an integral part in the metabolism and excretion of estrogens.

The pharmacokinetic profile of E₁S in man can best described by a two-compartmental model after intravenous injection of ³H-E₁S. The initial distribution phase exhibits a half-life and volume of distribution of 3 min and 7.2 L, respectively. The terminal half-life and the metabolic clearance rate of E₁S are 196 min and 90 L/day/m² (or 175 L/day), respectively (Longscope, 1972). In addition, E₁S binds rapidly to tissues and albumin and has a longer elimination half-life than that of E₁; thus, E₁S is an efficient storage form of estrogen in the circulation.

1.6.4.3 Metabolism and excretion

The liver is the principal site for metabolism of estrogens. There are two major groups of estrone metabolites (Fig. 1-4). The phase I metabolites are produced via metabolism catalyzed by cytochrome P450, 16α-hydroxylase, and 17β-hydroxysteroid dehydrogenase, as listed in Table 1-10. The phase II metabolites include estrone sulfate, estrone glucuronide, sulfate and glucuronide conjugates of the phase I metabolites. The principal urinary estrogens are the sulfate and glucuronide conjugates of estriol and catechol estrogens (Fishman et al., 1963). In addition to urinary excretion, there is significant enterohepatic circulation of the phase II metabolites. Takikawa et al. (1996) had shown that biliary excretion of sulfate and glucuronide conjugates of E₁ and E₂ in EHBR rat, a mutant rat that lacks Mrp2 at the canalicular membrane, were lower than normal rat. Thus, the biliary excretion of the phase II metabolites of E₁ is most likely mediated by Mrp2 (Takikawa et al., 1996).
Table 1-10. Principal enzymes involved in phase I metabolism of E₁

<table>
<thead>
<tr>
<th>Species</th>
<th>C2-OH</th>
<th>C4-OH</th>
<th>C10-OH</th>
<th>C16-OH</th>
<th>C17-OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>CYP1A2</td>
<td>CYP1B1</td>
<td>CYP1A1</td>
<td>16α-hydroxylase</td>
<td>17β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>CYP3A4</td>
<td></td>
<td>CYP2B6</td>
<td>CYP1A2</td>
<td>CYP3A4</td>
</tr>
<tr>
<td>Rat</td>
<td>CYP1A2</td>
<td>CYP1B1</td>
<td>CYP1B1</td>
<td>16α-hydroxylase</td>
<td>17β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>CYP3A1/A2</td>
<td>CYP2B1</td>
<td>CYP2C11</td>
<td>CYP2D</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Martucci and Fishman (1993), Yamazaki et al. (1998), and Ohe et al. (2000).

1.6.5 Estrone sulfate and estrone as model substrates to study the effect of futile cycling in hepatic clearance

The pharmacokinetics and biotransformation of E₁S and E₁ are well characterized in humans, but the kinetics of futile cycling between E₁S and E₁ has been neglected. E₁ is sulfated by estrone sulfotransferase, and the metabolite, E₁S, can be converted back to E₁ by estrone sulfatase. This futile cycling of E₁S and E₁ is a pharmacologically important biocycle, an efficient way of conserving and regulating the endogenous hormone, and may play an important role in the hepatic clearance of E₁S and E₁. Moreover, the disappearance of the futile cycling between E₁S and E₁ might affect the homeostasis of E₁S, E₁, and other estrogens in patients with multiple sulfatase deficiency and X-linked ichthyosis disease.
CHAPTER 2

STATEMENT OF PURPOSE OF INVESTIGATION
Estrone sulfate is used in hormone replacement therapy since it serves as a prodrug and storage form of active estrogens. Although many target organs possess estrone sulfatase, the key enzyme that activates E$_1$S, the liver contains the highest metabolic activities of the enzyme. The liver is considered one of the most important internal organs for regulating the levels of drugs and metabolites in the blood circulation. The factors influencing hepatic drug clearance include hepatic blood flow, protein and erythrocyte binding, transport into hepatocytes, tissue binding, metabolism, and biliary excretion. Recent investigations have found that futile cycling is an additional variable that influences drug and metabolite clearances. Futile cycling describes the interconversion of two substrates involving two different enzymatic reactions. The pharmacokinetic consequence of futile cycling is that both total drug clearance and net formation of the metabolite undergoing interconversion are reduced. Moreover, futile cycling also reduces the sequential metabolism of both compounds.

Although the pharmacokinetics and biotransformation of E$_1$S and E$_1$ have been well characterized in humans, the futile cycling between E$_1$S and E$_1$ has been neglected. E$_1$ is sulfated by estrone sulfotransferase, and the metabolite, E$_1$S, can be activated back to E$_1$ by estrone sulfatase. This futile cycling is a pharmacologically important biocycle and may play an important role in the hepatic clearance of E$_1$S and E$_1$. The futile cycling of E$_1$S and E$_1$ occurs along the length of the sinusoidal flow path in a distributed-in-space fashion within the liver. Since functional differences exist among hepatocytes within the liver acinus, in particular the enrichment of estrone sulfotransferase in the perivenous region, it is necessary to investigate the influence of zonal aspects on the futile cycling of E$_1$S and E$_1$. A thorough investigation of the zonal transport and metabolism of E$_1$S and E$_1$ is essential because these processes can regulate the local rates of sulfation and desulfation and also influence the overall hepatic clearance of E$_1$S and E$_1$. A concerted approach must be taken to scale up the in-vitro transport and metabolism parameters to an in-vivo scenario and
to develop a distributed-in-space liver model that can comprehensively describe the complex futile cycling and predict the hepatic clearance of \( E_1S \) and \( E_1 \). Thus, a systemic *in-vitro* to *in-vivo* approach was employed to study the importance of vascular binding, acinar localization of transporters and metabolic enzymes, tissue binding, excretion, and the futile cycling of \( E_1S \) and \( E_1 \) on the hepatic clearance of \( E_1S \) and \( E_1 \) (Fig 2-1).

**2.1 Hypotheses To Be Tested**

1) The processing of \( E_1S \) and \( E_1 \) in zonal hepatocytes differs and this is attributed to differences in zonal transport and metabolism.

2) Futile cycling kinetics result in parallel decay profiles of \( E_1S \) and \( E_1 \).

3) The *in-vitro* transport and metabolic parameters of \( E_1S \) and \( E_1 \) correspond to the *in-vivo* hepatic clearance parameters.

**2.2 Objectives**

1) To examine acinar transport and desulfation of \( E_1S \) and acinar sulfation of \( E_1 \) using isolated, zonal rat hepatocytes and their subcellular fractions.

2) To investigate the effects of the futile cycling of \( E_1S \) and \( E_1 \) on their disposition in the zonal intact hepatocytes.

3) To scale up the *in-vitro* transport and metabolic parameters of \( E_1S \) and \( E_1 \) to interpret the results of the recirculating *in-situ* liver perfusion via a distributed-in-space liver model.
Figure 2-1. Summary of the thesis

Futile cycling of E\textsubscript{1}S and E\textsubscript{1} is a pharmacologically important biocycle and may play an important role in the hepatic clearance of E\textsubscript{1}S and E\textsubscript{1} (Chapter 1 and 2).

The transport characteristics of E\textsubscript{1}S were investigated in the zonal hepatocytes because the uptake of E\textsubscript{1}S and zonal aspects may influence the futile cycling (Chapter 3).

The zonal metabolic activities of estrone sulfotransferase and sulfatase were examined, and the influence of transport and metabolism on the futile cycling of E\textsubscript{1}S and E\textsubscript{1} was studied in intact zonal hepatocytes (Chapter 4).

The binding of E\textsubscript{1}S and E\textsubscript{1} by protein and erythrocyte was examined, and the futile cycling was studied by simultaneous delivery of \textsuperscript{3}H]E\textsubscript{1}S and \textsuperscript{14}C]E\textsubscript{1} in a recirculating liver preparation. In addition, a distributed-in-space liver model was developed to explain the importance of the vascular and tissue binding, transport, zonal metabolism, and excretion on the hepatic clearance of E\textsubscript{1}S and E\textsubscript{1} (Chapter 5).

General discussion and conclusions (Chapter 6)
CHAPTER 3

LACK OF ZONAL UPTAKE OF ESTRONE SULFATE
IN ENRICHED PERIPORTAL AND PERIVENOUS ISOLATED RAT HEPATOCYTES

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3.1 Abstract

The zonal uptake of estrone sulfate or E1S (1 to 400 μM) was investigated in periportal and perivenous rat hepatocytes and cells isolated from whole liver (regular hepatocytes). Transport of E1S by periportal, perivenous, and regular hepatocytes was described by saturable ($K_m$'s of 24 to 26 μM and $V_{max}$'s of 1.8 nmol/min/mg protein) and nonsaturable components (2.5 to 3.2 μl/min/mg protein) that were not different among the zonal regions ($P > 0.05$. ANOVA). These kinetic constants represented pooled values for the entire complement of transporters for E1S, including two known transporters of E1S: Ntcp, Na$^+$-taurocholate cotransporting polypeptide, and oatp, organic anion transporting polypeptide cloned from rat liver. Uptake of E1S was significantly reduced by estradiol 17β-glucuronide (50 μM) and bumetanide (200 μM), and was inhibited strongly and competitively by pregnenolone sulfate with an inhibition constant of 6.7 μM. Further segregation of the kinetic constants as the sodium-dependent and sodium-independent systems was achieved through simultaneous fitting of data obtained in the presence and absence of sodium (145 mM) from parallel hepatocytic uptake studies. For the periportal, perivenous, and regular hepatocytes, two saturable systems: a sodium-dependent transport system, characterized by similar $V_{max}$'s (1.1 to 1.4 nmol/min/mg protein) and $K_m$'s (49 to 55 μM), a sodium-independent transport system of comparable $V_{max}$'s (0.70 to 0.84 nmol/min/mg protein) and $K_m$'s (16 to 22 μM), and a linear clearance of 1.7 to 2.7 μl/min/mg protein (ANOVA, $P > 0.05$) were obtained. The data suggest that hepatic uptake of E1S involved sodium-dependent and sodium-independent transporter systems. No zonal heterogeneity in transport for both sodium-dependent and sodium-independent transporter systems was observed.
3.2 Introduction

Estrone sulfate (E\textsubscript{1}S) serves as a storage form of estrogens in the human circulation and is used in hormone replacement therapy. It is metabolized primarily in liver to estrogens such as estrone, estradiol, and estriol. The influx of E\textsubscript{1}S into the liver could influence levels of estrogens in the body. Moreover, the presence of zonal distribution of transporters in the liver would affect the cell concentration and processing of E\textsubscript{1}S by desulfation or biliary excretion among zonal cells, thus altering the overall hepatic clearance. This aspect has been shown to impact drug levels in simulation studies (Sato et al., 1986; Hansel and Morris, 1996; Kwon and Morris, 1997).

The hepatic transport of E\textsubscript{1}S is mediated by the organic anion transporting polypeptide (oatp1 and oatp2) and the sodium-dependent taurocholate cotransporting polypeptide (Ntcp), two recently cloned sinusoidal transporters (Hagenbuch et al., 1991; Jacquemin et al., 1994; Noé et al., 1997). Oatp1 and oatp2, glycoproteins with twelve putative transmembrane domains, exist on the rat liver sinusoidal membrane (Jacquemin et al., 1994), the apical membrane of the kidney (Bergwerk et al., 1996), and the choroid plexus of the brain (Noé et al., 1997). The proteins are noted for their transport of bile acids and derivatives, the anionic dye bromosulfophthalein, ouabain, and anionic estrogen conjugates (Shi et al., 1995; Kanai et al., 1996; Bossuyt et al., 1996; Noé et al., 1997; Pang et al., 1998). Oatp1 mediated transport is sodium-independent, bidirectional (Shi et al., 1995), energy dependent (Shi et al., 1995), and involves bicarbonate (Satlin et al., 1997) and possibly glutathione (Li et al., 1998) as the counter ion. Ntcp, a glycoprotein with seven transmembrane domains, exists on the basolateral membrane of the liver. Ntcp preferentially mediates not only the hepatic uptake of bile acids and derivatives, but also anions such as E\textsubscript{1}S and bromosulfophthalein (Hagenbuch et al., 1991; Schroéder et al., 1998). The transport of anions such as the bile acid taurocholate by Ntcp is electrogenic and is
driven by the physiological sodium gradient (Hagenbuch et al., 1991) with an apparent Na\(^+\)-taurocholate stoichiometry of 2:1 (Weinman, 1997).

Although the nature of transport proteins is known for the uptake of E1S, there is virtually no information on its zonal uptake within the liver acinus. Heterogeneity in transport has been found for various endogenous and exogenous compounds. For example, the uptake of glutamate (Burger et al., 1989; Stoll et al., 1991), taurocholate (Stacey and Klaasen, 1981), ouabain (Stacey and Klaasen, 1981), and cysteine (Saiki et al., 1992) was reported to be greater in perivenous than in periportal hepatocytes, whereas the Na\(^+\)/K\(^+\) ATPase activity was implicated to be lower in the perivenous region (Silau et al., 1996). The intrinsic transport functions of oatp1 and Ntcp for E1S uptake has not been compared among zonal hepatocytes. although existing immunohistochemical evidence showed a lack of liver heterogeneity for Ntcp (Stieger et al., 1994) and for the mRNA of oatp1 in rat liver (Dubuisson et al., 1996). The objective of this study was to examine the heterogeneity in the uptake of E1S by investigating the intrinsic difference in transport velocity of E1S among periportal and perivenous hepatocytes. namely, for the sodium-dependent and sodium-independent uptake of E1S. The uptake of E1S in the presence of pregnenolone sulfate, a structural analog, was appraised to study the possible interactions between the hormonal sulfate conjugates.

### 3.3 Materials and Methods

#### 3.3.1 Materials

\(^{3}\text{H}\)E1S (ammonium salt, specific activity, 50 Ci/mmol), \(^{14}\text{C}\)L-glutamic acid (specific activity, 250 mCi/mmol), \(^{14}\text{C}\)sucrose (specific activity, 6.4 mCi/mmol) and \(^{3}\text{H}\)sucrose (specific activity, 11.9 Ci/mmol) were purchased from NEN Life Science Products (Boston,
MA). Unlabeled E$_1$S, glutamic acid, bumetanide, estradiol 17β-glucuronide, choline, bovine serum albumin (Fraction V), and pregnenolone sulfate were obtained from Sigma Chemical Co. (St. Louis, MO). Collagenase A (Clostridium histolyticum) was purchased from Boehringer Mannheim (Darmstadt, Germany). Digitonin was obtained from Fluka Chemie (Buchs, Switzerland). Silicone oil (Fluids 510 and 550) was purchased from Dow Corning (Mississauga, ON). All other reagents and solvents were of HPLC grade.

### 3.3.2 Isolation of rat hepatocytes

Male Sprague-Dawley rats (275-375 g, Charles River Canada, St. Constant, QC) were used for the preparation of isolated hepatocytes from whole liver (regular) by a previously established method (Hassen et al., 1996). Enriched periportal and perivenous hepatocytes were isolated by the digitonin/collagenase perfusion technique of Lindros and Pentilla (1985), with modifications. After anesthesia (50 mg pentobarbital/kg, intraperitoneally), the rat liver was perfused via the portal vein in a single pass fashion at 25 ml/min for 10 min with a Ca$^{2+}$- free buffer [consisting of Hank’s buffer, 10 mM HEPES, 0.5 mM EGTA, 4.2 mM NaHCO$_3$, 5 mM glucose, 0.65% bovine serum albumin, pregassed with carbogen (95% O$_2$, 5% CO$_2$), and buffered to pH 7.2]. The medium was then changed to the digitonin solution (3.25 mM digitonin, 150 mM NaCl, 6.7 mM KCl, and 50 mM HEPES) as described by Tosh et al. (1996). The digitonin solution was delivered at a lower perfusion rate of 5.6 ml/min for approximately 35 ± 9 sec (n = 8) progradely (flowing into the portal vein and exiting at the hepatic vein) or 77 ± 13 sec (n = 8) retrogradely (flowing into the hepatic vein and exiting at the portal vein). The infusion with digitonin was stopped immediately when maximal destruction of selective zonal cells was visually detected on the surface of the liver: appearance of white specks for destruction of perivenous cells and rings for destruction of periportal cells. The digitonin solution remaining in
the liver was flushed out by perfusion with calcium free buffer in the opposite direction (12 ml/min) for 2 min, then with collagenase buffer (Hanks' buffer plus 4 mM CaCl₂ and 0.06% w/w collagenase) for 8 min. All other subsequent steps involved were carried out as described previously (Hassen et al., 1996). Viability of the regular and zonal hepatocytes obtained was greater than 90%, as assessed by trypan blue exclusion.

3.3.3 Zonal cells

The enrichment of periportal and perivenous cells by the digitonin/collagenase perfusion method was monitored with alanine aminotransferase (ALT) with a Sigma diagnostics kit and with glutamine synthetase (GS) assayed by a standard UV method (Meister, 1985): the protein content was measured by the method of Lowry et al. (1951). Since the uptake of glutamate from blood was shown to be predominantly perivenous (Stoll et al., 1991; Burger et al., 1989), the periportal and perivenous cells were further differentiated by their ability to transport glutamate. After pre-incubation at 37°C for 10 min, hepatocyte suspensions were added to a mixture of unlabeled glutamate, [14C]glutamate and [3H]sucrose (an extracellular marker) to result in glutamate concentrations of 1 to 200 μM in 1.6 x 10⁶ cells/ml. Samples (100 μl) retrieved at 30, 60, 90 and 120 sec were placed into 300 μl polyethylene microfuge tubes containing silicone oil (100 μl of density 1.02 g/ml) atop 50 μl of 3N NaOH. The tubes were centrifuged immediately for rapid filtration of the cells through the silicone oil layer to sediment into the lower alkaline layer. The radioactivities of the incubation mixture, the supernatant (top layer), and the hepatocytes (residue) were quantified by liquid scintillation counting (Model 6800. Beckman Canada, Mississauga, ON).
3.3.4 Uptake of E₁S

For all studies, cell suspensions of 2 x 10⁶ cells/ml of the regular, periportal, or perivenous hepatocytes, pre-incubated at 37°C for 10 min, were added mixtures of E₁S. [³H]E₁S. and [¹⁴C]sucrose (an extracellular marker) to result in E₁S concentrations of 1 to 400 μM in 1.6 x 10⁶ cells/ml. Samples (100 μl) were retrieved at 15, 30, 45 and 60 sec for centrifugation as described above. The effect of estradiol 17β-glucuronide (50 μM) and the diuretic, bumetanide (200 μM) on E₁S (50 μM) uptake was also studied. The inhibitory effect of pregnenolone sulfate (0, 10, 25, and 100 μM) on the uptake of E₁S (1 to 200 μM) was investigated in parallel incubations. Since 1% ethanol was used for dissolution of pregnenolone sulfate in these studies. cell viability and uptake of estrone sulfate in 0% and 1% ethanol were first compared. Cell viability in 1% ethanol remained greater than 90% for at least 15 min, as determined by trypan blue exclusion. The preservation of hepatocytic viability in 1% ethanol was also observed by Sawyer et al. (1994) who demonstrated that both the liver cell viability and respiratory function were not adversely affected by 1% ethanol over a period of six hours. In another set of studies. parallel incubations were conducted in the presence or absence of sodium - choline chloride was substituted for sodium chloride, and potassium bicarbonate for sodium bicarbonate.

3.3.5 Kinetic analysis of E₁S uptake

The linear portion of the plot of accumulated amount vs. time yielded a positive intercept and a slope upon regression of the data. The positive intercept, that represents rapid and saturable binding of E₁S to hepatocyte membrane, had been described by Hassen et al. (1996). The slope furnished the initial velocity of uptake (v).

Various mechanisms, ranging from simple saturable Michaelis-Menten (Eq. 3-1) to multiple components (Eqs. 3-3 or 3-5), and presence of a nonsaturable system, denoted by $P_{\text{diff}}$
or the first-order clearance (see Eqs. 3-2 and 3-4), were used to describe the uptake of estrone sulfate by rat hepatocytes.

\[
v = \frac{V_{\text{max}} [S]}{K_m + [S]}
\]  
Eq. 3-1

\[
v = \frac{V_{\text{max}} [S]}{K_m + [S]} + P_{\text{diff}} [S]
\]  
Eq. 3-2

\[
v = \frac{V_{\text{max,1}} [S]}{K_{m,1} + [S]} + \frac{V_{\text{max,2}} [S]}{K_{m,2} + [S]}
\]  
Eq. 3-3

\[
v = \frac{V_{\text{max,1}} [S]}{K_{m,1} + [S]} + \frac{V_{\text{max,2}} [S]}{K_{m,2} + [S]} + P_{\text{diff}} [S]
\]  
Eq. 3-4

\[
v = \frac{V_{\text{max,1}} [S]}{K_{m,1} + [S]} + \frac{V_{\text{max,2}} [S]}{K_{m,2} + [S]} + \frac{V_{\text{max,3}} [S]}{K_{m,3} + [S]}
\]  
Eq. 3-5

The uptake data of glutamate and E₁S in periportal and perivenous hepatocytes were fitted to Eqs. 3-1 to 3-5 to determine the appropriate curve model for uptake.

For the inhibition study with pregnenolone sulfate, data on E₁S transport in the presence of pregnenolone sulfate (0, 10, 25, and 100 μM) were forced fitted to Eq. 3-6 or 3-7 which denoted competitive inhibition, or Eq. 3-8 or 3-9 which defined noncompetitive inhibition.

\[
v = \frac{V_{\text{max}} [S]}{K_m (1 + \frac{[I]}{K_i}) + [S]} + P_{\text{diff}} [S]
\]  
Eq. 3-6

\[
v = \frac{V_{\text{max}} [S]}{K_m (1 + \frac{[I]}{K_i}) + [S]} + \frac{P_{\text{diff}} [S]}{(1 + \frac{[I]}{K_i})}
\]  
Eq. 3-7

\[
v = \frac{V_{\text{max}} [S]}{(K_m + [S]) (1 + \frac{[I]}{K_i})} + P_{\text{diff}} [S]
\]  
Eq. 3-8
Previously, Hassen et al., (1996) have reported that Eq. 3-2, which described both a carrier mediated (saturable) and a linear (nonsaturable) component, was best in defining EiS uptake. However, the situation would change for the set of data on EiS transport in the presence and absence of sodium. Consequently, these data were simultaneously fitted to Eq. 3-2 (one saturable plus linear components for data obtained in absence of sodium) and Eq. 3-4 (two saturable components plus a linear component for data obtained in presence of sodium), or to Eq. 3-3 (two saturable components) and Eq. 3-5 (three saturable components), respectively.

\[ v = \frac{V_{\text{max}}[S]}{(K_m + [S]) (1 + \frac{[I]}{K_i})} + \frac{P_{\text{diff}}[S]}{(1 + \frac{[I]}{K_i})} \]  

Eq. 3-9

### 3.3.6 Fitting

The data were fitted with use of the software package SCIENTIST (version 2: MicroMath Scientific Software, Salt Lake City, UT) with the least squares method. The optimized parameters are summarized in Tables 3-1 to 3-4. The selection of the weighting scheme and goodness of fit were based on the coefficients of variation of the estimated parameters and residual plots. The kinetic parameters can be scaled-up to the whole liver with the scaling factor, \( \alpha \) (\( \alpha = 200 \text{ mg protein/g liver} \); Mahler and Cordes, 1996).

### 3.3.7 Statistical analysis

All data were presented as the mean ± standard deviation, and the means were compared by use of ANOVA, with the level of significance set at 0.05. The Model Selection Criterion (MSC) and the Akaike Information Criteria (AIC) (Akaike, 1974; Ludden et al., 1994) were used to select the appropriate model equation(s).
3.4 Results

3.4.1 Biochemical characterization of zonal hepatocytes

The activities of the two marker enzymes, alanine aminotransferase and glutamine synthetase, in the periportal and perivenous hepatocytes were summarized in Table 3-1. Significant difference ($P < 0.05$, ANOVA) was observed among the periportal and perivenous hepatocytes.

