HEPATIC CLEARANCE OF DRUGS: EFFECT OF ZONAL FACTORS.
TRANSPORT AND METABOLIC STUDIES OF ENALAPRIL WITH RAT, ZONAL
HEPATOCYTES

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Pharmacology, University of Toronto

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"And he sets his mind to work upon unknown arts"

-OVID, Metamorphoses, VIII., 18
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Hepatic Clearance of Drugs: Effect of Zonal Factors.

Transport and Metabolic Studies of Enalapril with Rat, Zonal Hepatocytes

ABSTRACT

Enalapril, an angiotensin converting enzyme inhibitor, was chosen as a model substrate to illustrate the effects of zonal factors on drug processing in rat liver. Zonation has been observed for enalapril hydrolysis in rat liver HAPV and HAHV perfusion studies, in which it was found that the perivenous (PV) zone exhibited a higher capacity than the periportal (PP) region in the conversion of enalapril to its active metabolite, enalaprilat (Pang et al., J. Pharmacol. Exp. Ther., 1991). Whether transport or metabolism is zonated is, however, unknown. The aims of the present investigation were to: i) determine the \textit{in vitro} kinetic parameters for enalapril uptake by intact hepatocytes from zonal and homogeneous hepatocytes and for enalapril hydrolysis in subcellular fractions prepared from whole rat liver and periportal or perivenous zones, and ii) assess the appropriateness of models of hepatic clearances with scaled up \textit{in vitro} parameters for uptake and metabolism for prediction of whole organ parameters. We wished to test the hypotheses that: i) metabolic and not transport activity is zonated and ii) a zonal-compartment model best describes the events \textit{in vivo} in the processing of enalapril. Studies were undertaken with the objective of furthering the development of hepatic clearance models by incorporation of zonal factors on transport and metabolism. Rat hepatocyte populations isolated from whole rat liver or enriched in PP or PV cells were prepared for the determination of kinetic parameters for enalapril uptake in intact hepatocytes and metabolism in subcellular fractions. It was found that enalapril uptake by rat hepatocytes was the same for all zones and was described by a single saturable component of \( V_{\text{uptake}}^{\text{max}} \) of 9.5 to 11.6 nmol/min/10^6 cells and \( K_m^{\text{uptake}} \) of 344 to 461 \( \mu \)mol/L. Metabolism of enalapril in S9 fractions exhibited differences in both affinity and maximal velocity, with the kinetic parameters \( V_{\text{max,PP}}^{\text{met}} \) (21.0 nmol/min/mg S9 protein) and \( K_m^{\text{met,PP}} \) (2612 \( \mu \)mol/L) being greatest in S9 prepared from...
PV hepatocytes and least in S9 prepared from PP hepatocytes ($V_{\text{max,PP}}$ of 5.5 nmol/min/mg S9 protein and $K_{m,PP}$ of 1049 μmol/L). The transport clearance was 4 to 23 times that of the metabolic intrinsic clearances among zones, and the overall hepatic removal of enalapril is rate-limited by intracellular metabolism and not transport. Enalapril removal by the whole liver is best described by a "zonal-compartment" model which incorporates a zonal distribution of metabolic enzymes with homogeneous transport. Results of the in vitro experiments, when scaled-up to the zonal model, predicted that metabolism of enalapril is a zonated process, resulting in a much reduced extraction ratio at the periportal region (0.16) vs. the whole liver (0.66). The present method of scale-up of in vitro parameters for use in a hepatic clearance model that incorporates zonal factors of transport and metabolism represents a further advancement of hepatic modeling.
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<td>ACEi</td>
<td>Angiotensin converting enzyme inhibitor</td>
</tr>
<tr>
<td>BSP</td>
<td>Bromosulfophthalein</td>
</tr>
<tr>
<td>BSP-GSH</td>
<td>Bromosulfophthalein-glutathione conjugate</td>
</tr>
<tr>
<td>$CL_{\text{bil, liver}}$</td>
<td>Biliary intrinsic clearance of whole liver</td>
</tr>
<tr>
<td>$CL_{\text{int}}$</td>
<td>Metabolic intrinsic clearance</td>
</tr>
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<td>$CL_{\text{int,liver}}$</td>
<td>Metabolic intrinsic clearance of whole liver</td>
</tr>
<tr>
<td>$CL_{\text{met, midZ}}$</td>
<td>Metabolic intrinsic clearance of mid-zone of liver</td>
</tr>
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<td>$CL_{\text{met, PP}}$</td>
<td>Metabolic intrinsic clearance of periportal zone of liver</td>
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<td>$CL_{\text{met, PV}}$</td>
<td>Metabolic intrinsic clearance of perivenous zone of liver</td>
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<td>$CL_{\text{total,liver}}$</td>
<td>Total intrinsic clearance of whole liver</td>
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<td>CYP 450</td>
<td>Cytochrome P450</td>
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<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>E</td>
<td>Extraction ratio</td>
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<tr>
<td>$E_{\text{s}}$</td>
<td>Steady-state extraction ratio</td>
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<tr>
<td>$E_{\text{s, HAHV}}$</td>
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<tr>
<td>$f_{\text{u}}$</td>
<td>Fraction unbound in plasma</td>
</tr>
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<td>GSH</td>
<td>Reduced glutathione</td>
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<td>HAHV</td>
<td>Hepatic artery, hepatic vein method of perfusion</td>
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<td>Hepatic artery, portal vein method of perfusion</td>
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<td>HEPES</td>
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<td>$K_m$</td>
<td>Michaelis-Menten constant</td>
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<td>$K_{\text{met}}$</td>
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<td>$K_{\text{met, HO}}$</td>
<td>Michaelis-Menten constant for intracellular metabolism of enalapril in whole liver</td>
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</table>
$K_{m,MZ}$ Michaelis-Menten constant for intracellular metabolism of enalapril in mid-zone of liver