The accumulation of $[^{14}\text{C}]$glutamate was linear with time up to 2 min among all of the concentrations investigated. The initial velocities of glutamate uptake ($v$), calculated from regression of data between 0.5 to 2 min, were best described by Eq. 3-2, and were statistically different among periportal and perivenous hepatocytes (Fig. 3-1). Saturable uptake of different $K_m$'s ($59 \pm 24$ and $136 \pm 23$ μM) and $V_{max}$'s ($9.4 \pm 5.6$ and $47 \pm 5.7$ nmol/min/mg protein) were obtained for glutamate uptake by periportal and perivenous hepatocytes (ANOVA, $P < 0.05$). and the rate of $[^{14}\text{C}]$glutamate uptake was significantly greater in perivenous cells at all concentrations. However, the nonlinear component was similar ($P_{diff}$ of $0.02 \pm 0.01$ vs. $0.03 \pm 0.003$, ANOVA, $P > 0.05$).
Table 3-1. Biochemical characterization of zonal hepatocytes by alanine aminotransferase (ALT) and glutamine synthetase (GS)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Periportal (PP)</th>
<th>Perivenous (PV)</th>
<th>PP/PV</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>391 ± 113</td>
<td>207 ± 110*</td>
<td>1.9</td>
</tr>
<tr>
<td>GS</td>
<td>0.81 ± 0.53</td>
<td>25 ± 15*</td>
<td>0.032</td>
</tr>
</tbody>
</table>

* Statistically significant from periportal (ANOVA, $P < 0.05$)

3.4.2 Uptake kinetics of E1S

The time course for the uptake of E1S was shown in Fig. 3-2. Accumulation of E1S in hepatocytes remained linear with time within 1 min for all of the concentrations of E1S (1 to 400 μM). When the velocity was plotted against the E1S concentration, concentration-dependent kinetics became evident (Fig 3-3A). Moreover, transport was temperature-dependent, and was reduced to a constant value (clearance of $2.1 ± 0.2 \mu l/min/mg$ protein) at 4°C. The data were best described by Eq. 3-2, as found by Hassen et al. (1996), and a weighting scheme of 1/observation was optimal for fitting. The uptake of E1S was not significantly different among regular, periportal, and perivenous hepatocytes (Fig. 3-3B; ANOVA, $P > 0.05$, Table 3-2). The $K_m$'s and $V_{max}$'s varied from 24 to 26 μM and 1.8 nmol/min/mg protein, respectively, and the linear clearance, $P_{diff}$, was 2.5 to 3.2 μl/min/mg protein. These kinetic constants were generally similar to those found by Hassen et al. (1996), but differed from those of Schwenk and del Pino (1980), who examined a much lower concentration range (0.1 to 10 μM) to arrive at lower estimates ($K_m$ of 0.8 μM and $V_{max}$ of 0.3 nmol/min/mg protein).
Figure 3-1. Glutamate transport by isolated rat periportal and perivenous hepatocytes

![Graph showing Glutamate Uptake Rate versus Glutamate Concentration](image)

Table 3-2. Uptake kinetic parameters of E_{i}S in isolated regular, periportal, and perivenous hepatocytes

<table>
<thead>
<tr>
<th>Hepatocytes Population</th>
<th>$K_{m}$ (μM)</th>
<th>$V_{max}$ (nmol/min/mg protein)</th>
<th>$P_{diff}$ (μl/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular (n = 6)</td>
<td>26 ± 8.3</td>
<td>1.8 ± 0.26</td>
<td>3.2 ± 1.0</td>
</tr>
<tr>
<td>Periportal (n = 8)</td>
<td>24 ± 13</td>
<td>1.8 ± 0.46</td>
<td>2.5 ± 1.1</td>
</tr>
<tr>
<td>Perivenous (n = 8)</td>
<td>24 ± 9.1</td>
<td>1.8 ± 0.36</td>
<td>2.7 ± 0.9</td>
</tr>
</tbody>
</table>

*Best fitted to Eq. 3-2 with a weighting of 1/prediction was optimal.*
Figure 3-2. Time course of $E_{1S}$ uptake: (A) 100 to 400 μM, and (B) 1 to 50 μM by isolated rat hepatocytes.
Figure 3-3. Uptake rates for $E_1S$: (A) in regular hepatocytes (whole liver) and (B) in regular, periportal, and perivenous hepatocytes.
3.4.3 Uptake of E₂S in the presence of inhibitors

Previous studies had shown inhibition of E₂S uptake by ouabain, energy depletors, other sulfate conjugates (Hassen et al., 1996), as well as by taurocholate (Schwenk and del Pino, 1980). Presently, the coadministration of estradiol 17β-glucuronide (50 μM) and bumetanide (200 μM) was found to reduce E₂S (50 μM) uptake significantly to 66 ± 5.2% and 64 ± 3.5% of control, respectively (n = 4, P < 0.05). The uptake of E₂S in the presence and absence of 1% ethanol was best described by Eq. 3-2. Upon comparison, the saturable component of E₂S transport in the presence and absence of 1% ethanol was similar, but the nonsaturable component of the uptake of E₂S was slightly higher in the presence of ethanol (Fig 3-4A, Table 3-3). The reason for the difference was not apparent. When the effect of pregnenolone sulfate (0.10, 25, and 100 μM dissolved in 1% ethanol) on E₂S uptake was examined in parallel incubations, the extent of inhibition intensified with increasing concentration of pregnenolone sulfate. The data were best fitted to the competitive inhibition equation (Eq. 3-6, Table 3-3): the overall inhibition constant was 6.7 μM, a value that is less than the overall $K_m$ for E₂S uptake (cf: values in Tables 3-2 and 3-3).

3.4.4 Uptake of E₂S in the presence and absence of sodium

The uptake parameters for E₂S by rat hepatocytes were significantly reduced in absence of sodium (Fig. 3-5A; Table 3-4, P < 0.05, ANOVA). Similar values as well as trends were observed for the data for periportal and perivenous hepatocytes in absence of sodium (Fig. 3-5B). The data were best fitted simultaneously to Eqs. 3-4 and 3-2, which described two saturable systems and a nonsaturable component, instead of Eqs. 3-5 and 3-3 due to the greater MSC, lower AIC values, and the smaller coefficient of variation. These “refined” uptake parameters, summarized in Table 3-4, revealed a slightly higher affinity ($K_m$ of 16 to 22 μM) and slightly
Figure 3-4. Uptake of E₁S: (A) in 0% and 1% ethanol and (B) in the presence of pregnenolone sulfate (PS; in 1% ethanol)
lower capacity ($V_{max}$ of 0.70 to 0.84 nmol/min/mg protein) system for the sodium-independent component, a lower affinity ($K_m$ of 49 to 55 μM) and slightly higher capacity ($V_{max}$ of 1.1 to 1.4 nmol/min/mg protein) system for the sodium-dependent component, and a linear component ($P_{diff}$ of 1.7 to 2.7 μl/min/mg protein).

### 3.5 Discussion

The marker enzymes, alanine aminotransferase and glutamine synthetase, verified that enriched periportal and perivenous hepatocytes were isolated from different lobular origin within the rat liver. Our finding that alanine aminotransferase activity was higher in periportal hepatocytes (with a ratio of 1.9 for periportal to perivenous activities. Table 3-1) was in agreement with histochemical evidence on the enzyme in rat liver (Gorgens et al., 1988) and what was observed in other studies (Silau et al., 1996; Tosh et al., 1996). The dramatic difference in glutamine synthetase activity between the perivenous and periportal regions (Table 3-1) was also shown by Stoll et al., (1991) and provided evidence on the successful preparation of enriched populations of periportal and perivenous cells. In addition, glutamate uptake, mediated by the sodium-dependent transporter, System G, in the perivenous region (Stoll et al., 1991) as well as the sodium-independent system in both periportal and perivenous hepatocytes (Burger et al., 1989; Stoll et al., 1991) was higher in perivenous hepatocytes (Fig. 3-1).

Although heterogeneity in transport was found for many endogenous and exogenous compounds: taurocholate (Stacey and Klaassen, 1981), ouabain (Stacey and Klaassen, 1981), and cysteine (Saiki et al., 1992), no significant difference in the uptake of E1S was found among the regular, periportal, and perivenous hepatocytes. Among these systems, E1S uptake is described by a saturable and nonsaturable system of similar magnitudes (Table 3-2). The constancy in the
Figure 3-5. Parallel incubations for the study of uptake of $E_1S$ in the presence and absence of sodium: (A) in regular hepatocytes and (B) in periportal and perivenous hepatocytes.
### Table 3-3. Uptake of E_{1}S in the presence of pregnenolone sulfate (PS) by isolated rat hepatocytes

<table>
<thead>
<tr>
<th></th>
<th>$K_{m}$ (μM)</th>
<th>$V_{max}$ (nmol/min/mg protein)</th>
<th>$P_{diff}$ (μl/min/mg protein)</th>
<th>$K_{i}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0% ethanol; n=6)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26 ± 8.3</td>
<td>1.8 ± 0.26</td>
<td>3.2 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Control (1% ethanol; n=4)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25 ± 9</td>
<td>1.8 ± 0.81</td>
<td>5.9 ± 1.6*</td>
<td></td>
</tr>
<tr>
<td>Added PS (1% ethanol; n=4)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37 ± 14</td>
<td>2.8 ± 0.72</td>
<td>2.7 ± 0.9</td>
<td>6.7 ± 0.9</td>
</tr>
</tbody>
</table>

* Different from 0% ethanol controls (ANOVA; $P < 0.05$)

<sup>a</sup> Best fitted to Eq. 3-2; a weighting of 1/observation was optimal 

<sup>b</sup> All data (0, 10, 25, 100 μM PS) were simultaneously fitted to Eq. 3-6; a weighting of 1/observation was optimal

### Table 3-4. Refinement of kinetic parameters for E_{1}S uptake in the presence and absence of sodium in parallel incubation studies in zonal hepatocytes

<table>
<thead>
<tr>
<th>Hepatocytes</th>
<th>Sodium-independent component</th>
<th>Sodium-dependent component</th>
<th>Linear component</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_{m}$ (μM)</td>
<td>$V_{max}$ (nmol/min/mg protein)</td>
<td>$K_{m}$ (μM)</td>
</tr>
<tr>
<td>Regular (n=5)</td>
<td>14 ± 7.2</td>
<td>0.80 ± 0.22</td>
<td>47 ± 17</td>
</tr>
<tr>
<td>Periportal (n=4)</td>
<td>22 ± 28</td>
<td>0.70 ± 0.32</td>
<td>50 ± 45</td>
</tr>
<tr>
<td>Perivenous (n=4)</td>
<td>18 ± 19</td>
<td>0.71 ± 0.32</td>
<td>55 ± 34</td>
</tr>
</tbody>
</table>

<sup>a</sup> Both data obtained in the presence and absence of sodium were forced fit to Eq. 3-4 and 3-2, respectively; a weighting of unity was optimal
values of $P_{\text{diff}}$ among the data sets in both absence and presence of sodium suggests that $P_{\text{diff}}$ is most likely passive diffusion, since a relatively high diffusive component could be predicted for E1S in view of its high (1.4) octanol:water partition at pH 7.4. However, the discrepancy in the nonsaturable component of E1S uptake, $P_{\text{diff}}$, in the presence of 1% ethanol may due to the fitting anomaly; the concentration used for this study was lower (up to 200 µM) and might not be high enough to fully reveal the linear component. In addition to the previously found inhibitors (Schwenk and del Pino, 1980; Hassen et al., 1996), the transport of E1S was reduced by estradiol 17β-glucuronide, a high affinity substrate for oatp1 (Kanai et al., 1996), bumetanide, a substrate for an organic anion transporter distinct from oatp1 and Ntcp (Horz et al., 1996), and was strongly inhibited by pregnenolone sulfate in a competitive fashion (Table 3-3, Fig. 3-4).

In light that oatp1, oatp2 and Ntcp mediate the transport of E1S in rat liver, it is reasonable to assume that data obtained with sodium-free media represent uptake by oatp1 and/or oatp2, the sodium-independent transporter, whereas data obtained in the presence of sodium encompass the entire spectrum of transporters in liver, including Ntcp. Thus, simultaneous fitting of data obtained in the presence and absence of sodium to Eqs. 3-4 and 3-2, respectively, yielded the best model. The hepatocellular entry of E1S was best described by three components: a sodium-dependent (saturable component), a sodium-independent (saturable component), and a nonsaturable or linear component (Fig. 3-5, Table 3-4). Furthermore, uptake of E1S by both sodium-dependent or sodium-independent systems was not significantly different among the periportal and perivenous hepatocytes (Table 3-4). The finding was in agreement with immunohistochemical evidence for Ntcp (Steiger et al., 1994) and for oatp1 in isolated, zonal hepatocytes (Abu-Zahra et al., 2000).

In this investigation, the $K_m$'s of the E1S transporters in rat hepatocytes were 14 and 47 µM, respectively, for the sodium-independent and sodium-dependent system, suggesting that the
sodium-independent pathway is of higher affinity. Indeed, transporters found in the rat liver, namely, oatp1 (Bossuyt et al., 1996), oatp2 (Noé et al., 1997), and Ntcp (Schroéder et al., 1998) expressed in the Xenopus laevis oocytes mediate the transport of E\textsubscript{i}S with corresponding $K_m$'s of 4.5, 11, and 27 $\mu$M, respectively, whereas the $K_m$ for the uptake of E\textsubscript{i}S uptake in COS cells expressing the human liver NTCP is 60 $\mu$M (Craddock et al., 1998). A low $K_m$ for E\textsubscript{i}S was inferred in the inhibition of studies of E\textsubscript{i}S on E\textsubscript{2-17β}-glucuronide transport (Kanai et al., 1996). It appears that the $K_m$'s obtained from rat hepatocytes in this study correlate well with those obtained in other purified or expression systems, and that separation of the contributions of oatp1 and oatp2 for the transport of E\textsubscript{i}S was not feasible since the $K_m$'s are close in values. The observation supports the view that, in absence of perturbation of the system and proper modeling, it becomes extremely difficult to separate the respective saturable components when the $K_m$'s are similar (Sedman and Wagner, 1974). It is further interesting to note that the sum of the $V_{max}$'s of the two saturable systems (Table 3-4) was similar to the overall $V_{max}$ obtained when data were treated as if it were a single saturable system (Table 3-2). Analogously, the $K_m$'s of the two saturable systems (Table 3-4), when averaged, matched the overall $K_m$ obtained when data were treated as if it were a single saturable system (Table 3-2).

In conclusion, two saturable systems, a sodium-dependent and a sodium-independent system that most likely represent Ntcp and oatp family, respectively, and a linear system, were found to mediate the transport of estrone sulfate. There was no difference in the uptake of E\textsubscript{i}S among regular, periportal, and perivenous hepatocytes. Pregnenolone sulfate was found to competitively inhibit the transport of E\textsubscript{i}S in rat liver.
3.5 Statement of Significance of Chapter 3

In this Chapter, we found that uptake of E1S in the liver was mediated by two saturable systems, a sodium-dependent and a sodium-independent system that most likely represent Ntcp and oatp family, respectively, and a linear system. In addition, we also found lack of acinar heterogeneity for the transport of E1S.

3.7 Acknowledgments

The biochemical characterization of zonal hepatocytes and the glutamate uptake studies were performed in collaboration with Dr. Rommel G. Tirona.
CHAPTER 4

SULFATION IS RATE-LIMITING IN THE FUTILE CYCLING BETWEEN ESTRONE SULFATE AND ESTRONE IN ENRICHED PERIPORTAL AND PERIVENOUS RAT HEPATOCYTES

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4.1 Abstract

The metabolic activities and tissue binding of estrone (E1) and estrone sulfate (E1S) on futile cycling were examined. Desulfation of E1S in the 9000g supernatant fraction (S9) of periportal (PP) and perivenous (PV) rat hepatocytes were of similar $V_{\text{max}}^{\text{E1S} \rightarrow \text{E1}}$ (2.9 ± 1.0 and 2.4 ± 0.9 nmol/min/mg S9 protein), $K_m^{\text{E1S} \rightarrow \text{E1}}$ (30.4 ± 8.3 and 34.8 ± 6.6 μM), and desulfation intrinsic clearances ($V_{\text{max}}^{\text{E1S} \rightarrow \text{E1}} / K_m^{\text{E1S} \rightarrow \text{E1}}$ of 77 and 55 μl/min/10^6 cells). The intrinsic clearance towards E1 sulfation (1 μM) in cytosolic preparations of PV hepatocytes was 4-times that of PP hepatocytes ($V_{\text{max}}^{\text{E1} \rightarrow \text{E1S}} / K_m^{\text{E1} \rightarrow \text{E1S}}$ of 26.4 ± 9.5 vs. 6.1 ± 2.2 μl/min/mg cytosolic protein or 13 ± 5 vs. 3.1 ± 1.1 μl/min/10^6 cells). The observation was consistent with the immunolocalization of estrogen sulfotransferase (PV/PP ratio of 3.4 ± 1.1) but not hydroxysteroid sulfotransferase (PV/PP ratio of 0.29 ± 0.21) nor phenol sulfotransferase (PV/PP ratio of 1.13 ± 0.23). Upon incubation of E1S (1 to 125 μM) with hepatocytes (30 min), higher concentrations of E1S and E1 were observed within PP than in PV cells, and saturation was evident at the higher concentrations. Based on the in vitro metabolic and tissue binding parameters for E1S and E1 and the published zonal uptake clearances of E1S (116 μl/min/10^6 cells), fitting revealed that uptake of E1 (1484 and 1463 μl/min/10^6 cells) by PP and PV cells was rapid and similar, and E1 sulfation was the slowest step in futile cycling. The greater metabolism of E1 in PV region led to higher levels E1 and E1S in PP hepatocytes, and the nonlinear uptake, binding, and vesicular accumulation of E1S resulted in different $t_{1/2}$'s for E1S and E1.
4.2 Introduction

Estrone sulfate (E₁S) is one of the compounds used in hormone replacement therapy. The activity of E₁S results from the release of active estrone (E₁) through desulfation in liver, and E₁ can be sulfated back to the inactive E₁S. Typically, the rate of sulfation of E₁ is measured but the observation may be erroneously based on the net rate of formation of E₁S. Since sulfoconjugation plays an important role in the deactivation and elimination of E₁, it is critical to consider the roles of sulfation and desulfation on the duration of activity of estrogens. When sulfation or desulfation is altered in disease states, the apparent formation of E₁S will be further modulated.

One of the key enzymes responsible for the sulfation of E₁ is estrogen sulfotransferase, a cytosolic enzyme that also sulfates estradiol (E₂) in the presence of the obligate cosubstrate, 3'-phosphoadenosine 5'-phosphosulfate (PAPS). Both human hydroxysteroid and phenol sulfotransferases are known to sulfate estrogens (Falany et al., 1994), but these possess lower affinities towards E₁ (Falany et al., 1994; 1995). Thus, under physiological conditions, E₁ is likely to be sulfated predominantly by estrogen sulfotransferase than by other isoforms of sulfotransferases. Previous studies had revealed that estrogen sulfotransferase was localized more abundantly in the PV than in the PP region of the rat liver. On the other hand, hydroxysteroid sulfotransferase was predominantly present in the PP hepatocytes, and the phenol sulfotransferase was evenly distributed in the liver acinus (Tosh et al., 1996).

Estrone sulfatase, a membrane-bound enzyme with a pH optimum of 7.4 is mainly responsible for the desulfation of E₁S and exhibits its highest activity in the liver (Milewich et al., 1984). Estrone sulfatase is also known as arylsulfatase C, a microsomal enzyme that copurifies with steroid sulfatase and cleaves the sulfate moiety of several 3-hydroxysteroid sulfates (Dolly et al., 1972). In female rats, an abundance of estrone sulfatase activity was found
in both the endoplasmic reticulum and nucleus (Zhu et al., 1998). Previous studies suggest that desulfation of 4-methylumbelliferyl sulfate (4MUS) by arylsulfatase C was homogeneous across the rat liver (Anundi et al., 1986) and human liver tissues (El Mouelhi and Kauffman, 1986).

The phenomenon of futile cycling between $E_1$ and $E_1S$ has yet to be viewed in conjunction with hepatic transport of $E_1$ and $E_1S$ and other metabolic pathways of $E_1$. Transport of $E_1S$ in rat liver is found to be mediated by the organic anion transporting polypeptides, Oatp1 (Jacquemin et al., 1994), Oatp2 (Noé et al., 1997), and Oatp4 (Cattori et al., 2000), the multispecific organic anion transporter 3, OAT3 (Kusuhara et al., 1999), and the sodium-dependent taurocholate cotransporting polypeptide, Ntcp (Hagenbuch et al., 1991). The values of the $K_m$ for the uptake of $E_1S$ mediated by Oatp1 (Bossuyt et al., 1996), Oatp2 (Noé et al., 1997), and Ntcp (Schroéder et al., 1998) expressed in the Xenopus laevis oocytes were around 4.5 to 27 µM, showing that transport is of high affinity. Transport of $E_1S$ was defined by these sinusoidal transporters and passive diffusion, and was found to be similar among perivenous (PV) and periportal (PP) rat hepatocytes (Tan et al., 1999).

The objective of this communication was to highlight the importance of transport, metabolism, and zonal aspects to understand their interplay on the futile cycling of estrogens in intact hepatocytes and ultimately, the whole organ. In this communication, metabolic activities in subcellular fractions of enriched PP and PV rat hepatocytes were assessed and in turn related to cellular expressions of rat liver estrone sulfatase and estrone sulfotransferase. These in vitro metabolic parameters and previously obtained transport parameters on hepatocyte uptake (Tan et al., 1999) were then used to describe the futile cycling between $E_1S$ and $E_1$ in intact cells when $E_1S$ (1, 5, 25, and 125 µM) was incubated with PP and PV hepatocytes.
4.3 Materials and Methods

4.3.1 Materials

$[6,7^{-3}\text{H}]E_1S$ (ammonium salt, specific activity, 53 Ci/mmol), $[6,7^{-3}\text{H}]E_1$ (specific activity, 40.6 Ci/mmol), and $[4^{-14}\text{C}]E_1$ (specific activity, 56.6 Ci/mol) were purchased from NEN Life Science Products (Boston, MA). The radiochemical purities, as found by high performance liquid chromatography (HPLC) or thin-layer chromatography (TLC), were greater than 95%. Unlabeled $E_1S$, $E_1$, PAPS and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase was obtained from Boehringer Mannheim (Oakville, ON). Digitonin was acquired from Fluka Chemie (Buchs, Switzerland). Antisera raised against rat liver phenol sulfotransferase (rSULT1A1), hydroxysteroid (bile acid) sulfotransferase (rSULT2A1), and estrogen sulfotransferase (rSULT1E1) were kindly provided by Dr. Charles N. Falany (University of Alabama, Birmingham, AL). The immunoblot Assay Kit (mini-Protean II systems) was obtained from Bio-Rad Laboratories (Mississauga, ON). Goat anti-rabbit horseradish peroxidase conjugate was obtained from Amersham (Oakville, ON). All other reagents were of the highest available grade.

4.3.2 Isolation of zonal rat hepatocytes, lysates and subcellular fractions of zonal hepatocytes

Male Sprague Dawley rats (275-325 g, Charles River Canada, St. Constant, QC) were used for the preparation of isolated hepatocytes. Rats were housed in accordance to Animal Protocols at the University of Toronto under a 12:12 h light:dark cycle, and were given food and water ad libitum.

Enriched PP and PV hepatocytes were isolated by the digitonin/collagenase perfusion
technique of Lindros and Pentilla (1985), with modifications (Tan et al., 1999). Viability of the zonal hepatocytes was greater than 90%, as assessed by Trypan blue exclusion. The enrichment of PP and PV cells was assessed by use of enzyme markers: alanine aminotransferase, which was measured by a Sigma diagnostics kit, and glutamine synthetase, which was assayed by a standard UV method (Meister, 1985). Cells were suspended in the incubation buffer consisting of NaCl (137 mM), KCl (5.4 mM), CaCl₂ (1 mM), MgCl₂·6H₂O (1 mM), MgSO₄·7H₂O (0.83 mM), NaH₂PO₄·2H₂O (0.5 mM), Na₂HPO₄·12H₂O (0.42 mM), NaHCO₃ (4.2 mM), glucose (5 mM) and HEPES (1 mM, pH 7.4). The 9000g supernatant (S9) and the 100,000g cytosolic fractions were obtained from zonal hepatocytes by homogenization (Ultra-Turrax T25 homogenizer, Janke & Kunkel, Staufen im Briesgau, Germany) and stepwise centrifugation at 9000g and 100,000g for 20 and 60 min, respectively, at 4°C.

Cell lysates from the most distal and proximal hepatocytes of the sinusoid were prepared by the dual-digitonin-pulse perfusion technique according to Quistorff and Brunnet (1987), with modifications (Tirona et al., 1999). In this case, rat livers were perfused with a perfusion buffer (Hank’s buffer [pH 7.2] containing 10 mM HEPES, 0.5 mM EGTA, 4.2 mM NaHCO₃ and 5 mM glucose).

4.3.3 Estrone sulfatase activity

Estrone sulfatase activity in the S9 of zonal hepatocytes was determined by formation of [³H]E₁ from [³H]E₁S. After pre-incubation of zonal S9 and E₁S separately at 37°C for 5 min, the solutions were combined to result in a mixture of S9 protein (1.4 mg) and E₁S (1 to 200 μM with 1 to 177 x 10⁵ dpm/ml of [³H]E₁S) in a final volume of 0.4 ml Tris-HCl buffer (25 mM) at pH 7.4. Samples (0.1 ml) were then removed at 2 min, a predetermined time in which product
formation was linear with time, into 0.5 ml of acetonitrile containing 4 μM danazol. the internal standard for HPLC analyses.

4.3.4 Estrone sulfotransferase activity

Estrone sulfotransferase activities in the zonal lysates and cytosolic fractions were estimated from that rates of formation of $[^3\text{H}]\text{E}_1\text{S}$ from $[^3\text{H}]\text{E}_1$. Lysates and cytosolic fractions of zonal hepatocytes, which were preincubated at 37°C for 5 min, were added to mixtures of PAPS, E$_1$, and $[^3\text{H}]\text{E}_1$ (2.0 ± 0.1 x 10$^5$ dpm/ml) to result in 1 μM of E$_1$ and 700 μM of PAPS in 1 ml Tris-HCl buffer (25 mM) at pH 7.4. To ensure sufficient cosubstrate for the reaction, a PAPS concentration higher than that reported for the rat liver - 70 nmol/g liver or 117 μM PAPS in cell water - was chosen (Brzeznicka et al., 1987). Samples (0.2 ml) were removed at 6 min. a predetermined time in which E$_1$ sulfation was linear with time. The samples were added to 0.8 ml of acetonitrile containing 4 μM of danazol.

4.3.5 Metabolism of E$_1$S in intact zonal hepatocytes

Zonal hepatocyte suspensions (2 x 10$^6$ cells/ml), preincubated at 37°C for 10 min in the incubation buffer, were added to equivolumes of unlabeled E$_1$S and $[^3\text{H}]\text{E}_1\text{S}$ (5.1 ± 0.3 x 10$^6$ dpm/ml) prepared in incubation buffer to result in 1, 5, 25, and 125 μM of E$_1$S in 10$^6$ cells/ml. Two samples were retrieved at various times (1 to 30 min) from the incubation mixture. The first sample (100 μl) was deproteinized immediately with 0.4 ml of acetonitrile containing 4 μM of danazol, and the second sample (150 μl) was placed immediately into a polyethylene microfuge tube (300 μl) containing 100 μl of 1-bromododecane. Upon centrifugation at 9000g (2 sec), hepatocytes were removed into the layer of 1-bromododecane, and 100 μl of the resultant
extracellular medium remaining on top was removed into a 1.5 ml microcentrifuge tube containing 4 μM danazol in 0.4 ml acetonitrile. Subsequently, the contents of E₁ and E₁S in the incubation mixture and extracellular medium were assayed by HPLC. The amount of E₁S or E₁ in the cellular space was estimated by the difference of the quantities in the incubation mixture and extracellular medium. The difference in mass between the administered amount and the sum of E₁S and E₁ provided an estimate of the formation of other E₁ metabolites. M (estrone glucuronide or E₁G, estradiol or E₂, and its conjugates) at various times.

### 4.3.6 Protein binding/Metabolism in extracellular medium

During the preparation of isolated hepatocytes, protein debris from suspending dead cells (0.16 ± 0.06 mg/10⁶ cells) was found to persist routinely in the incubation mixture despite the washings; centrifugation with percoll failed to remove the presence of the protein debris fragments. In view of the tight binding of E₁S and E₁ to albumin (Rosenthal et al., 1972; Rao, 1998), binding of E₁S and E₁ to the protein debris in the extracellular medium was estimated by ultrafiltration (Centricon 3, Amicon Inc, MA). Solutions containing [³H]E₁ (8.5 x 10⁵ dpm/ml) or [³H]E₁S (4.9 ± 2.4 x 10⁵ dpm/ml) plus E₁S (0.8 to 250 μM) were added to blank extracellular medium of the incubation mixture, which was prepared in absence of drug. After incubation of the mixture for 10 min at 37°C, an aliquot (2 ml) was removed into a Centicon tube and centrifuged at 2500g for 20 min at 37°C. Liquid scintillation fluor (5 ml, Ready Safe, Beckman Coulter, Canada, Mississauga, ON) was added to the original mixture (0.2 ml) and the resulting filtrate (0.2 ml) in different minicounting vials, then quantified by liquid scintillation spectrometry (Model LS6800, Beckman Coulter). Leakage of protein through the Centricon filter was less than 0.5% of the mixture solution. Desulfation of E₁S and metabolism of E₁ within the extracellular medium were less than 1% over the experimental time and were deemed
insignificant.

### 4.3.7 Immunoblot analysis

Lysates, centrifuged at 100,000g for 60 min at 4°C, and the cytosolic fraction of zonal hepatocytes, prepared as described by Tirona et al. (1999), were used for immunoblot analysis. Aliquots containing 10 μg of protein were resolved by SDS-PAGE in a 12% polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane. Primary rabbit antibodies (anti-rSULT1A1 at 1:20000, anti-rSULT2A1 at 1:10000, and anti-rSULT1E1 at 1:20000 dilution) were then incubated with the blots for 1 h at room temperature. Finally, goat anti-rabbit IgG horseradish peroxidase conjugate (1:40000) was used as the secondary antibody, and the immunonoconjugates were detected by chemiluminescence (ECL, Amersham). The intensity of the protein band was integrated using the NIH Image software (http://rsb.info.nih.gov/nih-image/).

### 4.3.8 Protein assay

In all preparations, protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard.

### 4.3.9 HPLC analysis

Liquid chromatography was performed on a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a SCL-10A system controller, a SPD-10A UV-VIS detector, a LC-10AT solvent delivery system, a SIL-10A-XL automatic injector, and a CR-4A
chromatopac integrator. Separation was carried out by a 10 μm μBondapak C<sub>18</sub> reversed-phase column (39 cm x 300 mm i.d.; Waters, Milford, MA). A binary gradient consisting of mobile phase A (10 mM ammonium acetate, pH 7.5) and mobile phase B (acetonitrile) was utilized at a constant flow of 1 ml/min for the separation of E<sub>1</sub>G, E<sub>1</sub>S, E<sub>1</sub>, E<sub>2</sub>, and danazol. Initially, the wavelength of detection was set at 270 nm for the detection of E<sub>1</sub>G and E<sub>1</sub>S, and the mobile phase was linearly increased from 10% B to 50% B in 15 min. Then the mobile phase was maintained at 50% B for 10 min, and the wavelength was altered to 285 nm at 20 min for the detection of E<sub>1</sub> and E<sub>2</sub>. At 25 min, the mobile phase was linearly increased to 75% B in 2 min, and was maintained there for another 10 min before being brought back down to 10% B over the next 5 min. The mobile phase was maintained at 10% B for a further 5 min to re-equilibrate the column. The retention times were: E<sub>1</sub>G, 14 min; E<sub>1</sub>S, 16 min; E<sub>1</sub>, 24 min; E<sub>2</sub>, 26 min; danazol, 34 min.

Good correspondence was observed between the eluted radioactivity ([<sup>3</sup>H]E<sub>1</sub>S and [<sup>3</sup>H]E<sub>1</sub>) which was collected at 0.5 min intervals after sample injection and UV absorbance. No carryover of radioactivity was observed for the compounds of interest despite that a delay of 0.5 min existed between UV detection and radioelution. Hence, the delay time was incorporated to the automated fraction collector (Model 202, Gilson Medical Electronics, WI). After the addition of 10 ml Ready Safe (Beckman Coulter, Canada, Mississauga, ON), the collected fractions were quantified by liquid scintillation spectrometry (Model LS6800, Beckman Counter, Canada).

4.3.10 Kinetic Analysis for extracellular binding of E<sub>1</sub>S and E<sub>1</sub>

The binding of E<sub>1</sub>S and E<sub>1</sub> to proteins that were present in the extracellular medium was
described by the Langmuir binding isotherm (Eq. 4-1), where the bound concentration of extracellular \( E_{1S} \) ([\( E_{1S_{\text{bound}}} \)\text{ec}] ) was related to unbound concentration ([\( E_{1S_{\text{unbound}}} \)\text{ec}] ) by the binding dissociation constant, \( K_{D}^{E_{1S}} \), and the product of number of binding site (\( n^{E_{1S}} \)) and the total protein concentration in the extracellular medium ([\( P_{\text{total}} \)\text{ec}] ). The kinetic constants were obtained by regression of [\( E_{1S_{\text{bound}}} \)\text{ec}] versus [\( E_{1S_{\text{unbound}}} \)\text{ec}] according to Eq. 4-1 with use of fitting software package, SCIENTIST (version 2; MicroMath Scientific Software, Salt Lake City, UT) and an optimized weighting scheme (1/observation^2).

\[
[E_{1S_{\text{bound}}} \text{ec}] = \frac{n^{E_{1S}} [P_{\text{total}} \text{ec}] [E_{1S_{\text{unbound}}} \text{ec}]}{K_{D}^{E_{1S}} + [E_{1S_{\text{unbound}}} \text{ec}]}
\]  
Eq. 4-1

After obtaining \( K_{D}^{E_{1S}} \) and \( n^{E_{1S}} [P_{\text{total}} \text{ec}] \) from Eq. 4-1, Eqs. 4-2 and 4-3 were used to estimate the tissue binding of \( E_{1S} \) in the hepatocyte from known, total protein concentration in cell-homogenate [\( P_{\text{total}} \)\text{c}].

\[
n^{E_{1S}} [P_{\text{total}} \text{c}] = n^{E_{1S}} [P_{\text{total}} \text{ec}] [P_{\text{total}} \text{c}] / [P_{\text{total}} \text{ec}]
\]  
Eq. 4-2

\[
[E_{1S_{\text{bound}}} \text{c}] = \frac{n^{E_{1S}} [P_{\text{total}} \text{c}] [E_{1S_{\text{unbound}}} \text{c}]}{K_{D}^{E_{1S}} + [E_{1S_{\text{unbound}}} \text{c}]}
\]  
Eq. 4-3

\[
[E_{1S_{\text{total}}} \text{c}] = [E_{1S_{\text{bound}}} \text{c}] + [E_{1S_{\text{unbound}}} \text{c}]
\]  
Eq. 4-4

4.3.11 Kinetic modeling of \( E_{1S} \) and \( E_{1} \) disposition in intact, zonal hepatocytes

A cellular, kinetic model that considered protein binding in both extracellular and cellular spaces, transport, metabolism, interconversion, and an intracellular vesicular compartment (Fig.
4-1), best described the concentration- and time-dependent data of E₁S and E₁ in enriched PP and PV hepatocytes. Inclusion of binding in both cellular and extracellular medium is justified in view of the demonstrable binding but lack of metabolism in extracellular medium, which contained protein debris from cells. E₁S, binding and debinding to protein are denoted by on- and off-rate constants, $k_{on}^{E₁S}$ and $k_{off}^{E₁S}$, respectively; these constants and the ratio $K_{D}^{E₁S}$ ($k_{off}^{E₁S}/k_{on}^{E₁S}$) are expected to be identical for both extracellular and cell media. However, the protein concentration or $[P_{total}]_{ec}$ in the extracellular medium is only a fraction of that in cells since the total protein concentration in cell $[P_{total}]_{c}$ is much higher. The effective binding concentration in cell was determined by multiplication of the ratio of protein concentrations in cell/debris ($[P_{total}]_{c}/[P_{total}]_{ec}$) with $(n^{E₁S}[P_{total}]_{ec})$ according to Eq. 4-2. For the sake of simplicity, the unbound concentration of E₁ is represented by the product of the unbound fractions of E₁ ($f_{e}^{E₁}$ and $f_{e}^{E₁}$ represent the unbound fraction of E₁ in the cell and the extracellular medium, respectively) and the total concentration of E₁. Since the canalicular membrane on the cell surface that mediates excretion of anions is known to internalize to form vesicles shortly after hepatocyte isolation (Oude Elferink et al., 1993), a similar phenomenon was postulated to exist for E₁S accumulation into vesicles. A vesicular compartment is included for E₁S since E₁S undergoes demonstrable biliary excretion in the perfused rat liver (unpublished observations).

Previously obtained parameters on transport were scaled-up and used in the fitting procedure. The transport parameters for E₁S ($V_{max}^{E₁S, in}$ and $P_{diff}^{E₁S}$) obtained from our previous hepatocyte uptake study (Tan et al., 1999) were scaled-up with the factor $\phi$ ($\phi = 1.6 \, \text{mg protein/10}^6 \, \text{cells}$; Mahler and Cordes, 1966; Lin et al., 1980). Based on these transport parameters of E₁S (Tan et al., 1999), the uptake of E₁S was mainly attributed to carrier-mediated transport, although the minor bi-directional flux of E₁S ($P_{diff}^{E₁S}$) also contributed to E₁S transport. In view of the fact that uptake of E₁ was too rapid for proper characterization of transport
constants in hepatocyte uptake studies, the observed linear transport clearance for $E_1$, $P_{\text{diff}}^E_1$, is assumed to exist for the permeation of $E_1$ at all concentrations. The in vitro parameter for the desulfation of $E_1$S ($V_{\text{max}}^{E_1S \rightarrow E_1}$) and the linear sulfation intrinsic clearance of $E_1$ ($CL_{\text{int}}^{E_1 \rightarrow E_1S}$ or $V_{\text{max}}^{E_1 \rightarrow E_1S} / K_m^{E_1 \rightarrow E_1S}$) were scaled-up with factors, $\alpha$ and $\beta$ ($\alpha = 0.8$ mg S9 protein/10$^6$ cells; $\beta = 0.5$ mg cytosolic protein/10$^6$ cells; Mahler and Cordes, 1966; Lin et al., 1980), respectively. It must be noted that the metabolic intrinsic clearance for estrone sulfation, $CL_{\text{int}}^{E_1 \rightarrow E_1S}$, could only be defined within a narrow concentration range of $E_1$ in vitro due to poor aqueous solubility, and this was used to relate $K_m^{E_1 \rightarrow E_1S}$ to the ratio $V_{\text{max}}^{E_1 \rightarrow E_1S} / CL_{\text{int}}^{E_1 \rightarrow E_1S}$. Since $E_1$ is known to form other metabolites ($M$, denoting the mixture of $E_1$G, $E_s$, estriol, and other estrogen conjugates: Holler et al., 1976), a simplified metabolic scheme (Fig. 4-1) was used to describe the formation of all $M$ with the pooled metabolic constants, $V_{\text{max}}^{E_1 \rightarrow M}$ and $K_m^{E_1 \rightarrow M}$. The intrinsic clearance of $E_1$S entry into vesicles is denoted by $CL_{\text{ves}}^{E_1S}$. The PP and PV cell volumes ($V_c$) were assumed to be 3.6 and 4.1 $\mu$l/10$^6$ cells, respectively (Garcia-Ruiz et al., 1994), and the extracellular volume ($V_{\text{cc}}$) was obtained by difference (total volume of the preparation [1 ml] - $V_c$).

4.3.12 Fitting

Mass balanced rate equations (see Appendix) were written to describe events of the cellular kinetic model (Fig. 4-1). Fitting was performed by a software package SCIENTIST (version 2; MicroMath Scientific Software, Salt Lake City, UT) based on experimentally obtained binding, metabolic, and transport parameters (Table 4-1). The parameters - transport clearance of $E_1$ ($P_{\text{diff}}^E_1$), the Michaelis-Menten constant for uptake of $E_1$S into hepatocytes ($K_m^{E_1S\text{in}}$), the vesicular intrinsic clearance for accumulation of $E_1$S ($CL_{\text{ves}}^{E_1S}$), the pooled kinetic constants for formation of the composite of $E_1$ metabolites $M$ ($V_{\text{max}}^{E_1 \rightarrow M}$ and $K_m^{E_1 \rightarrow M}$), the kinetic constants for $E_1$
Figure 4-1. A cellular kinetic model for the metabolism of estrone sulfate (EtS) and estrone (E₁) in intact zonal hepatocytes.
sulfation \( V_{\text{max}}^{E_1 \rightarrow E_1} \) and \( K_{m}^{E_1 \rightarrow E_1} \), the latter constrained as \( V_{\text{max}}^{E_1 \rightarrow E_1} / CL_{\text{int}}^{E_1 \rightarrow E_1} \) and the tissue binding of \( E_1 \) were optimized by least square fitting with appropriate weighting schemes of \( 1/\text{observation} \) (for data increasing in value) and \( 1/\text{observation}^2 \) (for data decreasing in value). The goodness of fit was viewed with respect to the coefficient of variation (standard deviation of parameter estimate/parameter value), the residual plot and the Model Selection Criterion (MSC).

4.3.13 Statistics

All data were presented as the mean ± standard deviation, and the means were compared by use of ANOVA, with the \( P \) value of 0.05 as the level of significance. A paired \( t \)-test was used to compare the means for the data on zonal lysates since the same liver was used for the preparation of both PP and PV lysates; the level of significance was set at 0.05.

4.4 Results

4.4.1 Biochemical characterization of zonal hepatocytes and lysates

The marker enzymes, alanine aminotransferase and glutamine synthetase, verified that enriched PP and PV hepatocytes and lysates were isolated from different acinar regions of the rat liver. The PP/PV ratio of the marker enzyme alanine aminotransferase for zonal hepatocytes and zonal lysates were 1.9 ± 0.9 and 7.4 ± 4.2, respectively. The steeper acinar gradient of alanine aminotransferase content in zonal lysates was due to the fact that the preparations were obtained from the most distal and proximal acinar regions of the rat liver. However, the PP/PV ratio of the marker enzyme glutamine synthetase for the zonal hepatocytes and lysates were 0.027 ± 0.023 and 0.029 ± 0.013, respectively, and were similar. These PP/PV ratios were in agreement
with those reported by others (Lindros and Pentilla, 1984; Quistorff and Brunnet, 1987; Tosh et al., 1996; Tirona et al., 1999).

**4.4.2 Desulfation of $E_1S$ in the zonal S9 preparations**

Preliminary study failed to show $E_1$ sulfation in absence of PAPS within the zonal S9 preparations. Hence, the rate of $E_1$ formation from $E_1S$ in S9 represented the true desulfation rate. The results were best fit to the Michaelis-Menten equation (Table 4-1 and Fig. 4-2), yielding similar $K_m^{E_1S\rightarrow E_1} (30$ to $34 \, \mu M)$ and $V_{max}^{E_1S\rightarrow E_1} (2.4$ to $2.9 \, \text{nmol/min/S9 protein})$ for estrone desulfation in the PP and the PV preparations (ANOVA, $P > .05$). The desulfation $K_m (K_m^{E_1S\rightarrow E_1})$ was much lower in relation to that reported for another well-studied sulfate conjugate, 4-methylumbelliferyl sulfate (Chiba and Pang, 1993).

| Table 4-1. Desulfation kinetic parameters of $E_1S$ in zonal S9 preparation |
|---------------------------------|-----------------|-----------------|
| S9                             | $K_m^{E_1S\rightarrow E_1}$ (µM) | $V_{max}^{E_1S\rightarrow E_1}$ (nmol/min/mg S9 protein) |
| Periportal (PP)                | 30.4 ± 8.3     | 2.9 ± 1.0       |
| Perivenous (PV)                | 34.8 ± 6.6     | 2.4 ± 0.9       |

**4.4.3 Estrone sulfation in zonal lysates and the cytosol of zonal hepatocytes**

Estrone sulfotransferase activity was detected in zonal lysates and cytosolic fractions of PP and PV hepatocytes in the presence of PAPS. Desulfation of $E_1S$ in zonal lysate and zonal
cytosol was less than 1% over the experimental time and was negligible. Hence in both lysate and cytosol, the observations reflected the true and not the net sulfation activity of Est sulfotransferases. The activity was significantly higher in the PV region than the PP region (Table 4-2). The PV/PP ratios of estrone sulfation activities in the cytosolic fractions and lysates were 4.3 ± 2.2 and 3.1 ± 1.8, respectively.

**Figure 4-2.** Desulfation rates of E_{1}S in the S9 fraction of the periportal (PP) and perivenous (PV) hepatocytes

![Desulfation rates graph](image)

**4.4.4 Immunoblot of rSULT1A1, rSULT2A1, and rSULT1E1 in zonal hepatocytes and zonal lysates**

The rSULT1E1 protein in the cytosolic fraction of PV hepatocytes was 3.4 ± 1.1 times that of PP, and paralleled the results obtained previously for CYP1A2, a PV marker within the
same cell preparations (PV/PP ratio of 4.1 ± 3.3; Tirona et al., 1999). On the other hand, the rSULT2A1 protein in PP hepatocytes was 3.5 ± 2.7 times that of the PV hepatocytes, and the rSULT1A1 protein was not significantly different (ANOVA, $P > 0.05$; PV/PP ratio of 1.13 ± 0.23) among zonal hepatocytes (Fig. 4-3). Trends for the zonal lysates remained similar to those of the zonal hepatocytes: the rSULT1E1 protein in the PV lysates was 4.0 ± 3.0 times that of the PP lysates. The rSULT2A1 protein was exclusively found in the PP lysates, and the rSULT1A1 protein was again present evenly among zonal lysates (Fig. 4-4).

Table 4-2. Sulfation of E₁ in the cytosolic fraction of zonal hepatocytes and zonal lysates in the presence of the cosubstrate PAPS (700 μM)

<table>
<thead>
<tr>
<th></th>
<th>Sulfation Intrinsic Clearance of Estrone (μl/min/mg protein)</th>
<th>PP/PV Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocytes:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Periportal (PP)</td>
<td>6.1 ± 2.2</td>
<td>0.23</td>
</tr>
<tr>
<td>Perivenous (PV)</td>
<td>26.4 ± 9.5*</td>
<td></td>
</tr>
<tr>
<td>Lysates:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Periportal (LP)</td>
<td>8.3 ± 4.3</td>
<td>0.32</td>
</tr>
<tr>
<td>Perivenous (LV)</td>
<td>25.9 ± 6.8**</td>
<td></td>
</tr>
</tbody>
</table>

* Statistically different from periportal data (ANOVA, $P < 0.05$)
** Statistically different from periportal data (Paired $t$-test, $P < 0.05$)
Figure 4-3. Immunoblot analysis of rSULT1E1, rSULT2A1, and rSULT1A1 in cytosols obtained from zonal hepatocytes

rSULT1E1

rSULT2A1

rSULT1A1

Periportal Hepatocytes (PP)  Perivenous Hepatocytes (PV)

Optical Density (Arbitrary Units)

0  500  1000  1500  2000  2500  3000  3500

PP PV  PP PV  PP PV  PP PV  PP PV

rSULT1E1  rSULT2A1  rSULT1A1  CYP1A2
Figure 4-4. Immunoblot analysis of rSULT1E1, rSULT2A1, and rSULT1A1 in zonal cell lysates obtained from dual-digitonin-pulse liver preparation.
4.4.5 Protein binding of $E_1S$ and $E_1$

The binding data of $E_1S$ (0.8 to 250 μM) in extracellular medium were best described by the Langmuir binding isotherm (Eq. 4-1). Assuming that the molecular weight of binding proteins were around 82 kDa (the molecular weight of estrogen binding protein: Rao. 1998), the maximum binding capacity or $(n^{E_1S}[P_{total}]_{ec})$ and the dissociation constant $(K_D^{E_1S})$ for $E_1S$ were estimated to be $23.5 \pm 3.0$ μM and $23.4 \pm 4.5$ μM, respectively, and the number of binding sites $n^{E_1S}$ was $12 \pm 1.5$, based on the measured extracellular protein concentration of $0.16 \pm 0.03$ mg/ml (Fig. 4-5A). The unbound fractions of $E_1S$ at 1 and 125 μM in the extracellular medium was around 0.5 and 0.8, respectively. In tissue, where the cellular protein concentration ($1.6 \pm 0.3$ mg/ml) was about 10 times the extracellular concentration, the unbound fractions of $E_1S$ in tissues were calculated according to Eq. 4-3, based on the $K_D^{E_1S}$ and $n^{E_1S}$. The unbound fractions of $E_1S$ in tissue were predicted to vary from 0.1 to 0.7, as shown in Fig. 4-5B.