$K_{m,PP}$ Michaelis-Menten constant for intracellular metabolism of enalapril in periportal zone of liver

$K_{m,PV}$ Michaelis-Menten constant for intracellular metabolism of enalapril in perivenous zone of liver

$K_{\text{suppk}}$ Michaelis-Menten constant for uptake of enalapril in whole liver

Oatp1 Organic anion transporting polypeptide 1 cloned from rat liver

PP Periportal zone of the liver

$P_{\text{in}}$ Influx permeability surface area product

$P_{\text{out}}$ Efflux permeability surface area product

PV Perivenous zone of the liver

Q Perfusate blood flow rate

$Q_{\text{bile}}$ Bile flow rate

$V_{\text{met max}}$ Maximum velocity for intracellular metabolism of enalapril

$V_{\text{met max,WH}}$ Maximum velocity for intracellular metabolism of enalapril in whole liver

$V_{\text{met max,MZ}}$ Maximum velocity for intracellular metabolism of enalapril in the mid-zone of liver

$V_{\text{met max,PP}}$ Maximum velocity for intracellular metabolism of enalapril in the periportal zone of liver

$V_{\text{met max,PV}}$ Maximum velocity for intracellular metabolism of enalapril in the perivenous zone of liver

$V_{\text{suppk max}}$ Maximum velocity for enalapril uptake in whole liver
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Chapter One

INTRODUCTION
1.1 THE LIVER

1.1.1 Structure and Function of the Liver

The liver is a vital organ responsible for the synthesis and regulation of important substrates such as proteins and endogenous and intermediary products in the body; it is also the main site for the detoxification of xenobiotics (Katz and Jungermann, 1993). Since the organ is strategically positioned between the gastrointestinal tract and the heart and lungs, absorbed solutes from the gastrointestinal tract must traverse the liver before these are transported to other tissues. Hepatocytes, the major parenchymal cell type, are able to remove incoming compounds from blood via metabolism and/or excretion into bile. Hence, the liver is capable of regulating blood concentrations of solutes leaving the organ (Gumucio and Guibert, 1993).

The unidirectional blood flow and structure of the hepatic vasculature contributes to the efficient removal of xenobiotics. The microvascular functional unit of the liver is the acinus (Rappaport, 1954), which extends from a terminal portal venule and hepatic arteriole, microvessels of the portal vein and hepatic artery, respectively, through a sinusoidal space to a central venule. About 80% of the blood entering the sinusoid originates from the portal vein which is relatively poorly oxygenated blood rich in absorbed solutes, while the remaining 20% is well oxygenated blood supplied by the hepatic artery. Blood from these two vessels mixes and enters specialized capillaries known as sinusoids and is channeled in a unidirectional fashion for the perfusion of single
sheets of hepatocytes. The sinusoid is lined with modified endothelial cells which possess fenestrated cell bodies capable of regulating the passage of solutes into the Space of Disse, the interstitial space. It is within the Space of Disse that close contact between solutes and hepatocytes occurs.

The hepatic microvasculature facilitates the delivery, removal and exchange of compounds between the blood and cell. The hepatocytes are organized in such a manner that solutes encounter successive cells which possess different functional capabilities, from portal to hepatic venules (Gumucio and Guibert, 1993). Blood-borne solutes enter hepatocytes through passive diffusion and/or by specific transporters found on the basolateral or sinusoidal membrane. Once inside the cell, a compound may be directly excreted into bile via proteins expressed on the canalicular membrane or biotransformed by a wide range of drug metabolizing enzymes. Intracellularly produced metabolites may also be excreted into bile, further metabolized within the same cell or effluxed into the sinusoid. The processes of uptake, metabolism, excretion and efflux are repeated among hepatocytes further downstream along the direction of flow. Thus, the removal of a drug by the liver is viewed as a distributed-in-space phenomenon, and the overall removal rate is the consequence of all of the processes occurring among the cells in series.

1.1.2 Zonation in Liver

During its passage through each acinus, the composition of blood changes as it releases oxygen and substrates to the tissue and accumulates metabolic products arising from hepatocytes. Blood in the periportal end is richer in O₂, substrates and hormones
while the perivenous region is richer in CO₂ and metabolic products, resulting in a changing microenvironment between the inlet and outlet (Katz and Jungermann, 1993). Three different zones exist within the acinus, and each exhibit a different level of oxygenation and metabolic activity. The first zone is termed the periportal zone (also zone 1) in reference to the proximity of the afferent blood flow from the portal vein. The distal region of the acinus, the perivenous region (or zone 3, or centrilocular) is named for its location with respect to the hepatic vein. The intervening zone, zone 2 or the midzonal region, is an ill-defined area between the periportal and perivenous zones. Thus, hepatocytes are classified as either periportal (PP) or perivenous (PV) hepatocytes. This distinction is not only based on the location of the hepatocytes within the microcirculation, difference in protein expression and hepatocyte function occurs between cells from different zones (Lindros 1997; Jungermann 1992; Oinonen and Lindros 1998).

Zonation of protein expression is of two types: the gradient-type or the compartment-type (Oinonen and Lindros, 1998). In both cases, expression is generally controlled at the level of transcription (Gebhardt, 1992). Gradient-type zonation, in which all of the cells express the same protein in varying amounts, arises due to differences in the concentrations of blood-borne solutes (e.g. O₂ and hormones) seen by cells across the acinus (Jungermann and Katz, 1989). A gradient of decreasing concentration results due to uptake of the compound, whereas an inverse gradient may arise because of efflux of metabolites from cells into the blood. The compartment-type zonation, with gene expression being restricted to certain regions, is thought to be
regulated by cell-cell, or cell-biomatrix interactions, and is more poorly understood than the gradient-type (Oinonen and Lindros, 1998). While the enzymes involved in the PP localization of ureagenesis arise due to gradient type zonation (Oinonen and Lindros, 1998), PV zonation of glutamine synthetase is a classic example of an enzyme whose zonal expression is of the compartment type - only a ring of two to three cells surrounding the central vein express this enzyme (Gebhardt and Mecke, 1983).