When the binding study was repeated for tracer $E_1$, a much higher unbound fraction of $0.84 \pm 0.03$ was found. Again, due to the poor aqueous solubility of $E_1$, binding at higher $E_1$ concentrations could not be studied.

4.4.6 Metabolism of $E_1S$ in intact zonal hepatocytes

Concentration-dependent metabolism of $E_1S$ was observed in intact PP and PV hepatocytes (Fig. 4-6A). For both PP and PV hepatocyte preparations, biphasic elimination patterns were observed for the lower concentrations of $E_1S$ (< 25 μM) whereas mono-exponential decay profiles were observed at the highest concentration of $E_1S$ used (125 μM). The patterns of $E_1S$ in extracellular medium paralleled those in the incubation mixture (Fig. 4-6B) and were similar for both PP and PV cells. However, the pattern differed dramatically in the cell wherein cellular concentrations of $E_1S$ were much higher than those extracellularly
Figure 4-5. (A) Protein binding profile of E₁S in extracellular medium, and (B) predicted unbound fractions of E₁S in extracellular medium and tissue.
Figure 4-6. Time- and concentration-dependent profiles for incubation study of $E_1S$ in intact periportal (PP) and perivenous (PV) hepatocytes.

(A) $E_1S$ in incubation mixture

(B) $E_1S$ in extracellular medium

(C) $E_1S$ in cell

(D) $E_1$ formed in incubation mixture

(E) $E_1$ formed in extracellular medium

(F) $E_1$ formed in cell

<table>
<thead>
<tr>
<th>PP</th>
<th>PV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 $\mu$M $E_1S$</td>
<td>■ ■</td>
</tr>
<tr>
<td>5 $\mu$M $E_1S$</td>
<td>■ ■</td>
</tr>
<tr>
<td>25 $\mu$M $E_1S$</td>
<td>■ ■</td>
</tr>
<tr>
<td>125 $\mu$M $E_1S$</td>
<td>■ ■</td>
</tr>
</tbody>
</table>
(Fig. 4-6C), yielding apparent tissue to medium partitioning ratios of greater than unity in both PP and PV cells (Fig. 4-7A). The apparent partition coefficients of E₁S at equilibrium decreased with increasing E₁S concentration, and were similar for both PP and PV cells (Fig. 4-7B). As shown in Table 4-3, nonlinear kinetics were shown to exist with increasing E₁S dose. Values of the AUC of E₁S were higher in PP than in PV hepatocytes, although statistical significance was not found (Table 4-3). The apparent clearance of E₁S [dose/AUC_{E₁S}^{E₁S}(0→∞)] decreased with increasing dose, and was lower in PP hepatocytes than in PV hepatocytes. Again, statistical difference was not found due to the large inter-animal variability.

Patterns of E₁ in the extracellular medium (Fig. 4-6E) and incubation mixture (Fig. 4-6D) were similar to that for the cell (Fig. 4-6F), but the decay of E₁ was faster than that of E₁S (Fig. 4-6). The terminal half-lives of E₁ differed for each dose (cf. Fig. 4-6C with Fig. 4-6F). At the lower initial concentrations of E₁S used for incubation with hepatocytes, greater E₁ levels existed in PP hepatocytes than in PV hepatocytes. Values of the AUC of E₁ were higher in PP than in PV hepatocytes, and statistical significance was found for AUC_{E₁}^{E₁}(0→30 min) for the lower concentrations of E₁S employed for study (Table 4-3). At higher initial concentrations of E₁S (> 5 μM), the decay half-life of E₁ in the cell was more prolonged, suggesting that the enzymes for the metabolism of estrone had become saturated.

### 4.4.7 Fitted results for E₁S and E₁ in intact zonal hepatocytes with the kinetic model

When simultaneous fitting was performed on the total, extracellular and cellular E₁S and E₁ data for each set of experiments consisting of four E₁S initial concentrations and the same pool of hepatocytes, good fits were obtained although high coefficients of variation were found
Figure 4-7. Partition coefficients of E₁S for incubation study of E₁S: (A) Time-dependent profiles for the partition coefficients of E₁S, cellular concentration/extracellular concentration, in relation to the different initial concentration of E₁S, and (B) partition coefficients of E₁S at equilibrium for the various initial concentrations of E₁S (1 to 125 μM)
Table 4-3. Clearances (CL) and area under the concentration-time profiles (AUC) of EiS and E1 in periportal (PP) and perivenous (PV) hepatocyte incubation systems (n = 5)

<table>
<thead>
<tr>
<th>EiS (µM)</th>
<th>AUC of EiS (µM.min/10^6 cells)</th>
<th>CL_{app} of EiS^a (ml/min/10^6 cells)</th>
<th>AUC of E1 (µM.min/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP</td>
<td>Cellular^b (0' – 30') Extracellular^b (0' – 30') Extracellular^c (0' – ∞)</td>
<td>Cellular^b (0' – 30') Extracellular^b (0' – 30')</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>74 ± 31 2.6 ± 0.9 3.0 ± 1.1</td>
<td>0.34 ± 0.12</td>
<td>60 ± 27 0.71 ± 0.24</td>
</tr>
<tr>
<td>5</td>
<td>560 ± 147 18 ± 3.3 20 ± 3.9</td>
<td>0.25 ± 0.05</td>
<td>300 ± 152 6.2 ± 1.7</td>
</tr>
<tr>
<td>25</td>
<td>3668 ± 1123 215 ± 27 236 ± 30</td>
<td>0.106 ± 0.014</td>
<td>2052 ± 1738 57 ± 14</td>
</tr>
<tr>
<td>125</td>
<td>18704 ± 7102</td>
<td>2372 ± 325 2634 ± 365</td>
<td>0.047 ± 0.007</td>
</tr>
<tr>
<td>PV</td>
<td>1</td>
<td>61 ± 14 2.5 ± 1.4 2.7 ± 1.6</td>
<td>0.37 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>432 ± 161 17 ± 8.2 19 ± 9.1</td>
<td>0.26 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>3366 ± 1340</td>
<td>212 ± 75 227 ± 81</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>18908 ± 4489</td>
<td>2363 ± 322 2586 ± 364</td>
</tr>
</tbody>
</table>

^a CL_{app} = \frac{\text{Dose}}{\text{AUC}_{EiS} (0->∞)}

^b AUC of 0 min to 30 min, estimated by trapezoidal method

^c AUC of 0 min to infinity; the AUC up to 30 min was estimated using trapezoidal method, and this was added to C_{30}/k (concentration at 30 min divided by the first order decay constant, k). assuming log-linear decline

* Statistically different from periportal data (ANOVA, P < 0.05)
associated with the fitted parameters. The optimized fit that considered tissue binding and vesicular storage of E₁S is presented in Fig. 4-6, and the mean of the optimized parameters of five experiments and the assigned parameters are summarized in Table 4-4.

The uptake constant of E₁S, $K_m^{E_1,S}$, when optimized (10 to 12 μM), was only half of the in vitro value (24 μM), showing that the hepatocyte uptake clearance ($CL_{uptake}^{E_1}$: 246 to 282 μl/min/10⁶ cells or 31 to 35 ml/min/g liver; Table 4-5), although of high value under first order conditions, was readily saturated. The hepatocyte uptake clearance for E₁ that is assumed to mediate bidirectional transport, was even faster (1463 to 1484 μl/min/10⁶ cells). These values suggest that under first-order conditions, transport of the estrogenic compounds are flow-rate limited in the rat liver.

The $V_{max}^{E_1 \rightarrow E_1 S}$ for estrone sulfation was low, differing in both PP and PV cells (0.014 to 0.077 nmol/min/10⁶ cells), but the $K_m^{E_1 \rightarrow E_1 S}$ was of high affinity (4 to 6 μM) and was similar in PP and PV cells. These values suggest that estrone sulfation, when compared to estrone sulfate desulfation, is a high affinity but low capacity pathway that would rapidly become saturated. By constraining the $K_m^{E_1 \rightarrow E_1 S}$ as $CL_{int}^{E_1 \rightarrow E_1 S} / V_{max}^{E_1 \rightarrow E_1 S}$, we optimized the $V_{max}^{E_1 \rightarrow E_1 S}$ value; the converse procedure of constraining the $V_{max}^{E_1 \rightarrow E_1 S}$ as $CL_{int}^{E_1 \rightarrow E_1 S} / K_m^{E_1 \rightarrow E_1 S}$ resulted in a higher coefficient of variation for the estimate of $K_m^{E_1 \rightarrow E_1 S}$. The $V_{max}^{E_1 \rightarrow M}$ and $K_m^{E_1 \rightarrow M}$ for formation of other metabolites. M, were 5.9 to 9.4 nmol/min/10⁶ cells and 18 to 19 μM, respectively. in PP and PV cells, showing that formation of other estrone metabolites greatly exceeds that of E₁S and exhibits a greater PV abundance. These fitted results indicate that both the sulfation of E₁ and the formation of M in PV hepatocytes are significantly higher than those in PP hepatocytes (Tables 4-4 and 4-5). The fitted tissue unbound fraction for E₁ was low (0.025 to 0.03) and was likely due to the presence of the estrogen binding protein in hepatocytes (Rao, 1998).
### Table 4-4. Assigned and fitted parameters for the cellular kinetic model of E,S and E₁ in zonal hepatocytes

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Description</th>
<th>Periportal</th>
<th>Perivenous</th>
</tr>
</thead>
<tbody>
<tr>
<td>( v_{\text{E,S}}^{\text{max}} ) (^{a})</td>
<td>Maximum uptake velocity for (\text{E,S} ) (nmol/min/10^6 cells)</td>
<td>2.9</td>
<td>2.9</td>
</tr>
<tr>
<td>( k_{m}^{\text{E,S}} ) (^{a})</td>
<td>Michaelis-Menten constant for (\text{E,S} ) uptake ((\mu)M)</td>
<td>11.8 ± 0.9</td>
<td>10.3 ± 4.1</td>
</tr>
<tr>
<td>( p_{\text{diff}}^{\text{E,S}} ) (^{a})</td>
<td>Bidirectional transport constant for (\text{E,S} ) ((\mu)l/min/10^6 cells)</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>( p_{\text{diff}}^{E_1} )</td>
<td>Bidirectional transport constant for (E_1 ) ((\mu)l/min/10^6 cells)</td>
<td>1484 ± 242</td>
<td>1463 ± 149</td>
</tr>
<tr>
<td>( v_{\text{E,S} \rightarrow E_1}^{\text{max}} ) (^{b})</td>
<td>Maximum desulfation rate for (\text{E,S} ) (nmol/min/10^6 cells)</td>
<td>2.2</td>
<td>1.9</td>
</tr>
<tr>
<td>( k_{m}^{E_1 \rightarrow E_1} ) (^{b})</td>
<td>Michaelis-Menten constant for desulfation of (\text{E,S} ) ((\mu)M)</td>
<td>30.4</td>
<td>34.8</td>
</tr>
<tr>
<td>( v_{\text{E,S} \rightarrow E_1}^{\text{max}} ) (^{c})</td>
<td>Maximum sulfation rate for (E_1 ) (nmol/min/10^6 cells)</td>
<td>0.014 ± 0.015</td>
<td>0.077 ± 0.052*</td>
</tr>
<tr>
<td>( k_{m}^{E_1 \rightarrow E_1} ) (^{c})</td>
<td>Michaelis-Menten constant for sulfation of (\text{E,S} ) ((\mu)M)</td>
<td>4.4</td>
<td>5.9</td>
</tr>
<tr>
<td>( v_{\text{E} \rightarrow M}^{\text{max}} ) (^{d})</td>
<td>Maximum metabolism(^d) rate for (\text{E} ) (nmol/min/10^6 cells)</td>
<td>5.9 ± 2.0</td>
<td>9.4 ± 2.4*</td>
</tr>
<tr>
<td>( k_{m}^{E_1 \rightarrow M} ) (^{d})</td>
<td>Michaelis-Menten constant for metabolism(^d) of (E_1 ) ((\mu)M)</td>
<td>18 ± 3.0</td>
<td>19 ± 0.6</td>
</tr>
<tr>
<td>( CL_{\text{E,S}}^{E_1} )</td>
<td>Excretion of (\text{E,S} ) to intracellular vesicle ((\mu)l/min/10^6 cells)</td>
<td>0.66 ± 0.25</td>
<td>0.49 ± 0.23</td>
</tr>
<tr>
<td>( n_{\text{E,S}}^{E_1} ) (^{e})</td>
<td>Number of binding sites for (\text{E,S} )</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>( k_{\text{E,S}}^{\text{on}} )</td>
<td>Association constant for (\text{E,S} ) (1/min/(\mu)M/10^6 cells)</td>
<td>0.0014 ± 0.0001</td>
<td>0.0020 ± 0.0009</td>
</tr>
<tr>
<td>( k_{\text{E,S}}^{\text{off}} ) (^{f})</td>
<td>Dissociation constant for (\text{E,S} ) (1/min/10^6 cells)</td>
<td>0.033</td>
<td>0.046</td>
</tr>
<tr>
<td>( f_{\text{E_1}} )</td>
<td>Unbound fraction of intracellular (\text{E_1} )</td>
<td>0.030 ± 0.005</td>
<td>0.025 ± 0.004</td>
</tr>
<tr>
<td>( f_{\text{E_1}} ) (^{g})</td>
<td>Unbound fraction of extracellular (\text{E_1} )</td>
<td>0.84</td>
<td>0.84</td>
</tr>
<tr>
<td>( V_{c} ) (^{h})</td>
<td>Cellular volume (ml/10^6 cells)</td>
<td>0.0036</td>
<td>0.0041</td>
</tr>
<tr>
<td>( V_{\text{ec}} )</td>
<td>Extracellular volume (ml/10^6 cells)</td>
<td>0.9964</td>
<td>0.9959</td>
</tr>
</tbody>
</table>

All fitted values were described in mean ± standard deviation; \(n = 5\)

* Statistically different from periportal parameter (ANOVA, \(P < 0.05\))

\(^{a}\) Obtained from Tan et al. (1999) and scaled up as described in the method section

\(^{b}\) Scaled up from the desulfation rate constants of \(\text{E,S} \) obtained from S9 of zonal hepatocytes

\(^{c}\) Constrained as \( v_{\text{E,S} \rightarrow E_1}^{\text{max}} \) divided by \( CL_{\text{E_1}}^{E_1} \) (the scaled up \textit{in-vitro} intrinsic clearance of \(\text{E}_1\))

\(^{d}\) Glucuronidation, hydroxylation, and oxidation of \(\text{E}_1\) (other than sulfation of \(\text{E}_1\))

\(^{e}\) Division of the effective binding concentration of \(\text{E,S} \) by the extracellular protein concentration

\(^{f}\) Constrained as \( k_{\text{E,S}}^{\text{on}} \) multiplied by \( K_{D}^{\text{E,S}} \) (the dissociate binding constant of \(\text{E,S} \))

\(^{g}\) Unbound fraction of \(\text{E}_1 \) obtained in the extracellular medium

\(^{h}\) Obtained from García-Ruiz et al. (1994)
<table>
<thead>
<tr>
<th>Parameters(^a) ((\mu L/\text{min}/10^6 \text{ cells}))</th>
<th>Description</th>
<th>Periportal</th>
<th>Perivenous</th>
</tr>
</thead>
<tbody>
<tr>
<td>(CL_{\text{uptake}}^{E_{1}S})</td>
<td>Uptake clearance for (E_{1}S)(^b)</td>
<td>246</td>
<td>282</td>
</tr>
<tr>
<td>(P_{\text{diff}}^{E_{1}})</td>
<td>Bidirectional diffusion constant for (E_{1})</td>
<td>1484</td>
<td>1463</td>
</tr>
<tr>
<td>(CL_{\text{int}}^{E_{1}S \rightarrow E_{1}})</td>
<td>Intrinsic clearance for (E_{1}S) desulfation(^c)</td>
<td>72</td>
<td>55</td>
</tr>
<tr>
<td>(CL_{\text{int}}^{E_{1} \rightarrow E_{1}S})</td>
<td>Intrinsic clearance for sulfation of (E_{1})(^d)</td>
<td>3.2</td>
<td>13</td>
</tr>
<tr>
<td>(CL_{\text{int}}^{E_{1} \rightarrow M})</td>
<td>Intrinsic clearance for glucuronidation, hydroxylation, and oxidation of (E_{1}) (other than sulfation of (E_{1}))(^e)</td>
<td>328</td>
<td>495</td>
</tr>
<tr>
<td>(CL_{\text{ext}}^{E_{1}S})</td>
<td>Intrinsic clearance for excretion of (E_{1}S) into cellular vesicle</td>
<td>0.66</td>
<td>0.49</td>
</tr>
</tbody>
</table>

\(^a\) Values become ml/min/g liver when multiplied by the factor (125/1000)

\(^b\) Obtained as \(\frac{V_{\text{max}}^{E_{1}S \rightarrow E_{1}}}{K_{m}^{E_{1}S \rightarrow E_{1}}} \cdot \frac{E_{1}S_{\text{in}}}{\text{in}} + P_{\text{diff}}^{E_{1}S}\)

\(^c\) Obtained as \(\frac{V_{\text{max}}^{E_{1}S \rightarrow E_{1}}}{K_{m}^{E_{1}S \rightarrow E_{1}}} \cdot \frac{E_{1}S_{\text{in}}}{\text{in}}\)

\(^d\) Obtained as \(\frac{V_{\text{max}}^{E_{1} \rightarrow E_{1}S}}{K_{m}^{E_{1} \rightarrow E_{1}S}} \cdot \frac{E_{1}_{\text{in}}}{\text{in}}\)

\(^e\) Obtained as \(\frac{V_{\text{max}}^{E_{1} \rightarrow M}}{K_{m}^{E_{1} \rightarrow M}}\)

The inclusion of the vesicular compartment in modeling appeared justified since cellular accumulation of \(E_{1}S\) followed by only a gradual depletion of \(E_{1}S\) was observed. Indeed, absence of the cellular storage compartment of \(E_{1}S\) furnished a slightly inferior fit, predicting a slightly faster decay of \(E_{1}S\) and greater formation of \(E_{1}\) in the liver cell. The contents of \(E_{1}S\) and \(E_{1}\) in the extracellular and total medium were, however, affected only slightly (data not shown), since the total accumulation of \(E_{1}S\) in the vesicular space at 30 min amounted to only 2% of the dose. By contrast, the binding of \(E_{1}S\) in debris and in tissue was found to be of paramount importance.
Absence of binding resulted in very poor fits that predicted mono-exponential decay rate constants for $E_S$ in the extracellular medium, and the accumulation of $E_S$ in the cell at early time points (Fig. 4-6C) was greatly attenuated in absence of binding (data not shown). The accumulation pattern of $E_S$ in cell is hence attributed mostly to tissue binding and less to vesicular storage.

4.5 Discussion

Estrone sulfate plays a vital role in the cycling of estrogens and serves as a storage form of estrogen (highly bound to albumin) in the circulation. $E_S$, a common substrate of Oatp1, Oatp2, Oatp4, Ntcp, and OAT3, gains ready access into the liver tissue where it is desulfated to yield the active $E_1$. The cycling between $E_S$ and $E_1$ is influenced not only by metabolic activities on desulfation and sulfation, but also presence of other competitive pathways for $E_1$ metabolism, excretion of $E_S$, the transmembrane characteristics, and tissue protein binding, with recognition that some of the proteins that mediate the processes may be zonated in the acinus. Hence, we assessed the zonal, metabolic activities in tissue, and integrated these with the transport and binding activities to examine the influence of metabolic heterogeneity on the futile cycling of estrogens in intact, zonal hepatocytes. A notable observation is that, in contrast to parallel decay profiles in both extracellular medium for both parent and metabolite (Ebling and Jusko, 1996), we observed different decay half-lives for $E_S$ and $E_1$ in the hepatocyte system (Fig. 4-6).

From *in vitro* values of the kinetic constants for $E_S$ desulfation ($K_m^{E_S-E_1}$ of 30 and 35 $\mu$M and $V_{\text{max}}^{E_S-E_1}$ of 2.2 and 1.9 nmol/min/10⁶ cells, respectively, for PP and PV hepatocytes), there was no difference in metabolic activity for both the proximal and distal regions of the rat
liver. The observation was in good agreement with other findings on the homogeneous distribution of arylsulfatase C activity (Anundi et al., 1986). The $K_m$ values were within the range of previous studies - 11 $\mu$M from Evans et al. (1992) to 32 $\mu$M from Iwamori et al. (1976). By contrast, estrone sulfation was of higher affinity ($K_m^{E_1\rightarrow E_1S}$ of 4.4 to 5.9 $\mu$M) in both PP and PV regions, but the $V_{max}^{E_1\rightarrow E_1S}$ was much lower in value and differed between PP and PV hepatocytes (0.014 to 0.077 nmol/min/10$^6$ cells, respectively; Table 4-4). The observation was consistent with the trends on sulfation of tracer estrone in both hepatocytes and lysates (Table 4-2) as well as with immunoblot analyses of rSULT1E1 (Figs. 4-3 and 4-4). The PP/PV ratio of rSULT1E1 protein was consistent with those reported by others (Tosh et al., 1996; Matsui et al., 1998). But the low PV/PP ratio of rSULT2A1 was opposite to the observation on sulfation activities, suggesting that hydroxysteroid sulfotransferase contributes little to estrone sulfation. The even distribution of rSULT1A1 protein in the zonal cells also infers that phenol sulfotransferase only plays a minor role in estrone sulfation. This evidence confirms that sulfation of estrone is predominantly catalyzed by estrogen sulfotransferase in the presence of PAPS.

The desulfation intrinsic clearance ($CL_{int}^{E_1S\rightarrow E_1}$) was about 4- to 23-times higher than the sulfation intrinsic clearance of $E_1$ ($CL_{int}^{E_1\rightarrow E_1S}$; Table 4-5), and $E_1$ sulfation was the rate-limiting step in the futile cycling. In addition to $E_1$ sulfation, $E_1$ was metabolized to other metabolites (M) with a much higher intrinsic clearance ($CL_{int}^{E_1\rightarrow M}$; 328 and 495 $\mu$l/min/10$^6$ cells, respectively for PP and PV cells; Table 4-5), that showed a PV preponderance. The competitive metabolism of $E_1$ represents both glucuronidation by the UDP-glucuronosyltransferases that are localized pericentrally (Tosh and Burchill, 1996) and oxidation of $E_1$ by CYP 1A2 and 3A that are concentrated in the PV region (Oinonen et al., 1996). This “pooled” metabolic intrinsic clearance, $CL_{int}^{E_1\rightarrow M}$, was 38- to 106-times higher than the sulfation intrinsic clearance of $E_1$. 
Consequently, little El is resulfated back to form ElS. The higher activities for El sulfation and formation of M in the PV region translates to the higher accumulation of El in PP cells, as observed under low concentrations (cf. AUC values Table 4-3).