1.2 ZONALLY DISTRIBUTED PROTEINS

1.2.1 Transporters

1.2.1.1 Sinusoidal Transporters. Entry across the sinusoidal membrane into the hepatocyte is the first barrier that a drug faces in its removal from the liver. Although some compounds are capable of entry through passive diffusion, many hydrophilic compounds do not exhibit adequate diffusion and enter hepatocytes via basolateral transmembrane carriers or transport proteins. In some cases, uptake by the hepatocyte is the rate-limiting step in the overall hepatic removal. This is exemplified by the glutathione (GSH) conjugation of ethacrynic acid (Tirona and Pang, 1999) and overall hepatic removal of pravastatin (Yamazaki et al. 1996). However, in other instances, metabolism and/or biliary excretion, which occur following entry into the cell, is rate-limiting.

Substrates for the cloned basolateral transport proteins have been identified in transfected expression systems. For example, endogenous compounds such as bile acids (Hagenbuch et al., 1991; Jacquemin et al., 1994; Noé et al., 1997) monocarboxylates
(Garcia et al., 1995) and catecholamines (Busch et al., 1996) as well as xenobiotics such as enalapril (Pang et al., 1998), digoxin (Noé et al., 1997), and bromosulfophthalein (Hagenbuch et al., 1991; Jacquemin et al., 1994) are all substrates of one or more of the cloned transporters expressed in adult rat hepatocytes (see Table 1-1). Zonal expression has been identified for some of these transporters (Meyer-Wentrup et al., 1998; Reichel et al., 1999; Table 1-1) while others are evenly distributed (Stieger et al., 1994; Staricoff et al., 1995; Table 1-1). In addition to the studies on heterogeneous expression of transporter proteins, specific compounds have been investigated for zonal uptake. Glutamate (Burger et al. 1989; Tan et al., 1999), aspartate, α-ketoglutarate (Stoll et al. 1989), cysteine (Saiki et al. 1992) and asialoglycoprotein (Casey et al. 1991) all show greater perivenous uptake, while epidermal growth factor is reported to be taken up more abundantly in the periportal region (Marti and Gebhardt, 1991).

1.2.1.2 Canalicular Transporters. Proteins expressed on the canalicular membranes are involved in the efflux of endogenous and xenobiotic compounds into bile. These canalicular transporters, often referred to as efflux pumps, are members of the ABC (ATP-binding cassette) superfamily of proteins which derive energy for activity through the hydrolysis of ATP. Efflux activity by specific pumps has been identified for endogenous compounds such as bilirubin mono- and di-glucuronides (Kamisako et al., 1999; Table 1-2), and the conjugated and unconjugated bile acids (Takikawa et al., 1991; Gerloff et al., 1998; Table 1-2). Several specific canalicular efflux pumps involved in biliary excretion of phase I (e.g. temocaprilat; Ishizuka et al., 1997) and phase II
metabolites (e.g. ethacrynic acid-GSH adduct; Evers et al., 1998) and intact drugs (e.g. pravastatin, Yamazaki et al., 1997 and vincristine, Watanabe et al., 1992) have been identified (Table 1-2). To date, none of the canalicular transport proteins present in rat liver has been found to be zonated (Table 1-2).

1.2.2 Metabolic Enzymes

Functional metabolic zonation differs between the periportal and perivenous regions for many enzymatic pathways. Many reviews on the zonation of metabolism of endogenous and exogenous compounds are available (see Pang and Xu 1988; Jungermann and Katz, 1989; Gebhardt 1992; Jungermann 1992 and 1995; Lindros 1997; Oinonen and Lindros, 1998). Gluconeogenesis, urea synthesis and fatty acid metabolism are examples of pathways present predominantly in the periportal zone whereas lipogenesis and glutamine synthesis are greater in the perivenous region (Jungermann and Katz, 1989; Gebhardt 1992; Jungermann 1992; Gebhardt and Mecke, 1983). Enzymes for phase I and II xenobiotic metabolism are also known to be zonated (Table 1-3). For example, glutathione peroxidase predominately occurs in the PP hepatocytes (Kera et al., 1987), while members of the cytochrome P450 family (Lindros, 1997), and carboxylesterases (Pohl et al., 1991; Huang et al., 1993; Yan et al., 1994) are localized in the PV region.
1.3 TECHNIQUES TO STUDY ZONATION

Several techniques exist for the study of zonation in the liver, each with its advantages and limitations. Generally, three types of techniques have been used to study zonation of function and/or expression of hepatic proteins: i) whole organ perfusions, ii) immunohistochemical localization studies and iii) hepatocytes or subcellular fraction made from PP or PV zones.

1.3.1 Whole Organ Perfusions

Various organ perfusions have been developed to study zonated events of the liver. These include antegrade/retrograde and HAHV/HAPV perfusions (Pang and Terrell, 1981; Pang et al., 1988). Ante- and retrograde studies involve perfusing the substrate in the normal (antegrade) and reverse (retrograde) directions and assessment of differences in the outflow profiles or tissue concentrations for inferences on zonation of the cellular processes such as tissue binding, uptake and metabolism (Pang et al., 1991; Groothuis and Meijer, 1992). The method details the relative distribution of the overall activity, but rarely identifies the rate-limiting step. The HAHV/HAPV technique is a more sophisticated perfusion method (Pang et al., 1988). In the HAPV case, blank perfusate enters the liver through the portal vein in the antegrade direction, while drug is perfused through the hepatic artery, exposing the drug to essentially the entire acinus. In the HAHV case, however, blank perfusate enters the liver in a retrograde fashion through the hepatic vein while drug is perfused through the hepatic artery, limiting the drug to the
Table 1-1: Summary of cloned rat sinusoidal transporters and their zonation.

<table>
<thead>
<tr>
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<th>Substrates</th>
<th>Zonation</th>
<th>Technique for study</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Glucose transporter 1 (GLUT1)</td>
<td>glucose</td>
<td>PV</td>
<td>immunofluorescence of protein</td>
<td>Tal et al., 1990</td>
</tr>
<tr>
<td>Glucose transporter 2 (GLUT2)</td>
<td>glucose</td>
<td>PP</td>
<td>isolated zonal hepatocytes and immunohistochemistry</td>
<td>Ogawa et al., 1996</td>
</tr>
<tr>
<td>Monocarboxylic acid transporter (MCT2)</td>
<td>lactate, pyruvate</td>
<td>even</td>
<td>isolated zonal hepatocytes</td>
<td>Garcia et al., 1995; Staricoff et al., 1995</td>
</tr>
<tr>
<td>Sodium-dependent taurocholic acid cotransporting polypeptide (ntcp)</td>
<td>bile acids, estrone sulfate BSP</td>
<td>even/even</td>
<td>immunofluorescence immunohistochemistry</td>
<td>Hagenbuch et al., 1991; Meier et al., 1997; Stieger et al., 1994; Anatharayanam et al., 1994</td>
</tr>
<tr>
<td>Organic anion transporting polypeptide 1 (Oatp1)</td>
<td>bile acids, BSP, enalapril, BSP-GSH</td>
<td>even/even</td>
<td>in situ hybridization immunohistochemistry</td>
<td>Jacquemin et al., 1994; Pang et al., 1998; Dubuisson et al., 1996; Bergwerk et al., 1996</td>
</tr>
<tr>
<td>Organic anion transporting polypeptide 2 (Oatp2)</td>
<td>digoxin, ouabain, bile acids pravastatin, fenofenadine</td>
<td>MZ to PV</td>
<td>in situ hybridization immunohistochemistry</td>
<td>Noé et al., 1997; Tokui et al. 1999; Cvetkovic et al., 1999; Reichel et al., 1999; Kanyak et al., 1999a</td>
</tr>
<tr>
<td>Organic anion transporter 2 (out2)</td>
<td>NSAIDS, PAH</td>
<td>unknown</td>
<td>unknown</td>
<td>Endou, 1998; Sekine et al., 1998</td>
</tr>
<tr>
<td>rat liver-specific transporter 1 (rlst-1)</td>
<td>taurocholate</td>
<td>unknown</td>
<td>unknown</td>
<td>Kanyak et al., 1999b</td>
</tr>
<tr>
<td>rat organic cation transporter 1 (rOCT1)</td>
<td>tetraethylammonium, catecholamines</td>
<td>PV</td>
<td>immunohistochemistry and in situ hybridization</td>
<td>Gründemann et al., 1994; Busch et al., 1996; Meyer- Wentrup et al., 1998</td>
</tr>
<tr>
<td>sodium/dicarboxylate transporter 2 (sDCT2)</td>
<td>α-ketoglutarate, dicarboxylates</td>
<td>PV</td>
<td>in situ hybridization</td>
<td>Chen et al., 1999</td>
</tr>
</tbody>
</table>