Upon comparison of the metabolic intrinsic clearances of El sulfation and E1S desulfation to those for transport, the hepatic uptake clearances greatly exceed the metabolic intrinsic clearances (Table 4-5). The transport clearance of E1S is rapid, but that for El is even faster. The transport clearance of E1S (CL_{uptake}^{E1S}; Table 4-5) is substantial. Under physiological and first-order conditions where both El and E1S exist in low concentrations (nM), transport should remain very rapid and unsaturated. At high concentrations of E1S, however, transport may become saturated at concentrations comparable to, or exceeding $K_m^{E1S}$. The value of the fitted $K_m^{E1S}$ is within the range of the $K_m$'s (4.5 to 27 μM) reported for the various transporters and was similar to the value of $K_m^{E1S}$ (24 μM) obtained in-vitro (Tan et al., 1999). Adoption of the in-vitro $K_m^{E1S}$ value (24 μM), however, furnished poorer fits. We found that the parameters for the transport systems of E1S obtained from fitting were similar for both PP and PV hepatocytes, and the finding suggests the uniform distribution of transporters in rat liver. Uniform acinar distributions were found for Ntcp (Stieger et al., 1994), Oatp1 (Abu-Zahra et al., 2000), and Oatp2 (Timna et al., 2000) in rat liver, and uptake of E1S was similar in zonal hepatocytes (Tan et al., 1999). Saturation in uptake had occurred within the concentration range studied in the hepatocyte system, and this was shown by the decreasing partition coefficients of E1S with increasing concentrations (Fig 4-7B). Consistent with lack of zonation in uptake, values of the equilibrium partition coefficients of E1S were similar for both PP and PV hepatocytes.

Although previous evidence has suggested that transport of El across the membrane might involve carriers (Rao et al., 1977), our data were consistent with a linear, transmembrane flux for El ($P_{diff}^{E1}$). The bidirectional uptake clearance for El (1463 to 1484 μl/min/10^6 cells) was
even faster than that for \( E_1 S \), and no difference was found among PV and PP hepatocytes. The rapid transport clearance of \( E_1 \) was congruent with parallel trends of \( E_1 \) in cellular and extracellular medium that suggest rapid equilibration (Figs. 4-6E and 4-6F). The high transmembrane flux of \( E_1 \) \((P_{\text{diff}}^{E_1})\) is probably due to the high lipophilicity of \( E_1 \); indeed, the octanol/water \( \log P \) value of \( E_1 \) is 3.1 (Howard and Meylan 1997).

The study is the first account on binding of substrates to cell debris that resulted during hepatocyte preparation. The presence of extracellular binding of \( E_1 S \) and \( E_1 \) has lead to the conclusion that an even tighter tissue binding exists (Fig. 4-5B). Extracellular binding would decrease the uptake of \( E_1 S \) and \( E_1 \), whereas cellular binding of \( E_1 S \) and \( E_1 \) entraps the species within the cell and impedes cellular elimination. Tissue binding of \( E_1 S \) and \( E_1 \) therefore exerts an important influence on the cellular kinetics of futile cycling of estrogens. Another issue that needs to be addressed with respect to tissue binding and metabolism is nonlinear tissue binding of \( E_1 S \), and \( K_{D}^{E_1 S} < K_{m}^{E_1 S \rightarrow E_1} \) (24 vs. 30 to 34 \( \mu M \)). The comparison of the \( K_m \) values suggests that, with increasing cellular concentrations of \( E_1 S \), saturation of tissue binding precedes the saturation of the metabolic enzymes for desulfation. A similar scenario - with \( K_D \) for vascular binding of a flow-limited substrate < the \( K_m \) - had resulted in nonlinearity in drug clearance (Chiba and Pang, 1993; Xu et al., 1993). The same consequence will result here with nonlinearity in tissue binding.

To understand the interplay among the nonlinearity in transport, tissue binding, and the presence of vesicular accumulation of \( E_1 S \) on the different \( t_{1/2} \)’s of \( E_1 S \) and of \( E_1 \), simulations were further performed with the fitted parameters, with the substitution single. nonsaturable uptake clearance of \( E_1 S \) \((CL_{\text{uptake}}^{E_1 S} \) of 246 \( \mu l/min/10^6 \) cells), then a 10x higher dissociation binding constant \((K_{D}^{E_1 S} \) was increased to 230 \( \mu M \)), and ultimately, an absence of vesicular accumulation of \( E_1 S \). When only linear transport was introduced, linear decay of extracellular \( E_1 S \) was
observed. But the difference in $t_{1/2}$'s of $E_1S$ and $E_1$ persisted (data not shown). The similarity in decay $t_{1/2}$'s of $E_1S$ and of $E_1$ for any given dose could only be attained when $CL_{\text{uptake}}^{E_1S}$ was high and linear (246 $\mu$l/min/10$^6$ cells), when $K^E_{E_1S}$ greatly exceeded $K^E_{m,E_1S-E_1}$, and when there was lack of vesicular accumulation of $E_1S$. The extracellular and cellular $E_1S$ contents would now decay in unison with those of $E_1$, and similar half-lives were attained for both drug and metabolite species, as expected of futile cycling phenomenon (Fig. 4-8). The pattern conforms to other reversible metabolic systems that describe the futile cycling between methylprednisolone and methylprednisone for which similar in vivo elimination half-lives were observed for both drug and metabolite (Ebling and Jusko, 1986). It may be thus be concluded that the nonlinearity in uptake and tissue binding, and the presence of vesicular accumulation of $E_1S$ had resulted in different decay half-lives for $E_1S$ and $E_1$ in the hepatocyte system.

In conclusion, both $E_1$ and $E_1S$ are rapidly taken up evenly into rat zonal hepatocytes. The sulfation of $E_1$ by estrogen sulfotransferase and the metabolism of estrone to other metabolites were more abundant in PV than in PP hepatocytes, although the desulfation of $E_1S$ was evenly distributed. The rate limiting factor for the futile cycling of $E_1S$ and $E_1$ was sulfation, since transport was rapid and the intrinsic clearance of $E_1S$ desulfation was higher than that of $E_1$ sulfation. The higher levels of $E_1$ and $E_1S$ in PP hepatocytes were due to the higher PV metabolic activity towards $E_1$ sulfation and the formation of other metabolites. Different decay half-lives for $E_1$ and $E_1S$ were observed, and this was due to nonlinear uptake, tissue binding, and vesicular uptake of $E_1S$ in the cell.
Figure 4-8. Simulated profiles of $E_1$S and $E_1$ in the hepatocyte incubation system: Profiles were based on the PP parameters and substitution of a single, linear transport clearance of $E_1$S (was set constant as 246 $\mu$l/min/10^6 cells), then a 10x higher binding dissociation constant (was increased to 230 $\mu$M), and absence of vesicular accumulation of $E_1$S.
Appendix

A cellular kinetic model was presented (Fig. 4-1). \([E_{1}S]_{c}, [E_{1}], [M],\) and \([P]_{c}\) denote the concentrations of \(E_{1}S, E_{1},\) metabolites of \(E_{1}\) other than \(E_{1}S,\) and protein in various compartments; subscripts \(ec, c,\) and \(ves\) represent the extracellular medium, the cellular space, and vesicular compartment, respectively. Description of the parameters was given in Table 4-3 and in the Experimental Procedure section.

The equations describing extracellular space (ec) for \(E_{1}\) and \(E_{1}S\) are as follows:

\[
\frac{d[E_{1}S_{\text{unbound}}]_{ec}}{dt} = \left\{ P_{\text{diff}}^{E_{1}S} [E_{1}S_{\text{unbound}}]_{c} + k_{\text{off}}^{E_{1}S} [E_{1}S_{\text{bound}}]_{ec} V_{ec} \right. \\
- \left. \left( \frac{V_{\text{max}}^{E_{1}S_{in}}}{K_{m}^{E_{1}S_{in}} + [E_{1}S_{\text{unbound}}]_{ec}} + P_{\text{diff}}^{E_{1}S} + k_{\text{on}}^{E_{1}S} [P_{\text{unbound}}]_{ec} V_{ec} \right) [E_{1}S_{\text{bound}}]_{ec} \right\} / V_{ec}
\]

\[
\frac{d[E_{1}S_{\text{bound}}]_{ec}}{dt} = k_{\text{on}}^{E_{1}S} [P_{\text{unbound}}]_{ec} [E_{1}S_{\text{unbound}}]_{ec} - k_{\text{off}}^{E_{1}S} [E_{1}S_{\text{bound}}]_{ec}
\]

The total concentration of extracellular \(E_{1}S\) is the sum of unbound and bound \(E_{1}S\) and is given by

\[
\frac{d[E_{1,\text{total}}]_{ec}}{dt} = \left\{ P_{\text{diff}}^{E_{1}} s_{E_{1}} [E_{1,\text{total}}]_{c} - P_{\text{diff}}^{E_{1}} s_{E_{1}} [E_{1,\text{total}}]_{ec} \right\} / V_{ec}
\]

The equation describing the amount of \(E_{1}S\) (\(E_{1}S_{ves}\)) effluxed into the vesicular space (ves) is:

\[
\frac{dE_{1}S_{ves}}{dt} = CL_{ves}^{E_{1}S} [E_{1}S_{\text{unbound}}]_{ec}
\]
The equations describing cellular space (c) for $E_1$, $E_1$ and $M$ are as follows:

\[
\frac{d[E_1S_{\text{unbound}}]_c}{dt} = \left\{ \left( \frac{V_{E_1S}^{in}}{K_m^E + [E_1S_{\text{unbound}}]_c} + P_{diff}^{E_1} \right) [E_1S_{\text{unbound}}]_c + k_{off}^{E_1S} [E_1S_{\text{bound}}]_c \right\} \frac{V_c}{V_c}
\]

\[
\frac{d[E_1S_{\text{bound}}]_c}{dt} = k_{on}^{E_1S} [P_{\text{unbound}}]_c \frac{[E_1S_{\text{unbound}}]_c}{[E_1S_{\text{bound}}]_c} - k_{off}^{E_1S} [E_1S_{\text{bound}}]_c \]

The total amount of intracellular $E_1S$ is the sum of unbound, bound, and vesicular contents of $E_1S$, and the total concentration of intracellular $E_1S$ is obtained upon division of the total amount of intracellular $E_1S$ by the cellular volume.

\[
\frac{d[E_1\text{total}]_c}{dt} = \left\{ P_{diff}^{E_1} f_c^{E_1} [E_1\text{total}]_c + \frac{V_{max}^{E_1S \rightarrow E_1} [E_1S_{\text{unbound}}]_c}{K_m^{E_1S \rightarrow E_1} + [E_1S_{\text{unbound}}]_c} \right\} \frac{V_c}{V_c}
\]

The total intracellular concentration of $M$ formed from estrone metabolism is

\[
\frac{d[M]_c}{dt} = \left\{ \frac{V_{max}^{E_1 \rightarrow M} f_c^{E_1} [E_1\text{total}]_c}{K_m^{E_1 \rightarrow M} + f_c^{E_1} [E_1\text{total}]_c} \right\} \frac{V_c}{V_c}
\]

The metabolic intrinsic clearance for desulfation is

\[
CL_{int}^{E_1 \rightarrow E_1S} = \frac{V_{max}^{E_1 \rightarrow E_1S}}{K_m^{E_1 \rightarrow E_1S}}
\]
Binding of E₁S in extracellular medium is described by the binding capacity ($n^{E₁S}[P_{total}]_ec$) (where $n^{E₁S}$ is the number of binding sites and $[P_{total}]_ec$ is the total protein concentration in the extracellular medium) and the binding dissociation constant ($K_D^{E₁S}$) as described below. We assumed that the cellular and the extracellular binding proteins have the same $n^{E₁S}$ and $K_D^{E₁S}$.

Thus, the binding capacity of cellular E₁S is obtained from the multiplication of the ratio of protein concentrations ($[P_{total}]_c /[P_{total}]_ec$) with the binding capacity ($n^{E₁S}[P_{total}]_ec$).

$$[P_{total}] = [P_{bound}] + [P_{unbound}]$$

The binding dissociation constant $K_D$ is the ratio of the on and off rate constants for binding.

$$K_D^{E₁S} = \frac{k_{off}^{E₁S}}{k_{on}^{E₁S}}$$
4.6 Statement of Significance of Chapter 4

In this chapter, we found that the sulfation of E₁ was the rate limiting step in the futile cycling and was mainly localized in the PV hepatocytes. In addition, lack of heterogeneity was found for both the desulfation of E₁S and the uptake of E₁. Furthermore, we found that the greater metabolism of E₁ in the PV region led to lower levels of E₁ and E₁S in the PV hepatocytes, and the nonlinear uptake and binding and the vesicular accumulation of E₁S resulted in different decay half-lives for E₁S and E₁.

4.8 Acknowledgments

We thank Dr. Charles N. Falany (University of Alabama, Birmingham, AL) for providing us with antibodies to rat liver phenol (minoxidil) sulfotransferase (rSULT1A1), hydroxysteroid (bile acid) sulfotransferase (rSULT2A1), and estrogen sulfotransferase (rSULT1E1). The assistance of Dr. Rommel G. Tirona in preparing zonal hepatocytes and lysates is gratefully acknowledged.
CHAPTER 5

FUTILE CYCLING OF ESTRONE SULFATE AND ESTRONE
IN THE PERFUSED RAT LIVER

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5.1 Abstract

The futile cycling of estrone sulfate (\(E_1S\)) and estrone (\(E_1\)) was investigated in the recirculating rat liver preparation. Although \(E_1S\) was not distributed into erythrocytes, the compound was highly bound to albumin (4% BSA, unbound fraction of 0.03 ± 0.01). By contrast, \(E_1\) was bound and metabolized to estradiol (\(E_2\)) by bovine erythrocytes of the perfusion medium, with metabolic clearances of 0.061 to 0.069 ml/min when normalized to the hematocrit. The presence of 4% BSA greatly reduced the red cell clearance of \(E_1\) to a minimum (0.0024 to 0.0031 ml/min normalized per unit hematocrit) due to strong binding to albumin (plasma unbound fraction of 0.05 ± 0.01). Despite the low unbound fractions of \(E_1S\) (0.027 ± 0.004) and \(E_1\) (0.036 ± 0.006) in perfusate (4% BSA and 20% erythrocytes), clearances of the simultaneously delivered tracers - [\(^3\)H]\(E_1S\) and [\(^14\)C]\(E_1\) - were high (0.53 ± 0.08 and 0.85 ± 0.2 ml/min/g liver, respectively) at the blood flow rate of 0.91 ± 0.1 ml/min/g liver. Although low levels of [\(^3\)H]\(E_1\) were detected following the tracer [\(^3\)H]\(E_1S\), both parent and metabolite species displayed similar decay half-lives characteristic of compounds undergoing futile cycling. The same decay profile was observed for [\(^14\)C]\(E_1\), but the half-life of the metabolite, [\(^14\)C]\(E_1S\) was more prolonged in comparison. A distributed-in-space liver model that included both the zonal and subcompartmentalization of metabolic enzymes in the cytosol (for sulfation) and endoplasmic reticulum (for desulfation and glucuronidation) was needed to interpret the data. In this model, the liver was divided into the perportal and perivenous units, within which higher perivenous distributions of \(E_1\) sulfation and glucuronidation activities and an even distribution of \(E_1S\) desulfation activity were described. The distributed-in-space liver model adequately described the perfusion data. However, in the absence of \(E_1\) partitioning into the endoplasmic reticulum compartment, parallel elimination profiles for \(E_1\) and \(E_1S\), characteristic of compounds undergoing futile cycling, were observed in the simulation study.
5.2 Introduction

The hepatic clearance of a drug is regulated by hepatic blood flow, vascular binding, transport, tissue binding, metabolism, and biliary excretion. Futile cycling, or the metabolic interconversion of two substrates involving different enzymes, is an additional variable that influences drug and metabolite clearances. Futile cycling has been noted between 4-methylumbelliferone (4-MU) and 4-methylumbelliferyl sulfate (4-MUS; Ratna et al., 1993) and methylprednisone and methylprednisolone (Ebling and Jusko, 1986). The futile cycling of estrone sulfate (E1S) and estrone (E1), which represents a pharmacologically important biocycle that conserves and regulates endogenous estrogens, however, has not been thoroughly investigated.

Since hepatic processing is a distributed-in-space phenomenon with uptake, metabolism, efflux occurring repeatedly in hepatocytes along the direction of flow, it is expected that the removal of E1S and E1 would be affected by a dual set of transporters and enzymes and their associated acinar heterogeneities in liver. Uptake of E1S into the liver involves passive diffusion and transport mediated by the organic anion transporting polypeptides (Oatp1, Oatp2 and Oatp4) (Jacquemin et al., 1994; Noé et al., 1997; Cattori et al., 2000), the multispecific organic anion transporter (Oat3) (Kusuhara et al., 1999), and the sodium-dependent taurocholate cotransporting polypeptide (Ntcp) (Hagenbuch et al, 1991). It is suspected that E1 rapidly diffuses through the cell membrane due to its high lipophilicity (Tan and Pang, 2000). However, a lack of acinar heterogeneity has been found for the transport of E1S (Tan et al., 1999) and E1 (Tan and Pang, 2000) in rat liver.

In rat hepatocytes, E1S and E1 are highly bound to liver tissue (Tan and Pang, 2000), and consequently, the high and nonlinear binding reduces the cellular unbound concentrations of E1S
and E₁ for metabolism and excretion. Estrone sulfate is mainly deconjugated by arylsulfatase C, a microsomal enzyme, to E₁, which can be further metabolized to estrone glucuronide (E₁G), estradiol (E₂), estriol, their glucuronide and sulfate conjugates, and other minor metabolites (Roy et al., 1987). Although arylsulfatase C is found to be evenly dispersed in the liver acinus, estrogen sulfotransferase, which is responsible for the sulfation of E₁, is predominantly localized in the perivenous region (Tosh et al., 1996, Tan and Pang, 2000). Both UDP-glucuronosyltransferase-1 (UGT1) and UGT2 families are found to glucuronidate estrone (Tukey and Strassburg, 2000), and UGT is predominantly localized in the perivenous region (Tosh and Burchill, 1996). The conjugates are found in bile, with Mrp2/cmoat mediating the biliary excretion of E₁G (Takikawa et al., 1996). But the transporter(s) involved in E₁S excretion into bile is yet to be investigated.

Drugs bound to vascular components, namely to plasma protein and red blood cells (RBC), are expected to be associated with reduced hepatic drug clearances (Gillette et al., 1973b: Pang and Rowland, 1977; Pang et al., 1995; Hinderling, 1997). Estrone is bound to both plasma protein and erythrocytes. Consequently, only 3% of estrone exist in the unbound form in human blood (Koefoed and Brahm, 1994). In addition, E₁ is also metabolized by 17β-hydroxysteroid dehydrogenase, a cytosolic enzyme in erythrocytes of animals (Tsang, 1976) and human (Mulder et al., 1972), to estradiol (E₂). By contrast, E₁S is highly bound to human plasma protein (1.6% unbound in plasma; Rosenthal et al., 1972), but not to erythrocytes. The determination of vascular binding and metabolism of E₁ and of protein binding of E₁S is another important aspect towards the understanding of factors impacting the hepatic clearances of E₁ and E₁S.

In this communication, the metabolic disposition of simultaneously delivered [³H]E₁S and [¹⁴C]E₁ was investigated with the recirculating perfused rat liver preparation. Use of dual radiolabeling of the precursor-product pair allows for full characterization of the differential
metabolism of $[^3\text{H}]E_1S$ and $[^{14}\text{C}]E_1$. The strategy is suitable for investigating the various influences of vascular and tissue binding, red blood cell (RBC) metabolism of $E_1$, transport, metabolism, and the zonal aspects on the futile cycling between $E_1S$ and $E_1$ in the liver, especially when the RBC distribution and metabolism of $E_1$ were fully characterized. Finally, a distributed-in-space model that embodied zonal and subcellular distribution of metabolic enzymes was developed to interpret the perfusion results.

5.3 Methods and Materials

5.3.1 Materials

$[6,7-^3\text{H}]E_1S$ (ammonium salt, specific activity, 53 Ci/mmol), $[6.7-^3\text{H}]E_1$ (specific activity, 40.6 Ci/mmol), and $[4-^{14}\text{C}]E_1$ (specific activity, 56.6 Ci/mol) were purchased from NEN Life Science Products (Boston, MA). All radiochemical purities, as found by high performance liquid chromatography (HPLC) or thin-layer chromatography (TLC), were greater than 95%. $E_1S$, $E_1$, $E_2$, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were the highest available grade.

5.3.2 Protein assay and hematocrit count

In all preparations, protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard. The hematocrit was measured by the capillary centrifugation method in a microhematocrit centrifuge (IEC MB Centrifuge, Damon).
5.3.3 BSA binding of tracer E₁, E₂, and E₃S

BSA binding of E₁, E₂, and E₃S were investigated by using a commercial ultrafiltration kit (Centricon 3, Amicon Inc, MA). Tracer [³H]E₁ (3.7 ± 0.1 x 10⁶ dpm/ml) [³H]E₃S (1.7 ± 0.1 x 10⁶ dpm/ml) or [³H]E₂ (1.1 ± 0.1 x 10⁶ dpm/ml) was added to 4% BSA (v/v) in Krebs Henseleit bicarbonate buffer. After incubating the mixture for 10 min at 37°C, an aliquot of the mixture (2 ml) was removed into a Centricon tube and then centrifuged at 2500g for 20 min. The radioactivities in the original mixture (0.2 ml) and the resulting filtrate (0.2 ml) were quantified by liquid scintillation spectrometry (model LS6800, Beckman Instruments Canada, Mississauga, ON). Leakage of BSA in the filtration device was less than 1% of the original protein solution.

5.3.4 Distribution and metabolism of E₁ in erythrocytes

Bovine erythrocytes (a generous gift from Ryding Regency Meat Packers Ltd, Toronto, On, Canada) were washed three times with saline and then twice with Lactated Ringer’s Solution (Baxter Corporation, Toronto, On, Canada). The distribution and metabolism of tracer [³H]E₁ (2.5 ± 0.4 x 10⁵ dpm/ml or 27 ± 4.2 nM) were studied with perfusate of different compositions: 20% and 60% RBC, in absence and presence of 4% BSA. Erythrocytes (20 or 60% v/v) and plasma (Krebs Henseleit bicarbonate buffer at pH 7.4 containing 0 or 4% BSA and [³H]E₁) were mixed and incubated under oxygenation (carbogen, 95% oxygen and 5% carbon dioxide, Canox Gas, Mississauga, ON) at 37°C in the reservoir of the TWO-TEN perfuser. Blood perfusate samples were taken at 1, 30, 60, 120, and 180 min, and the hematocrit of the blood perfusate was measured. The 20% and 60% RBC yielded hematocrits (HCT) of 0.15 ± 0.01 and 0.5 ± 0.03, respectively.
5.3.5 Rat liver perfusion

Male Sprague-Dawley rats (290-330 g; Charles River Canada, St. Constant, QC, Canada), which were fed *ad libitum*, were used for recirculation at 10 ml/min. and the temperature of the liver was maintained at 37°C with a heating lamp. Surgery was performed under pentobarbital anesthesia (50 mg/kg, intraperitoneal injection), and the surgical procedure and the perfusion apparatus were identical to those described by de Lannoy et al. (1993). The perfusion medium (pH 7.4) consisted of 20% washed bovine erythrocytes, 4% BSA, and 300 mg/dl glucose (50% dextrose injection USP; Travenol Canada, Mississauga, Ontario) in Krebs-Henseleit bicarbonate solution (v/v/v). The rat liver was recirculated with blank perfusate in the equilibration period (20 min) prior to receiving perfusion from a second reservoir (200 ml) containing $[^3]$H]E$_1$S (initial concentrations of $2.85 \pm 0.23 \times 10^5$ dpm/ml or $2.4 \pm 0.20$ nM) and $[^1]$C]E$_1$ (initial concentrations of $1.04 \pm 0.12 \times 10^5$ dpm/ml or $848 \pm 94$ nM). Reservoir perfusate (1-2 ml) was sampled at 0, 2.5, 10, 30, 60, 90, 110, 130, 150 min. The total volume removed from the reservoir was 7% (14 ml) of the initial volume (200 ml) and no attempt was made to correct for the loss in volume. Bile was collected at 5-10 min intervals, such that the mid-time of the interval corresponded to the sampling time of the reservoir.


The blood and its derived plasma obtained by instantaneous centrifugation (1.5 ml each) were immediately extracted into ethyl acetate (1:2, v/v). One aliquot (1 ml) of the ethyl acetate extract was directly subjected to liquid scintillation counting and the total count of the sample was determined against a calibration curve constructed of standards containing varying known
counts of $[^3\text{H}]E_1$ in perfusate and processed in the same fashion. Since both $[^3\text{H}]E_1$ and $[^3\text{H}]E_2$ were completely extracted into ethyl acetate (>$99\%$), the extraction method furnished a mixture of $[^3\text{H}]E_1$ and $[^3\text{H}]E_2$ in each sample except for time zero, when only $[^3\text{H}]E_1$ was present. The ratio of $[^3\text{H}]E_1/[^3\text{H}]E_2$ was given by TLC. A second aliquot (1 ml) of the ethyl acetate was spotted onto the Silica Gel GF (250 μm) TLC plate (Analtech, Newark, DE) which had been preloaded with $E_1$ and $E_2$ at the origin to separate $[^3\text{H}]E_1$ and $[^3\text{H}]E_2$. The plates were developed in a system of toluene:ethanol (9:1, v/v). Regions for $E_1$ and $E_2$ were visualized under UV light and scraped into mini-counting vials. After the addition of water (0.5 ml) and liquid scintillation fluid (5 ml, Ready Safe, Beckman Instruments, ON) into mini-counting vials, the radioactivity was quantified by liquid scintillation spectrometry (model LS6800, Beckman Instruments, Palo Alto, CA). Hence, the amounts of $[^3\text{H}]E_1$ and $[^3\text{H}]E_2$ in plasma and blood perfusate were quantified by the combined extraction – TLC method. The amounts of $E_1$ and $E_2$ in RBC were, however, calculated as differences between the quantities in plasma and blood perfusate of known hematocrit. The radioactivities were expressed as a percentage of the initial concentration of $[^3\text{H}]E_1$ used.

5.3.7 HPLC assays for quantitation of $E_1$G, $E_1$S, and $E_1$ in the liver perfusion studies

Acetonitrile, which contained 4 μM of danazol (the internal standard), was used to terminate any metabolic reactions, with 1:4 (v/v) volume ratio. All perfusate samples (1 to 2 ml) were immediately transferred to acetonitrile (4 to 8 ml). Contents of the deproteinized samples were dried under nitrogen (Canox Gas, Mississauga, ON) and analyzed by HPLC as described by Tan and Pang (2000). Standards of the calibration curve prepared with samples containing varying known counts of $[^3\text{H}]E_1$S and $[^{14}\text{C}]E_1$ were processed in the same fashion. Bile samples
were diluted 1:1 (v/v) with water, and 20 µl aliquots were directly counted. A portion of the
diluted bile (20 µl) was subjected to HPLC with internal standardization. The radioactivities in
bile and from HPLC radioelution were quantified by liquid scintillation spectrometry. Eluted
radioactivities of less than 3-times the background counts were treated as zeroes. All $^3$H- and
$^{14}$C-radioactivities quantified in the samples were higher than 3000 and 1000 dpm, respectively.

5.3.8 Kinetic modeling of [$^3$H]E$_1$ metabolism in erythrocytes and fitting

A series of cellular models were tested for their abilities to predict the disposition of
[$^3$H]E$_1$ and [$^3$H]E$_2$ in erythrocytes. The cellular, kinetic model that included plasma protein
binding and red cell binding and metabolism of E$_1$ (Fig. 5-1) best described the kinetics of E$_1$ and
E$_2$. Mass balanced rate equations (Eqs. 5-1 to 5-4) were written to describe the RBC distribution
and metabolism of E$_1$ and E$_2$ (Fig. 5-1). Oxidation of E$_2$ to E$_1$ was not included since preliminary
study revealed less than 1% metabolism of E$_2$ to E$_1$ over 3 h. The same was observed by Tsang
(1976).

We assumed that protein and red cell binding and debinding of E$_1$ and E$_2$ occur almost
instantaneously. Binding to plasma albumin results in plasma unbound fractions of E$_1$ ($f_{p E_1}^{ub}$) and
E$_2$ ($f_{p E_2}^{ub}$) whereas distribution in erythrocytes (RBC) yields unbound fractions of E$_1$ ($f_{RBC E_1}^{ub}$) and E$_2$
($f_{RBC E_2}^{ub}$). These unbound fractions in plasma and RBC allowed the estimation of the unbound
fraction in blood.

$$f_{blood} = \frac{f_p}{C_{blood} / C_p}$$  \hspace{1cm} \text{Eq. 5-1}

$$f_{blood} = \frac{1}{(1 - \text{HCT}) / f_p + \text{HCT} / f_{RBC}}$$  \hspace{1cm} \text{Eq. 5-2}

where $C_{blood}$ and $C_p$ are the blood and plasma concentrations, respectively.
Figure 5-1. Cellular kinetic model for the disposition of $E_1$ and $E_2$ in blood perfusate.
For estimation of the bound concentrations of $E_i$ in plasma ($[E_{i,\text{bound}}]_p$), we assume that $K_D^{E_i}$ (the binding dissociation constant of $E_i$) $> [E_{i,\text{unbound}}]_p$, and this exists for the tracer condition (Tan and Pang, 2000). The Langmuir binding isotherm simplifies to

$$[E_{i,\text{bound}}]_p = \frac{n^{E_i} [P\text{\text{total}}]_p [E_{i,\text{unbound}}]_p}{K_D^{E_i}}$$

Eq. 5-3

where $n^{E_i} [P\text{\text{total}}]_p$ is effective binding concentration, with $n^{E_i}$ being the number of binding site on BSA, and $[P\text{\text{total}}]_p$, the total protein concentration in plasma, and

$$[E_{i,\text{total}}]_p = [E_{i,\text{unbound}}]_p + [E_{i,\text{bound}}]_p$$

Eq. 5-4

The unbound fraction of $E_i$ in plasma ($f_{p}^{E_i}$) is derived from substitution of Eq.5-3 into Eq. 5-4:

$$f_{p}^{E_i} = \frac{[E_{i,\text{unbound}}]_p}{[E_{i,\text{total}}]_p} = \frac{1}{1 + \frac{n^{E_i} [P\text{\text{total}}]_p}{K_D^{E_i}}}$$

Eq. 5-5

The fixed protein concentration (4% BSA) for total perfusate in the in-vitro incubation study will change, however, when the concentration is expressed in relation to plasma when the hematocrit (0, 20% and 60% RBC) is modified. In order to relate to the in-vitro binding data of 4% BSA in plasma, the following correction was made. The plasma protein concentration ($[P\text{\text{total}}]_p$) is first related to the total protein concentration defined with respect to the whole blood perfusate, $[P\text{\text{total}}\text{\text{perfsuse}}]$, as given below:

$$[P\text{\text{total}}]_p = \frac{[P\text{\text{total}}\text{\text{perfsuse}}]}{(1-\text{HCT})}$$

Eq. 5-6

The corrected unbound fraction of $E_i$ in plasma of the 4% BSA perfusate is equivalent to the following unbound fraction in plasma from the in-vitro binding study (obtained from substitution of Eq.5-6 into Eq. 5-5):
Analogously, the plasma unbound fraction of $E_2$ was described by Eq. 5-8:

$$f_{p, \text{cor}}^E_2 = \frac{f_p^E_2 (1 - \text{HCT})}{1 - \text{HCT} f_p^E_2}$$  \quad \text{Eq. 5-8}

For the 20% RBC and 60% RBC, albumin-free perfusate, the unbound fractions of $E_1$ and $E_2$ equal unity. In the presence of 4% BSA, the plasma unbound fractions of $E_1$ and $E_2$ are related to the \textit{in-vitro} unbound plasma fractions of $E_1$ and $E_2$ ($f_{p, \text{cor}}^E_1$ and $f_{p, \text{cor}}^E_2$) as described by Eq. 5-7 and Eq. 5-8, respectively.

Clearance terms were normalized to the hematocrit for comparisons since different hematocrits were used for study. Analogously, the transmembrane clearances of $E_1$ and $E_2$ obtained from Koefoed and Brahm (1994) were also normalized to hematocrit (HCT) to provide $CL_{\text{diff, rbc}}^{E_1}$ and $CL_{\text{diff, rbc}}^{E_2}$, respectively. The hematocrit normalized intrinsic clearance of $E_1$, $CL_{\text{int, rbc}}^{E_1 \rightarrow E_2}$, is expressed as follows

$$CL_{\text{int, rbc}}^{E_1 \rightarrow E_2} = \frac{CL_{\text{int, rbc}}^{E_1 \rightarrow E_2}}{\text{HCT}}$$  \quad \text{Eq. 5-9}

Analogously, the hematocrit normalized permeation clearances of $E_1$ and $E_2$ across the red cell membrane are,

$$CL_{\text{diff, rbc}}^{E_1} = \frac{CL_{\text{diff, rbc}}^{E_1}}{\text{HCT}}$$  \quad \text{Eq. 5-10}

$$CL_{\text{diff, rbc}}^{E_2} = \frac{CL_{\text{diff, rbc}}^{E_2}}{\text{HCT}}$$  \quad \text{Eq. 5-11}

The hematocrit normalized clearances were multiplied back to the hematocrit to yield the corresponding diffusion and intrinsic clearance for various compositions of the perfusate, namely
20% and 60% RBC (see Eqs. 5-12 to 5-15). The equations that describe the rates of change of $E_1$ and $E_2$ in the plasma ($p$) are:

$$\frac{d[E_{1,\text{total}}]}{dt} = \frac{C_L^{E_1}_{\text{diff, rbc}} \text{ HCT} \left\{ f_{\text{hbc}}^{E_1} [E_{1,\text{total}}]_{\text{hbc}} - \left( \frac{f_p^{E_1} - \text{HCT} \ f_p^{E_1}}{1 - \text{HCT} \ f_p^{E_1}} \right) [E_{1,\text{total}}]_p \right\}}{V_p}$$

Eq. 5-12

$$\frac{d[E_{2,\text{total}}]}{dt} = \frac{C_L^{E_2}_{\text{diff, rbc}} \text{ HCT} \left\{ f_{\text{hbc}}^{E_2} [E_{2,\text{total}}]_{\text{hbc}} - \left( \frac{f_p^{E_2} - \text{HCT} \ f_p^{E_2}}{1 - \text{HCT} \ f_p^{E_2}} \right) [E_{2,\text{total}}]_p \right\}}{V_p}$$

Eq. 5-13

The equations that describe the changes of $E_1$ and $E_2$ in the RBC space (rbc) are:

$$\frac{d[E_{1,\text{total}}]}{dt} = \left\{ \frac{C_L^{E_1}_{\text{diff, rbc}} \text{ HCT} \left( \frac{f_p^{E_1} - \text{HCT} \ f_p^{E_1}}{1 - \text{HCT} \ f_p^{E_1}} \right) [E_{1,\text{total}}]_p}{V_{\text{rbc}}} - (C_L^{E_1}_{\text{diff, rbc}} + C_L^{E_1 \rightarrow E_2}_{\text{int, rbc}}) \text{ HCT} \ f_{\text{hbc}}^{E_1} [E_{1,\text{total}}]_{\text{rbc}} \right\}$$

Eq. 5-14

$$\frac{d[E_{2,\text{total}}]}{dt} = \left\{ \frac{C_L^{E_2}_{\text{diff, rbc}} \text{ HCT} \left( \frac{f_p^{E_2} - \text{HCT} \ f_p^{E_2}}{1 - \text{HCT} \ f_p^{E_2}} \right) [E_{2,\text{total}}]_p + C_L^{E_1 \rightarrow E_2}_{\text{int, rbc}} \text{ HCT} \ f_{\text{hbc}}^{E_1} [E_{1,\text{total}}]_{\text{rbc}}}{V_{\text{rbc}}} - C_L^{E_2}_{\text{diff, rbc}} \text{ HCT} \ f_{\text{hbc}}^{E_2} [E_{2,\text{total}}]_{\text{rbc}} \right\}$$

Eq. 5-15

The RBC unbound fractions of $E_1$ ($f_{\text{hbc}}^{E_1}$) and $E_2$ ($f_{\text{hbc}}^{E_2}$) and the metabolic intrinsic clearances were estimated by the least squares fitting procedure (SCIENTIST version 2: MicroMath Scientific Software, Salt Lake City, UT) with the weighting schemes of unity. The goodness of fit was viewed with respect to the coefficient of variation (standard deviation of parameter estimate/parameter value), the residual plot and the model selection criterion (MSC).

5.3.9 Kinetic modeling of $E_1$S and $E_1$ disposition in the recirculating rat liver preparation

A distributed-in-space model containing two sequential units, representing the periportal (PP) and perivenous (PV) regions of the liver, is the most adequate model that predicted the
disposition of \(E_1S\) and \(E_1\) in the perfused liver preparation (Fig. 5-2). In light of the known acinar localizations of estrone sulfotransferase and estrone sulfatase (Tan and Pang, 2000) and of UDP-glucuronosyltransferase (Tosh and Burchill, 1996) in the liver, the zonal metabolic activities could be assigned for each of the enzymes to reflect their enrichment patterns (Fig. 5-2, bottom panel). In this model, a reservoir compartment was included for recirculating of perfusate. Transfer of substrates occurs unidirectionally along the direction of flow from PP to PV compartment; linear transport is expected to prevail in view of the tracer conditions (Tan and Pang, 2000). Species such as \(E_1S\), \(E_1\), and \(E_1G\) were modeled. Other metabolites which were formed from \(E_1\) and \(E_1S\) \([E_2\) and estriol \((E_3)\), and their glucuronide and sulfate conjugates, and \(E_2S, E_2-3S-17G, E_3S,\) and \(E_3-3S-16G\)] were grouped as \(M'\).

A new feature of the model is the addition of an endoplasmic reticulum compartment, as proposed by Tirona and Pang (1996). This was necessary since levels of \([^3H]E_1\) were low but a greater extent of glucuronidation was observed subsequent to the \([^3H]E_1S\) dose. The added compartment segregates the cytosol from the endoplasmic reticulum where microsomal enzymes are found. Estrone sulfotransferase is placed in the cytosolic compartment, whereas estrone sulfatase and UDP-glucuronosyltransferase are placed in the endoplasmic reticulum compartment. The assigned volume of sinusoid \((V_s)\), cytosol \((V_c)\), endoplasmic reticulum \((V_{cr})\), and biliary compartment \((V_{bile})\) were 1.4 ml (Schwab et al., 1990), 7.3 ml (Pang et al., 1988), 0.2 ml (Tirona et al., 1996), and 0.07 ml, respectively. The volume of the biliary compartment was the summation of the biliary volume (0.044 ml; Reichen and Paumgartner 1980) and the void volume (about 0.026 ml) in the bile-duct cannula (PE50, Becton Dickinson, MD). The apparent biliary excretion clearance of \(E_1S\) or \(E_1G\) was calculated as the biliary excretion rate divided by the midpoint reservoir concentration of each respective species.
Figure 5-2. Schematic representation of the liver by a distributed-in-space model that embodied zonal and subcellular distribution of metabolic enzymes.
5.3.10 Fitting of data to the distributed-in-space model

Mass balanced rate equations (see Appendix) were written to describe events of the distributed-in-space model (Fig. 5-2). The amounts of drug and metabolite in both perfusate and bile were normalized by the dose. Binding to red cell and albumin is assumed to be rapidly equilibrative such that use of on- and off-rate constants is not necessary. Under this instance, the unbound fractions of E1S and E1 in whole blood perfusate equals that in plasma and in RBC. The unbound fraction in blood may be calculated from Eq. 5-1 and Eq. 5-2.

The clearance of E1 in erythrocytes (\(CL_{\text{rbc}}^{E1}\)) was determined as dose/area from the in-vitro RBC metabolism study. Values of the sinusoidal bidirectional transmembrane clearance of E1S (\(CL_{\text{er}}^{E1S}\)) and E1 (\(CL_{\text{er}}^{E1}\)) and the cytosolic unbound fraction of E1S (\(f_{\text{cyt}}^{E1S}\)) and E1 (\(f_{\text{cyt}}^{E1}\)) were taken from a previous study (Tan and Pang, 2000). Fitting was performed by a software package SCIENTIST (version 2; MicroMath Scientific Software, Salt Lake City, UT) based on experimentally obtained binding, metabolic, and transport parameters (Table 5-6). The fitted parameters – sinusoidal bidirectional transmembrane clearance of E1G (\(CL_{\text{er}}^{E1G}\)) the endoplasmic reticulum influx (\(CL_{\text{er}}^{E1 \text{ in}}\)) and efflux (\(CL_{\text{er}}^{E1 \text{ out}}\)) clearances of E1, the bidirectional transmembrane clearances of E1S (\(CL_{\text{er}}^{E1S}\)) and E1G (\(CL_{\text{er}}^{E1G}\)) for the endoplasmic reticulum compartment. the biliary intrinsic clearances of E1S (\(CL_{\text{bile}}^{E1S}\)) and E1G (\(CL_{\text{bile}}^{E1G}\)), the total sulfation intrinsic clearance of E1 (\(CL_{\text{int}}^{E1 \text{ S \rightarrow E1S}}\)), the total glucuronidation intrinsic clearance of E1 (\(CL_{\text{int}}^{E1 \text{ G \rightarrow E1G}}\)), the intrinsic clearance of E1 for the pooled metabolites (\(CL_{\text{int}}^{E1 \text{ M \rightarrow E1M}}\)), the total desulfation intrinsic clearance of E1S (\(CL_{\text{int}}^{E1S \rightarrow E1}\)), and other intrinsic clearances of E1S (\(CL_{\text{int}}^{E1S \rightarrow E1}\)) were optimized by least square fitting with appropriate weighting schemes of \(1/\text{observation}\) (for data increasing in value) and \(1/\text{observation}^2\) (for data decreasing in value). The goodness of fit was viewed with respect to the coefficient of variation (standard deviation of parameter estimate/parameter value). the residual plot and the model selection criterion (MSC).
5.3.11 Statistical analysis

All data were presented as the mean ± standard deviation, and the means were compared by use of ANOVA or the paired t-test, with the level of significance set at 0.05. The Model Selection Criterion (MSC) and the Akaike Information Criteria (AIC) (Akaike, 1974; Ludden et al., 1994) were used to select the appropriate model(s).

5.4 Results

5.4.1 Plasma binding of E₁S, E₁, and E₂

The unbound fraction of E₁S in 4% BSA plasma was 0.03 ± 0.01 (n = 3). However, the unbound fractions of E₁ and E₂ in perfusate with different compositions are summarized in Table 5-1.

5.4.2 Incubation of a tracer dose of [³H]E₁ in blood perfusate

The time courses for [³H]E₁ and [³H]E₂ in erythrocytes are shown in Fig. 5-3. Different areas under the concentration-time curves (AUC) for [³H]E₁ in the presence and absence of BSA were observed, and similar observations were found for [³H]E₂ in the presence and absence of BSA (Table 5-2). The RBC clearance of E₁ (CL\textsubscript{rbc\rightarrow E₂}; 0.035 ± 0.02 ml/min) in 60% RBC blood-perfusate was higher than that of the 20% RBC blood-perfusate (0.0092 ± 0.006 ml/min), and the rate of E₂ formation decreased in the presence of 4% BSA (Table 5-2, Fig. 5-3). The hematocrit normalized RBC clearances of E₁ (CL\textsubscript{rbc\rightarrow E₂} \div HCT) in 20% and 60% RBC perfusates were 0.061 ± 0.04 and 0.069 ± 0.04 ml/min/HCT, respectively, and these were dramatically reduced to 0.0031 ± 0.001 and 0.0024 ± 0.001 ml/min/HCT, respectively, in the presence of 4% BSA. The RBC to plasma partitioning ratios of E₁ and E₂ in the 20% and 60% RBC albumin-free perfusate were
higher than those in the presence of 4% BSA. In the presence of 4% albumin in perfusate, values of approximately unity was obtained for the RBC to plasma partitioning ratio for $E_1$ and $E_2$ (Fig. 5-4). Moreover, the values reached their equilibrium value almost immediately.

Table 5-1. Unbound fractions of $E_1$ and $E_2$ in perfusate of different compositions

<table>
<thead>
<tr>
<th>Composition of perfusate</th>
<th>Unbound fraction of $E_1$ in blood perfusate</th>
<th>Unbound fraction of $E_2$ in blood perfusate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% RBC 4% BSA$^a$</td>
<td>0.05 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>20% RBC 4% BSA$^{b,c}$</td>
<td>0.045 ± 0.043</td>
<td>0.034 ± 0.009</td>
</tr>
<tr>
<td>60% RBC 4% BSA$^{b,d}$</td>
<td>0.036 ± 0.008</td>
<td>0.029 ± 0.013</td>
</tr>
<tr>
<td>20% RBC 0% BSA$^{b,c}$</td>
<td>0.34 ± 0.12</td>
<td>0.38 ± 0.24</td>
</tr>
<tr>
<td>60% RBC 0% BSA$^{b,d}$</td>
<td>0.13 ± 0.06</td>
<td>0.17 ± 0.12</td>
</tr>
</tbody>
</table>

$^a$ Obtained from in-vitro binding study (mean ± SD, n = 3)
$^b$ Calculated from Eq. 5-1 (mean ± SD, n = 3)
$^c$ Hematocrit (HCT) was 0.15 ± 0.01
$^d$ Hematocrit (HCT) was 0.5 ± 0.03
Figure 5-3. Time-dependent profiles for incubation of tracer \([^{3}\text{H}]E_{1}\) in blood perfusates

(A) \(E_{1}\) in blood perfusate

(B) \(E_{1}\) in plasma

(C) \(E_{1}\) in erythrocyte

(D) \(E_{2}\) formed in blood perfusate

(E) \(E_{2}\) in plasma

(F) \(E_{2}\) formed in erythrocyte
5.4.3 Fitted results for the kinetic model of $E_1$ and $E_2$ in erythrocytes

Upon simultaneous fitting of the composite data for plasma and RBC, $E_1$ and $E_2$ rapidly reached equilibrium between plasma and erythrocytes in less than a minute (Fig. 5-4). The same observation was found by Koefoed and Brahm (1994). The fitted RBC unbound fractions of $E_1$ ($f^{E_1}_{nc}$) and $E_2$ were $0.073 \pm 0.032$ and $0.10 \pm 0.07$, respectively, showing that both $E_1$ and $E_2$ were highly bound to erythrocytes. The fitted RBC metabolic intrinsic clearance of $E_1$ ($\text{CL}_{int, nc}^{E_1\rightarrow E_2}$) was $0.11 \pm 0.07$ ml/min/HCT. Good fits were obtained although high coefficients of variation were found associated with the fitted parameters. The optimized fit that considered red cell and plasma binding $E_1$ and $E_2$ and metabolism of $E_1$ was presented in Fig. 5-3, and optimized parameters of five experiments are summarized in Table 5-3.

5.4.4 Metabolism of a tracer dose of $[^3\text{H}]E_1\text{S}$ in the perfused rat liver preparation

Since the plasma and RBC unbound fractions are known (Table 5-3), the unbound fraction of $E_1\text{S}$ in the blood perfusate ($f^{E_1\text{S}}_{\text{blood}}$) was estimated to be $0.027 \pm 0.004$ and $0.037 \pm 0.01$ according to Eqs. 5-1 and 5-2 ($f^{E_1\text{S}}_{nc} = 1$), respectively, and the apparent hepatic clearance of $[^3\text{H}]E_1\text{S}$ in the recirculating perfusion was $5.8 \pm 0.9$ ml/min (Table 5-4). Within 150 min of recirculation, a monoexponential decline of $[^3\text{H}]E_1\text{S}$ ($t_{1/2} = 27 \pm 1$ min) to around 1 % of its initial concentration was observed (Fig. 5-5A). Accumulation of $[^3\text{H}]E_1\text{G}$ was higher than that of $[^3\text{H}]E_1$ in perfusate within the first hour, followed by a gradual descent (Table 5-4: Fig. 5-5A).
Table 5-2. Clearances (CL) and area under the concentration-time profiles (AUC) of E₁ and E₂ in plasma and RBC after incubation with a tracer concentration of [³H]E₁ in blood perfusates

<table>
<thead>
<tr>
<th>Per fusates (n=5)</th>
<th>AUC of E₁ (nM.min)</th>
<th>CL&lt;sub&gt;rbc&lt;/sub&gt;&lt;sup&gt;E₁→E₂&lt;/sup&gt; of E₁&lt;sup&gt;a&lt;/sup&gt; (ml/min)</th>
<th>CL&lt;sub&gt;rbc&lt;/sub&gt;&lt;sup&gt;E₁→E₂&lt;/sup&gt; of E₁&lt;sup&gt;b&lt;/sup&gt; (ml/min/HCT)</th>
<th>AUC of E₂ (nM.min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RBC&lt;sup&gt;c&lt;/sup&gt;</strong></td>
<td><strong>Plasma&lt;sup&gt;c&lt;/sup&gt;</strong></td>
<td><strong>Plasma&lt;sup&gt;d&lt;/sup&gt;</strong></td>
<td><strong>RBC&lt;sup&gt;c&lt;/sup&gt;</strong></td>
<td><strong>Plasma&lt;sup&gt;c&lt;/sup&gt;</strong></td>
</tr>
<tr>
<td>20% RBC, 0% BSA&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1353 ± 118 ± 40*</td>
<td>419 ±</td>
<td>0.009.2 ±</td>
<td>0.061 ± 0.04*</td>
</tr>
<tr>
<td>60% RBC, 0% BSA&lt;sup&gt;f&lt;/sup&gt;</td>
<td>445 ± 169</td>
<td>41 ± 19*</td>
<td>79 ± 46*</td>
<td>0.035 ± 0.02*</td>
</tr>
<tr>
<td>20% RBC, 4% BSA&lt;sup&gt;e&lt;/sup&gt;</td>
<td>617 ± 527</td>
<td>521 ± 139</td>
<td>7949 ±</td>
<td>0.00046 ±</td>
</tr>
<tr>
<td>60% RBC, 4% BSA&lt;sup&gt;f&lt;/sup&gt;</td>
<td>297 ± 76</td>
<td>580 ± 311</td>
<td>2722 ±</td>
<td>0.0012 ±</td>
</tr>
</tbody>
</table>

<sup>a</sup> \[ \text{CL}_{rbc}^{E₁→E₂} = \frac{\text{Dose}}{\text{AUC}_{p}(0→\infty)} \]

<sup>b</sup> \[ \text{CL}_{rbc}^{E₁→E₂} = \frac{\text{CL}_{rbc}^{k₁→k₂}}{\text{HCT}} \]

<sup>c</sup> AUC of 0 min to 180 min, estimated by the trapezoidal method.

<sup>d</sup> AUC of 0 min to infinity; the AUC up to 180 min was estimated using the trapezoidal method, and this was added to C<sub>180/k</sub> (concentration at 180 min divided by the first order decay constant k), assuming log-linear decline.

<sup>e</sup> Hematocrit (HCT) was 0.15 ± 0.01

<sup>f</sup> Hematocrit (HCT) was 0.5 ± 0.03

* Statistically different from the value obtained in the presence of 4% BSA
Figure 5-4. RBC to plasma partitions of (A) $E_1$ and (B) $E_2$ in blood perfusates
Table 5-3. Assigned and fitted parameters for the cellular kinetic model that described the distribution and metabolism of E₁ and E₂ in blood

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$CL_{diff, rbc}^{E_1}$</td>
<td>Hematocrit normalized bidirectional RBC transmembrane constant for E₁ (l/min/HCT)</td>
<td>1.092&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$CL_{diff, rbc}^{E_2}$</td>
<td>Hematocrit normalized bidirectional RBC transmembrane constant for E₂ (l/min/HCT)</td>
<td>1.008&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$CL_{int, rbc}^{E_1 \rightarrow E_2}$</td>
<td>Hematocrit normalized metabolic intrinsic clearance of E₁ (ml/min/HCT)</td>
<td>0.11 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>$f_{pE_1}$</td>
<td>Unbound fraction of E₁ in 4% BSA plasma</td>
<td>0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>$f_{pE_2}$</td>
<td>Unbound fraction of E₂ in 4% BSA plasma</td>
<td>0.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>$f_{E_1}^{E_1}$</td>
<td>Unbound fraction of E₁ in RBC</td>
<td>0.073 ± 0.032&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>$f_{E_2}^{E_2}$</td>
<td>Unbound fraction of E₂ in RBC</td>
<td>0.10 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Obtained from Koefoed and Brahms (1994)
<sup>b</sup> Fitted value (mean ± SD, n = 5) from the cellular kinetic model (Fig. 5-1)
<sup>c</sup> Obtained from in-vitro binding study

During recirculation, the excreted amounts of $[^3]$H]E₁S and $[^3]$H]E₁G in bile increased with time and reached asymptotic levels at 150 min (Fig. 5-5B), and the total amounts of $[^3]$H]E₁S and $[^3]$H]E₁G found in bile were 2.5 ± 0.4 and 6.5 ± 0.6 % dose, respectively (Table 5-5). However, very little $[^3]$H]E₁ was detected in the bile (below the detection sensitivity). When the biliary excretion clearances for $[^3]$H]E₁S and $[^3]$H]E₁G were plotted against time, time-dependent declining profile was observed for $[^3]$H]E₁G (Fig. 5-6B); the bile flow declined with perfusion time, as expected of the rat liver preparation with depletion of bile salts (Fig. 5-6A). The excretion clearance of preformed $[^3]$H]E₁S reached asymptotic levels at 150 min, after reaching
distribution equilibrium in the system. At the end of the experiment, the radioactivities in reservoir, bile, and liver accounted for $3.5 \pm 0.4$, $54 \pm 3$, and $43 \pm 6\%$ dose, respectively.

### 5.4.5 Metabolism of a tracer dose of $[^{14}\text{C}]\text{E}_1$ in the perfused rat liver preparation

Estrone was highly cleared upon the recirculation of $[^{14}\text{C}]\text{E}_1$ with an apparent hepatic clearance of $9.4 \pm 2.2$ ml/min (Table 5-4), although the unbound fraction of $\text{E}_1$ in the blood ($f_{\text{blood}}^{\text{E}_1}$) was very low ($0.036 \pm 0.006$ and $0.053 \pm 0.02$ according to Eqs. 5-1 and 5-2, respectively). The unbound fraction of $\text{E}_1$ in blood ($0.036 \pm 0.006$) that was estimated according to Eq. 5-1 agreed well with that ($0.053 \pm 0.02$) based on (Eq. 5-2), suggesting that soundness in the fitted $f_{\text{unc}}^{\text{E}_1}$ ($0.073$). The concentrations of $[^{14}\text{C}]\text{E}_1$ declined monoexponentially with a half-life of $20 \pm 1.6$ min (Fig. 5-5C), but the elimination profiles of the metabolite, $[^{14}\text{C}]\text{E}_1\text{S}$. failed to decay in union within the observed time-frame. The concentrations of $[^{14}\text{C}]\text{E}_1\text{S}$ and $[^{14}\text{C}]\text{E}_1\text{G}$ were comparable and increased in the perfusate at the first hour, followed by gradual declines. During the time course of the experiment, the dose-corrected AUC of $[^{3}\text{H}]\text{E}_1\text{G}$. when extrapolated to time infinity, was not different from that of $[^{14}\text{C}]\text{E}_1\text{G}$, although large variations were observed (Table 5-4).
Figure 5.5. Time-dependent profiles of $[^3H]E_1S$ and $[^{14}C]E_1$ in the recirculating rat liver preparation.
Figure 5-6. (A) Bile flow rates of the recirculating rat liver preparation, and (B) apparent biliary excretion clearances of E₁S and E₁G.
Table 5-4. Hepatic clearances (CL) and area under the concentration-time profiles (AUC) of \[^3\text{H}\]\text{E}_1\text{S}, \[^{14}\text{C}\]\text{E}_1, and their metabolites in the recirculating rat liver preparation

<table>
<thead>
<tr>
<th></th>
<th>\text{E}_1</th>
<th>\text{E}_1\text{S}</th>
<th>\text{E}_1\text{G}</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>After [^3\text{H}]\text{E}_1\text{S} dose (n=4)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC(0-∞)(^a) (nM.min)</td>
<td>0.48 ± 0.14</td>
<td>85 ± 12</td>
<td>39 ± 19</td>
</tr>
<tr>
<td>AUC (0-∞)/Dose (min/ml)</td>
<td>0.0010 ± 0.0004</td>
<td>0.056 ± 0.036</td>
<td></td>
</tr>
<tr>
<td>CL(_\text{hepatic}^\text{E}_1\text{S}) (ml/min)</td>
<td>5.8 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>After [^{14}\text{C}]\text{E}_1 dose (n=4)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC(0-∞)(^a) (μM.min)</td>
<td>18.7 ± 5.5</td>
<td>3.5 ± 0.5</td>
<td>5.1 ± 1.6</td>
</tr>
<tr>
<td>AUC (0-∞)/Dose (min/ml)</td>
<td>0.021 ± 0.002</td>
<td>0.030 ± 0.006</td>
<td></td>
</tr>
<tr>
<td>CL(_\text{hepatic}^\text{E}_1) (ml/min)</td>
<td>9.4 ± 2.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) AUC of 0 min to infinity; the AUC up to 150 min was estimated using the trapezoidal method, and this was added to \(C_{150}/k\) (concentration at 150 min divided by the first order decay constant, \(k\)), assuming log-linear decline.

During recirculation, the amounts of \[^{14}\text{C}\]\text{E}_1\text{S} and \[^{14}\text{C}\]\text{E}_1\text{G} excreted in bile increased with time and reached asymptotic levels at 150 min (Fig. 5-5D). The total amounts of \[^{14}\text{C}\]\text{E}_1\text{S} and \[^{14}\text{C}\]\text{E}_1\text{G} found in the bile were 1.7 ± 0.01 and 8.2 ± 0.2 % dose, respectively (Table 5-5). Again, little \[^{14}\text{C}\]\text{E}_1 was detected in the bile (below the detection limit). When the biliary excretion clearances of \[^{14}\text{C}\]\text{E}_1\text{S} and \[^{14}\text{C}\]\text{E}_1\text{G} were plotted against time, time-dependent declining excretion clearances were observed for both \[^{14}\text{C}\]\text{E}_1\text{S} and \[^{14}\text{C}\]\text{E}_1\text{G} (Fig. 5-6B). At the
end of the experiment, the radioactivities in reservoir, bile, and liver radioactive accounted for 3.3 ± 0.7, 54 ± 6, and 43 ± 8 % dose.

5.4.6 Fitted results for the kinetic model of E₁ and E₁S in the perfused liver preparation

Upon simultaneous fitting of perfusate and bile data for each experiment consisting of [³H]E₁, [³H]E₁S, [³H]E₁G, [¹⁴C]E₁, [¹⁴C]E₁S, and [¹⁴C]E₁G in the same liver preparation, good fits were obtained although high coefficients of variation were found associated with the fitted parameters. The optimized fit that considered both zonal and subcellular localization of metabolic enzymes is presented in Fig. 5-5, and the mean of the optimized parameters of four experiments and the assigned parameters are summarized in Table 5-6. Inclusion of the endoplasmic reticulum compartment in modeling appeared justified since high partitioning of E₁ into the endoplasmic reticulum space was observed by Zakim and Vessey (1977). In fact, absence of the endoplasmic reticulum compartment furnished an inferior fit, predicting a higher formation of [³H]E₁ (Fig. 5-7). The fitted sinusoidal bidirectional transmembrane clearance for E₁G (\( CL_{E₁G} \)) was 339 ± 22 ml/min. The endoplasmic reticulum influx (\( CL_{E₁,m}^{E₁} \)) and efflux (\( CL_{E₁,out}^{E₁} \)) clearances of E₁ were 86 ± 40 and 17 ± 2 ml/min, respectively. The bidirectional transmembrane clearances of E₁S (\( CL_{E₁S}^{E₁} \)) and E₁G (\( CL_{E₁G}^{E₁} \)) for the endoplasmic reticulum were 742 ± 146 and 0.018 ± 0.001 ml/min, respectively. The biliary intrinsic clearances of E₁S (\( CL_{bile}^{E₁S} \)) and E₁G (\( CL_{bile}^{E₁G} \)) were 8.0 ± 0.1 and 1.8 ± 0.2 ml/min, respectively. The total sulfation intrinsic clearance of E₁ (\( CL_{int}^{E₁→E₁S} \)), the total glucuronidation intrinsic clearance of E₁ (\( CL_{int}^{E₁→E₁G} \)), and the total desulfation intrinsic clearance of E₁S (\( CL_{int}^{E₁S→E₁} \)) were 318 ± 38, 105 ± 30, and 332 ± 44 ml/min, respectively. Lastly, the “pooled” metabolic intrinsic clearances of E₁ (\( CL_{int}^{E₁→M'} \)) and E₁S (\( CL_{int}^{E₁S→M'} \)) were 255 ± 60 and 214 ± 57 ml/min, respectively.
Table 5-5. Biliary excretion of \([^3\text{H}]\text{E}_1\text{S}, \ [^{14}\text{C}]\text{E}_1,\) and their metabolites during their simultaneous delivery to the recirculating perfused rat liver preparation

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Bile</th>
<th>[^3\text{H}]\text{E}_1\text{S} (% \text{dose})</th>
<th>Bile</th>
<th>[^{14}\text{C}]\text{E}_1 (% \text{dose})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>(\text{E}_1\text{S})</td>
<td>(\text{E}_1\text{G})</td>
<td>(\text{M}^\prime)</td>
</tr>
<tr>
<td>1</td>
<td>54</td>
<td>2.1</td>
<td>7.3</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>57</td>
<td>2.3</td>
<td>5.9</td>
<td>49</td>
</tr>
<tr>
<td>3</td>
<td>49</td>
<td>2.8</td>
<td>6.2</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>54</td>
<td>2.8</td>
<td>6.8</td>
<td>44</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>54 ± 3</td>
<td>2.5 ± 0.4*</td>
<td>6.5 ± 0.6</td>
<td>45 ± 4</td>
</tr>
</tbody>
</table>

\(\text{M}^\prime\) denotes "pooled metabolites" of \(\text{E}_1\) excepting \(\text{E}_1\text{G}\) and \(\text{E}_1\text{S}\)

* Statistically different from the data of \([^{14}\text{C}]\text{E}_1\text{S}\)
Table 5-6. Assigned and fitted parameters for the distributed-in-space model

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Description</th>
<th>Assigned Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Q_{blood}$</td>
<td>Total blood flow rate (ml/min)</td>
<td>10</td>
</tr>
<tr>
<td>$Q_{bile}$</td>
<td>Bile flow rate (ml/min)</td>
<td>Regression $^a$</td>
</tr>
<tr>
<td>$V_r$</td>
<td>Volume of the reservoir (ml)</td>
<td>200</td>
</tr>
<tr>
<td>$V_s$</td>
<td>Volume of the sinusoidal compartment (ml)</td>
<td>1.4$^b$</td>
</tr>
<tr>
<td>$V_{cyst}$</td>
<td>Volume of the cytosolic compartment (ml)</td>
<td>7.3$^c$</td>
</tr>
<tr>
<td>$V_{er}$</td>
<td>Volume of the endoplasmic reticulum compartment (ml)</td>
<td>0.2$^d$</td>
</tr>
<tr>
<td>$V_{bile}$</td>
<td>Volume of the biliary compartment (ml)</td>
<td>0.07$^e$</td>
</tr>
<tr>
<td>$CL_{E_i}^{E_i}$</td>
<td>Sinusoidal bidirectional transmembrane clearance for $E_i$ (ml/min)</td>
<td>2026$^f$</td>
</tr>
<tr>
<td>$CL_{E_i}^{E_iS}$</td>
<td>Sinusoidal bidirectional transmembrane clearance for $E_iS$ (ml/min)</td>
<td>368$^f$</td>
</tr>
<tr>
<td>$CL_{E_i}^{E_iG}$</td>
<td>Sinusoidal bidirectional transmembrane clearance for $E_iG$ (ml/min)</td>
<td>339 ± 22$^g$</td>
</tr>
<tr>
<td>$CL_{er}^{E_i}$</td>
<td>Endoplasmic reticulum influx clearance for $E_i$ (ml/min)</td>
<td>86 ± 40$^g$</td>
</tr>
<tr>
<td>$CL_{er}^{E_i}$</td>
<td>Endoplasmic reticulum efflux clearance for $E_i$ (ml/min)</td>
<td>17 ± 2$^e$</td>
</tr>
<tr>
<td>$CL_{er}^{E_iS}$</td>
<td>Endoplasmic reticulum bidirectional transmembrane clearance for $E_iS$ (ml/min)</td>
<td>742 ± 146$^g$</td>
</tr>
<tr>
<td>$CL_{er}^{E_iG}$</td>
<td>Endoplasmic reticulum bidirectional transmembrane clearance for $E_iG$ (ml/min)</td>
<td>0.018 ± 0.001$^g$</td>
</tr>
<tr>
<td>$CL_{bile}^{E_iS}$</td>
<td>Biliary intrinsic clearance for $E_iS$ (ml/min)</td>
<td>8.0 ± 0.1$^g$</td>
</tr>
<tr>
<td>$CL_{bile}^{E_iG}$</td>
<td>Biliary intrinsic clearance for $E_iG$ (ml/min)</td>
<td>1.8 ± 0.2$^g$</td>
</tr>
<tr>
<td>$f_{blood}^{E_i}$</td>
<td>Unbound fraction of $E_i$ in blood</td>
<td>0.036$^h$</td>
</tr>
<tr>
<td>$f_{blood}^{E_iS}$</td>
<td>Unbound fraction of $E_iS$ in blood</td>
<td>0.027$^h$</td>
</tr>
<tr>
<td>$f_{cyst}^{E_i}$</td>
<td>Cytosolic unbound fraction of $E_i$</td>
<td>0.03$^i$</td>
</tr>
<tr>
<td>$f_{cyst}^{E_iS}$</td>
<td>Cytosolic unbound fraction of $E_iS$</td>
<td>0.09$^i$</td>
</tr>
<tr>
<td>$CL_{rbc}^{E_i}$</td>
<td>RBC clearance of $E_i$ (ml/min)</td>
<td>0.00046$^j$</td>
</tr>
<tr>
<td>$CL_{int}^{E_i}$</td>
<td>Total sulfation intrinsic clearance of $E_i$ (ml/min)</td>
<td>318 ± 38$^g$</td>
</tr>
<tr>
<td>$CL_{int}^{E_i}$</td>
<td>Total glucuronidation intrinsic clearance of $E_i$ (ml/min)</td>
<td>105 ± 30$^g$</td>
</tr>
<tr>
<td>$CL_{int}^{E_i}$</td>
<td>Other intrinsic clearance of $E_i$ (ml/min)</td>
<td>255 ± 60$^g$</td>
</tr>
<tr>
<td>$CL_{int}^{E_iS}$</td>
<td>Total desulfation intrinsic clearance of $E_iS$ (ml/min)</td>
<td>332 ± 44$^g$</td>
</tr>
<tr>
<td>$CL_{int}^{E_iS}$</td>
<td>Other intrinsic clearance of $E_iS$ (ml/min)</td>
<td>214 ± 57$^g$</td>
</tr>
</tbody>
</table>

$^a$ Based on regression of the bile flow rate vs time profile ($Q_{bile} = 0.0114 - 0.000029t$);
$^b$ obtained from Schwab et al (1990); $^c$ obtained from Pang et al (1988); $^d$ obtained from Tirona et al (1996); $^e$ the summation of the biliary volume (0.044 ml; Reichen and Paumgartner, 1980) and the void volume (about 0.026 ml) in the bile-duct cannula; $^f$ scaled-up from Tan and Pang (2000); $^g$ fitted value (mean ± SD, n = 4); $^h$ calculated from in-vitro binding study; $^i$ obtained from in-vitro RBC metabolism study.
5.4.7 Further simulation for understanding the futile cycling kinetics of E₁ and E₁S in the perfused liver preparation.

Simulations were further performed based on the fitted and assigned parameters shown in Table 6. If rapid equilibration of the species existed between the cytosolic and endoplasmic reticulum compartments (transport clearances of 1000 ml/min for E₁), similar elimination profile for E₁S and E₁ would result pursuant to [³H]E₁S and [¹⁴C]E₁ dosing as expected of futile cycling (Fig. 5-7), and the observed, discrepant half-lives of E₁S resulting from tracer [¹⁴C]E₁ dose and not the [³H]E₁S dose now disappeared.

For understanding the effect of futile cycling on the clearances of estrone and estrone sulfate, the total sulfation intrinsic clearance of E₁ (\(CL_{int}^{E₁S}E₁\)) was set to zero to eliminate futile cycling of E₁S. The result was the accumulation of [³H]E₁ upon elimination of the re-sulfation pathway after the [³H]E₁S dose (Fig. 5-8A), whereas the profiles of [³H]E₁S and [³H]E₁G remained virtually unchanged. When the total desulfation intrinsic clearance of E₁S (\(CL_{int}^{E₁S}E₁\)) was set as zero to prevent futile cycling of E₁, a greater accumulation of the [¹⁴C]E₁S resulted and formation of [¹⁴C]E₁G was reduced after the [¹⁴C]E₁ dose; the elimination profile of [¹⁴C]E₁ remained the same (Fig. 5-8B).
Figure 5-7. Simulated profiles of $[^{3}H]{E}_1S$, $[^{14}C]{E}_1$, and their metabolites in the recirculating rat liver preparation:
Profiles were based on rapid equilibration of all species between the cytosolic and endoplasmic reticulum compartments.
Figure 5-8. Simulated profiles of $E_1S$, $E_1$, and $E_1G$ following the administration of $[^{3}H]E_1S$ in absence of sulfation, with the sulfation intrinsic clearance of $E_1$ equal to zero (A) and following the administration of $[^{14}C]E_1$ with the desulfation intrinsic clearance of $E_1S$ equal to zero (B), in the recirculating rat liver preparation.
5.5 Discussion

The phenomena of erythrocyte binding and metabolism have often been neglected in pharmacokinetics. However, there is increasing evidence that erythrocytes play an important role in drug disposition since they are strong binders of drugs. Extensive erythrocyte binding of the carbonic anhydrase inhibitor, acetazolamide (Wallace et al., 1977) and the immunosuppressive agent, tacrolimus (Piekoszewski et al., 1993), have been demonstrated. In addition, it has been reported that when the binding capacities of plasma proteins become saturated, erythrocytes can also bind significant amounts of estrogens (Challis et al., 1973). Hence, in the presence of drug-drug interaction or in the disease-state, the erythrocytes play an important role in the vascular binding and metabolism of E₁ and E₂.

In this communication, we had characterized the erythrocyte distribution of E₁ and E₂ and the RBC metabolism of E₁ in the presence and absence of 4% BSA. Rapid equilibrium was reached for E₁ and E₂ between plasma and RBC (Fig. 5-3), justifying use of the unbound fractions of E₁ and E₂ in blood instead of the discrete association and dissociation rate constants in the fitting procedure. The observation was consistent with rapid exchange of E₁ and E₂ into erythrocytes due to their high lipophilicity (the octanol/water log P value of E₁ and E₂ are 3.1 and 4.0, respectively; Howard and Meylan, 1997) and their rapid dissociation from erythrocytes as suggested by Koefoed and Brahm (1994). Two conclusions may be made regarding the metabolism of E₁ in the blood perfusate. Firstly, the presence of a higher hematocrit in blood increased the conversion of E₁ to E₂ and rendered a higher RBC clearance of E₁ due to the presence of higher quantities of 17β-hydroxysteroid dehydrogenase (Table 5-2). Upon normalization to the hematocrit, the same RBC clearance of E₁ was obtained for both 60% and 20% RBC perfusate. Secondly, E₁ was bound more strongly to BSA than to erythrocytes (Fig. 5-4) and the presence of BSA greatly decreased the distribution of E₁ into erythrocytes and reduced
the conversion of E₁ to E₂ (Table 5-2). Expectedly, the RBC/plasma partition coefficients of E₁ and E₂ decreased in the presence of 4% BSA (Fig. 5-4) due to the stronger binding to BSA.

Since BSA greatly reduced the RBC metabolism of E₁ in blood, we chose the perfusate of 4% BSA and 20% RBC that exhibited minimum RBC metabolism of E₁ but delivered adequate oxygenation to the perfused rat liver preparation. The simultaneous delivery of [³H]E₁S and [¹⁴C]E₁ to the same liver allowed for a full characterization of the differential metabolism of [³H]E₁S and [¹⁴C]E₁. It was expected that, under tracer conditions, the nonlinearity observed previously in hepatocytes (Tan and Pang, 2000) would be obviated, and parallel decay patterns for E₁ and E₁S would surface. The study design was expected to reveal the underlying influence of vascular binding, transport, tissue binding, metabolism, and excretion on the hepatic clearances of E₁S and E₁. The unbound fractions of E₁S and E₁ in the blood were constant (0.027 and 0.036, respectively) for the recirculating perfusion of the rat liver preparation. The transmembrane clearances of E₁S (33 ml/min/g liver) and E₁ (184 ml/min/g liver) found in a previous study (Tan and Pang, 2000) were higher than their respective hepatic clearances (0.53 ± 0.08 ml/min/g liver for E₁S and 0.85 ± 0.2 ml/min/g liver for E₁ at the blood flow rate of 0.91 ± 0.1 ml/min/g liver), suggesting that transport is not rate limiting for E₁S and E₁ elimination.

The series compartmental approach that embodied transport and metabolic heterogeneity for the prediction of hepatic drug clearances (Tirona and Pang, 1999, Abu-Zahra et al., 2000) was inadequate. An extended, series compartmental model (distributed-in-space model in Fig. 5-2) that incorporated zonal and subcompartmentalization of metabolic enzymes in both cytosol and the endoplasmic reticulum (Tirona and Pang, 1996) was needed to interpret the results of the recirculating liver perfusion of [³H]E₁S and [¹⁴C]E₁. Since the distributed-in-space model was very complex, the fitting software only allowed a maximum capacity of two zonal units only.
Thus, more complicated models with higher zonal units were not examined. However, the use of two zonal units in the liver appeared adequate to describe the perfusion data.

The segregation of the endoplasmic reticulum from the cytosolic compartment implies not only distinction in metabolic enzymes but drug partitioning in the endoplasmic reticulum compartment composed of lipoidal membranes (Tirona and Pang, 1996). Creation of the endoplasmic reticulum compartment can be viewed by the following scenario: $E_1S$ was rapidly transported into the endoplasmic reticulum compartment (transport clearance of $740 \pm 146$ ml/min) and was desulfated (total desulfation intrinsic clearance of $332 \pm 44$ ml/min) by estrone sulfatase to $E_1$. The metabolite $E_1$ is lipophilic and is likely to be highly bound to membranes of the endoplasmic reticulum (Zakim and Vessey, 1977; Rao, 1998), leading to an imbalance in influx (clearance of $86 \pm 40$ ml/min) and efflux (clearance of $17 \pm 2$ ml/min). Similar high partitioning of $E_1$ into the endoplasmic reticulum was observed by Zakim and Vessey (1977). The close proximity of the membrane bound $E_1$ and UDP-glucuronosyltransferase would readily promote the sequential glucuronidation of $E_1$ (total glucuronidation intrinsic clearance of $105 \pm 30$ ml/min). Since UDP-glucuronosyltransferase is facing the luminal side (Iyanagi et al., 1986). the glucuronide conjugate of $E_1$ is released into the endoplasmic reticulum lumen and transported out to the cytosolic side ($0.018 \pm 0.001$ ml/min) and subsequently excreted into bile. In addition, $E_1$ and $E_1S$ in the endoplasmic reticulum space were also being metabolized by the cytochrome P450s (1A1, 3A4, and 2D; Martucci and Fishman, 1993) to the “pooled metabolites” $M'$, with metabolic intrinsic clearances of $255 \pm 60$ and $214 \pm 57$ ml/min, respectively. By contrast, if $E_1$ was administered, it is immediately sulfated upon its entry into the cytosolic space before $E_1$ partitioned into the endoplasmic reticulum. Due to the presence of the endoplasmic reticulum compartment, the total sulfation intrinsic clearance ($318 \pm 38$ ml/min) was found to differ from previous values ($11.2$ ml/min; Tan and Pang, 2000) that was estimated in absence of the
endoplasmic reticulum compartment. Rapid equilibration for the species between the cytosolic and endoplasmic reticulum compartments would effectively merge these subcompartments. As shown by the simulations (achieved with high endoplasmic reticulum bidirectional transmembrane clearance for E₁), the different elimination half-lives of E₁S and E₁ (Fig. 5-5) now disappeared in absence of the endoplasmic reticulum compartment, rendering similar decay profiles for E₁S and E₁ (Fig. 5-7). Upon further probing of the partitioning species, E₁ and not E₁S was found to be important (simulation not shown). The pattern is characteristic of both drug and metabolite undergoing futile cycling (Ebling and Jusko, 1986; Tan and Pang, 2000). It may thus be concluded that the partitioning of E₁S and E₁ into the endoplasmic reticulum had resulted in different elimination half-lives for E₁S and E₁ in the recirculating liver preparation.

In order to understand the influence of the futile cycling of E₁S and E₁ on the hepatic clearance of E₁S and E₁, simulations were further performed with obliterating the reversible pathway in the futile cycle. The simulation showed a greater accumulation of [³H]E₁ from [³H]E₁S in the absence of the futile cycling of E₁S (no sulfation pathway; Fig. 5-8A), and a greater formation of [¹⁴C]E₁S from [¹⁴C]E₁ in the absence of the futile cycling of E₁ (no desulfation pathway; Fig. 5-8B). One should further be aware that these sinusoidal transmembrane, endoplasmic reticulum transmembrane, metabolic intrinsic, and biliary intrinsic clearances are highly interrelated, and the set of values is not unique because other combinations could possibly be consistent with the data.

The fit also revealed that the sinusoidal bidirectional transmembrane clearance of E₁G was similar to that for E₁S (Table 5-6). Although Kanai et al (1996) had suggested that E₁G transport was mediated by Oatp1 in a set of inhibition studies, direct data describing E₁G uptake in rat liver was lacking. The biliary intrinsic clearances of E₁S and E₁G were 8.0 ± 0.1 and 1.8 ± 0.2 ml/min, respectively. Upon multiplying the biliary intrinsic clearance with the cytosolic
unbound fraction, the “effective” biliary intrinsic clearance of $E_1S$ (0.7 ml/min) becomes smaller than that of $E_1G$ (1.8 ml/min), whose biliary excretion appeared to be mediated by Mrp2 (Takikawa et al., 1996). In addition, a time-dependent decline in the biliary excretion clearances for the metabolites - $[^3H]E_1G$, $[^{14}C]E_1S$, and $[^{14}C]E_1G$ - was observed (Fig 5-6B). Since these metabolites were formed in liver and were immediately excreted into the bile, this led to a component of biliary clearance that was not accounted for by the concentration of the metabolite in the circulation. A similar phenomenon was observed for the liver perfusion of enalapril and enalaprilat (deLannoy et al., 1993). However, the biliary excretion profiles of metabolites, $E_1S$ and $E_1G$, were similar for both $[^3H]E_1S$ and $[^{14}C]E_1$ doses (Figs. 5-5 and 5-6). As for the preformed $[^3H]E_1S$, the excretion clearance reached asymptotic levels at 150 min. after reaching distribution equilibrium in the system. The bile flow rate declined with perfusion time due to the depletion of bile salts in the liver.

In conclusion, $E_1$ was highly bound to red blood cells and albumin and was metabolized by bovine erythrocytes to $E_2$. There were at least two factors governing the RBC metabolism of $E_1$ - a higher hematocrit in blood increased the metabolism whereas BSA greatly attenuated RBC metabolism. The hepatic clearances of the simultaneously delivered tracer $[^3H]E_1S$ and $[^{14}C]E_1$ were high in the rat liver preparation. Moreover, $E_1S$ and $E_1$ were highly partitioned into the endoplasmic reticulum compartment, yielding different elimination profiles for $E_1S$ and $E_1$. The notion was substantiated upon degeneration of endoplasmic reticulum with the cytosolic space, obliterating the partitioning of $E_1S$ and $E_1$ in the cell and rendering parallel decay profiles of $E_1S$ and $E_1$. The extended distributed-in-space model provided a complex but reasonable interpretation of the futile cycling between $E_1S$ and $E_1$. 
5.6 Appendix

Based on mass balance, the terminologies used to describe differential equations for the distributed-in-space model (Fig 5-2) are given as follows; n denotes the zonal unit in the rat liver where n=1 (periportal) or 2 (perivenous). \([X]^n_s\), \([X]^n_{cyt}\), \([X]_er^n\), and \([X]_{bile}^n\) denote the total concentrations of species X in the reservoir, sinusoidal, cytosolic, endoplasmic reticulum, and biliary compartments, respectively. \(A_{bile}^X\) denotes the amount of species X excreted in bile. \(Q_{blood}\) and \(Q_{bile}\) are the total blood flow rate and the bile flow rate, respectively. \(V_r\), \(V_s\), \(V_{cyt}\), \(V_{er}\), and \(V_{bile}\) represent the volume of the reservoir, sinusoidal, cytosolic, endoplasmic reticulum and bile compartments, respectively. \(CL_s^X\), \(CL_{er}^X\), and \(CL_{bile}^X\) denote the bidirectional transmembrane clearance of species X at the sinusoidal, endoplasmic reticulum, and canalicular membranes, respectively. However, \(CL_{er}^{X,in}\) and \(CL_{er}^{X,out}\) represent the influx and efflux clearances, respectively, for the species X in the endoplasmic reticulum space. \(CL_{int}^{X\rightarrow Y}\) denotes the total metabolic intrinsic clearance of species X which is being metabolized to species Y. In addition, \(CL_{rbc}^{E_1\rightarrow E_2}\) is the RBC metabolic clearance of \(E_1\), which is being reduced to \(E_2\). \(M^r\) represents all metabolites of \(E_1\) except \(E_1S\) and \(E_1G\). The fractions, \(f_{blood}^X\) and \(f_{ext}^X\), are the unbound fraction of species X in the perfusate and cytosol, respectively.

In reservoir:

\[
\frac{d[E_1]}{dt} = \left\{ \frac{Q_{blood} ( [E_1]^2_s - [E_1]_r ) - CL_{rbc}^{E_1\rightarrow E_2} f_{blood}^{E_1} [E_1]_r }{V_r} \right\}
\]

\[
\frac{d[E_1S]}{dt} = \left\{ \frac{Q_{blood} ( [E_1S]^2_s - [E_1S]_r ) }{V_r} \right\}
\]

\[
\frac{d[E_1G]}{dt} = \left\{ \frac{Q_{blood} ( [E_1G]^2_s - [E_1G]_r ) }{V_r} \right\}
\]

\[
\frac{d[M^r]}{dt} = \left\{ \frac{Q_{blood} ( [M^r]^2_s - [M^r]_r ) + CL_{rbc}^{E_1\rightarrow E_2} f_{blood}^{E_1} [E_1]_r }{V_r} \right\}
\]
For the zonal distribution of estrogen sulfotransferase, the periportal (n = 1) intrinsic sulfation clearance is 20% of the total intrinsic sulfation clearance ($CL_{int}^{E_i \rightarrow E_1}$), whereas the perivenous (n = 2) intrinsic sulfation clearance is 80% of the total intrinsic sulfation clearance ($CL_{int}^{E_i \rightarrow E_1}$).
For the zonal distribution of glucuronosyltransferase, the periportal (n = 1) intrinsic glucuronidation clearance is 33% of the total intrinsic glucuronidation clearance \( (CL_{er}^{E_i} \rightarrow E_i^{G}) \), whereas the perivenous (n = 2) intrinsic glucuronidation clearance is 67% of the total intrinsic glucuronidation clearance \( (CL_{int}^{E_i} \rightarrow E_i^{G}) \). As for the zonal distribution of estrone sulfatase, the periportal (n = 1) intrinsic desulfation clearance is 50% of the total intrinsic sulfation clearance \( (CL_{er}^{E_i} \rightarrow E_i^{S}) \), and the perivenous (n = 2) intrinsic desulfation clearance is 50% of the total intrinsic sulfation clearance \( (CL_{int}^{E_i} \rightarrow E_i^{S}) \).

**Bile compartment:**

Since the bile flow rate decreased with the perfusion time, the bile flow rate used in the fitting procedure was based on the regression of the bile flow rate versus time profile \( (Q_{bile} = 0.0114 - 0.000029t) \). When n = 1:
\[
\frac{d[E_1S]_{\text{bile}}^1}{dt} = \left\{ CL_{\text{bile}} f_{\text{cyst}} E_{1S}^s [E_1S]_{\text{cyst}}^1 + Q_{\text{bile}} ( [E_1S]_{\text{bile}}^2 - [E_1S]_{\text{bile}}^1 ) \right\} / V_{\text{bile}}
\]

\[
\frac{d[E_1G]_{\text{bile}}^1}{dt} = \left\{ CL_{\text{bile}} f_{\text{cyst}} E_{1G}^s [E_1G]_{\text{cyst}}^1 + Q_{\text{bile}} ( [E_1G]_{\text{bile}}^2 - [E_1G]_{\text{bile}}^1 ) \right\} / V_{\text{bile}}
\]

When \( n = 2 \):

\[
\frac{d[E_1S]_{\text{bile}}^2}{dt} = \left\{ CL_{\text{bile}} f_{\text{cyst}} E_{1S}^s [E_1S]_{\text{cyst}}^2 - Q_{\text{bile}} [E_1S]_{\text{bile}}^2 \right\} / V_{\text{bile}}
\]

\[
\frac{d[E_1G]_{\text{bile}}^2}{dt} = \left\{ CL_{\text{bile}} f_{\text{cyst}} E_{1G}^s [E_1G]_{\text{cyst}}^2 - Q_{\text{bile}} [E_1G]_{\text{bile}}^2 \right\} / V_{\text{bile}}
\]

The rate of change of \( E_1S \) and \( E_1G \) in bile:

\[
\frac{dA_{\text{bile}}^{E_1S}}{dt} = Q_{\text{bile}} [E_1S]_{\text{bile}}^1
\]

\[
\frac{dA_{\text{bile}}^{E_1G}}{dt} = Q_{\text{bile}} [E_1G]_{\text{bile}}^1
\]
5.7 Statement of Significance of Chapter 5

In this chapter we found $E_1S$ and $E_1$ were highly bound to BSA. In addition to BSA binding, $E_1$ was metabolized to $E_2$ in bovine-erythrocytes. However, the presence of 4% BSA greatly reduced RBC metabolism of $E_1$ since $E_1$ and $E_2$ were bound more strongly to BSA than to erythrocytes. The hepatic clearances of the simultaneously delivered tracer $[^3H]E_1S$ and $[^{14}C]E_1$ were high. Moreover, $E_1S$ and $E_1$ were highly partitioned into the endoplasmic reticulum compartment. The distributed-in-space liver model that included the zonal and subcompartmentalization of metabolic enzymes in the cytosol and endoplasmic reticulum adequately described the perfusion data. However, in the absence of drug partitioning into the endoplasmic reticulum compartment, parallel elimination profiles for $E_1$ and $E_1S$, characteristic of compounds undergoing futile cycling, were observed in the simulation study.

5.8 Acknowledgments

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS
We have chosen to study the reversible reaction between E₁S and E₁ because this futile cycle represents a pharmacologically important biocycle that conserves and regulates levels of endogenous estrogens. The disappearance of the backward pathway will affect the homeostasis of E₁S, E₁, and other estrogens in patients with multiple sulfatase deficiency and X-linked ichthyosis diseases. Moreover, futile cycling is an added complexity that influences the residence times of E₁S and E₁ in the liver. Thus, thorough understanding of the impact of the futile cycle is necessary.

Hepatic drug removal is a distributed-in-space process that is affected by vascular and tissue binding, acinar localization of transporters and metabolic enzymes, and excretion. Some of these factors can be assessed through \textit{in-vitro} experimentation. However, it is often found that \textit{in-vitro} data, though appropriately scaled-up, are not able to predict the complex function of the liver, even though many traditional models of hepatic drug clearance, namely the “Well-Stirred,” “Parallel Tube,” “Dispersion,” and “Series Compartment” models, are available for integration of the \textit{in-vitro} data (Abu-Zahra and Pang, 2000). The explanation is zonal heterogeneity in transport and metabolism. Thus, we employed the strategy of scaling the \textit{in-vitro}, zonal parameters to predict hepatic drug clearances. A complex distributed-in-space liver model that incorporates all of the determinants of hepatic clearance is needed to describe the kinetics of futile cycling between E₁S and E₁.

In this research project, we employed various \textit{in-vitro} and liver perfusion approaches to study zonal transport, zonal metabolism, and the futile cycling of E₁S and E₁. The experimental observations support the following findings:

1) Futile cycling decreases the formation of the metabolite which undergoes interconversion but increases formation of other, noncycling metabolites.

2) Nonlinear transport, metabolism, tissue binding, and/or partitioning into the endoplasmic reticulum contribute to different elimination half-lives for both species undergoing futile
cycling.

3) The complex kinetics of futile cycling are described adequately by a series compartment (distributed-in-space) model that encompasses transport, metabolic heterogeneity, vascular and tissue binding, and nonlinear behaviour.

Results of the experiments support the hypotheses stated in Section 2.1. The first hypothesis states that the processing of E1S and E1 in zonal hepatocytes differs and that this is attributed to differences in zonal transport and metabolism. Although uptake (both saturable and nonsaturable components) of E1S and E1 and the desulfation of E1S were not different among PP and PV hepatocytes, the sulfation intrinsic clearance of E1 in the cytosolic preparation of PV hepatocytes was four times that of PP hepatocytes, validating the hypothesis.

The second hypothesis states that futile cycling kinetics result in parallel decay profiles of E1S and E1. Parallel elimination profiles of E1S and E1 were not observed in *in-vitro* incubation study (Chapter 4) due to nonlinear tissue binding, saturable zonal uptake and vesicular accumulation of E1S. However, upon removal of the nonlinear uptake, binding, and vesicular accumulation of E1S, parallel elimination profiles of E1S and E1 were observed in the simulation study under first order condition. Parallel elimination profiles of E1S and E1 were observed in the liver perfusion preparation after dosing with tracer E1S (Chapter 5). However, due to the high partitioning of E1 into the endoplasmic reticulum space, different elimination profiles for E1S and E1 resulted after dosing with tracer E1 (Chapter 5). The discrepancy disappeared under rapid equilibration of E1 between the cytosolic and the endoplasmic reticulum spaces.

The final hypothesis states that the *in-vitro* transport and metabolic parameters of E1S and E1 correspond to the *in-vivo* hepatic clearance parameters. When the *in-vitro* tissue binding, zonal uptake and metabolic parameters of E1S and E1 were utilized to describe data in the
recirculating liver preparation for the simultaneous delivery of \([{}^3\text{H}]\text{E}_1\)S and \([{}^{14}\text{C}]\text{E}_1\). The perfusion data of \(\text{E}_1\)S and \(\text{E}_1\) were well described by a distributed-in-space liver model (Chapter 5) only when zonal and subcellular distribution of the metabolic enzymes in the cytosolic and endoplasmic reticulum compartments were considered.

The understanding of futile cycling of \(\text{E}_1\)S and \(\text{E}_1\) in the animal model brought to light that nonlinear transport, metabolism, tissue binding, and/or partitioning into the endoplasmic reticulum can contribute to different elimination half-lives \(\text{E}_1\)S and \(\text{E}_1\). These developed concepts impact of our understanding of futile cycling to man. Sandberg and Slaunwhite (1957) found that the elimination half-life of \(\text{E}_1\) is 70 min after intravenous injection of \({}^{14}\text{C}-\text{E}_1\) into women, and if the half-life of the metabolite, \({}^{14}\text{C}-\text{E}_1\)S, were measured, \(\text{E}_1\)S would have exhibited a longer half-life. Longscope (1972) found that the elimination half-life of \({}^3\text{H}-\text{E}_1\)S is 196 min after its intravenous injection of into women but failed to measure the \({}^3\text{H}-\text{E}_1\) formed. The developed concepts therefore await validation in man.

Several questions have arisen during the course of my research work. One question is whether the biliary excretion of \(\text{E}_1\)S is mediated by Mrp2 (cmoat), Mdr1 (Pgp), or Bsep (sPgp) in the rat liver. One could study the excretion characteristics of \(\text{E}_1\)S using Mrp2, Mdr1, or Bsep expressed \(\text{Xenopus laevis}\) oocytes. In addition, Eisai hyperbilirubinemic rats (EHBR), a mutant rat that lacks Mrp2 at the canalicular membrane, may be used as an animal model to study the excretion of \(\text{E}_1\)S. Moreover, one could investigate the excretion characteristics of \(\text{E}_1\)S using canalicular vesicles of EHBR and normal rats.

Another question is on the transport and excretion of \(\text{E}_1\)G in the rat liver. Although \(\text{E}_1\)G transport has been found to be mediated by rat liver Oatp1 (Kanai et al., 1996) and excretion is mediated by the canalicular Mrp2 (Takikawa et al., 1996), the roles of other basolateral transporters (Oatp2, Oatp3, Oatp4, Oat3, Lst1, Ntcp, and Mrp3) and canalicular transporters
(Mdr1 and Bsep) on the hepatic biliary transport of E\textsubscript{1}G in the rat liver are unknown. Thus, one could study the transport and excretion characteristics of E\textsubscript{1}G by using Oatp2, Oatp3, Oatp4, Oat3, Lst1, Ntcp, Mrp3, Mdr1, and Bsep expressed Xenopus laevis oocytes.

Finally, the glucuronidation of E\textsubscript{1} by different rat UDP-glucuronosyltransferase (UGT) isoenzymes remains to be investigated. Although human UGT1A1, 1A3, 1A4, 1A7, 1A8, 1A9, 1A10, 2A1, 2B7, and 2B15 has been found to glucuronidate estrogens (Tukey and Strassburg, 2000), the involvement of rat UGT isoenzymes in the glucuronidation of E\textsubscript{1} has not been fully characterized. One could study the glucuronidation activity of E\textsubscript{1} by different rat UDP-glucuronosyltransferase (UGT) isoenzymes in the presence of UDP-glucuronic acids (co-substrate). In addition, zonal glucuronidation of E\textsubscript{1} has not been investigated. Thus, one could investigate the glucuronidation activity of E\textsubscript{1} in the presence of UDP-glucuronic acids in the subcellular fraction (9000g) of zonal hepatocytes. I had neglected to measure the E\textsubscript{1}G formed in the incubation study of E\textsubscript{1}S in zonal hepatocytes (Chapter 4), otherwise the E\textsubscript{1}G results may provide a better understanding on the zonal transport and metabolism of E\textsubscript{1}G.
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Tirona RG, Tan E, Meng LJ, Novikoff PM, Wang PJ, Wolkoff AW, Kim RB and Pang KS (2000) Oatp2 is functionally distributed throughout the rat liver lobule and is a high affinity transporter of digoxin and sulfotlothycholyglysurine. Abstract #1109. 50th Annual Meeting of American Association for the Study of Liver Diseases, Dallas, Texas, USA.


PUBLICATIONS


ABSTRACTS


5. Tirona RG, Tan E, Meng LJ, Novikoff PM, Wang PJ, Wolkoff AW, Kim RB and Pang KS (2000) Oatp2 is functionally distributed throughout the rat liver lobule and is a high affinity transporter of digoxin and sulfodihydrocholate. 50th Annual Meeting of American Association for the Study of Liver Diseases, Dallas, Texas, USA.

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