*BSP, bromosulphophthalein; BSP-GSH, bromosulphophthalein-glutathione conjugate, NSAID, non-steroidal anti-inflammatory drug; PAH, p-aminohippurate

even: even distribution across the acinus; PP, periportal zonation; PV, perivenous zonation; MZ to PV, mid-zonal to perivenous zonation
Table 1-2: Summary of cloned rat canalicular transporters and their zonation.

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Substrates*</th>
<th>Zonation</th>
<th>Technique for study</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>bile salt efflux pump (Bsep)</td>
<td>taurocholate, bile acids</td>
<td>even</td>
<td>immunohistochemistry</td>
<td>Gerloff et al., 1998</td>
</tr>
<tr>
<td>multidrug resistance associated-protein 2; (Mrp2)</td>
<td>bilirubin mono- and di-glucuronides, pravastatin, temocaprilat</td>
<td>even</td>
<td>Western blotting</td>
<td>Paulusma et al., 1996</td>
</tr>
<tr>
<td>or canalicular multispecific organic anion transporter (cMOAT)</td>
<td>ethacrynic acid-GSH, bile acid sulfates and glucuronides</td>
<td></td>
<td></td>
<td>Kamisako et al., 1999</td>
</tr>
<tr>
<td>Multiple drug resistance protein 1 (Mdr 1)</td>
<td>fenofenadine, estradiol 17-β-glucuronide, cationic anticancer drugs</td>
<td>even</td>
<td>C219 Western Blotting</td>
<td>Yamazaki et al., 1997</td>
</tr>
<tr>
<td>or P-glycoprotein (Pgp)</td>
<td></td>
<td></td>
<td></td>
<td>Ishizuka et al., 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Evers et al., 1998</td>
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<td></td>
<td>Takikawa et al., 1991</td>
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<td>Even et al., 1998</td>
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<td></td>
<td></td>
<td>Takikawa et al., 1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tirona et al., 1999</td>
</tr>
</tbody>
</table>

* ethacrynic acid-GSH, ethacrynic acid-glutathione conjugate
**Table 1-3: Zonation of xenobiotic drug metabolizing activities in rat liver.**

<table>
<thead>
<tr>
<th>Metabolic Reaction&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Zonation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Substrate</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phase I Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP 450 Monooxygenation</td>
<td>PV</td>
<td>carbon tetrachloride</td>
<td>Ingelman-Sundberg <em>et al.</em>, 1988;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lindros <em>et al.</em>, 1990</td>
</tr>
<tr>
<td></td>
<td>PV</td>
<td>enalapril</td>
<td>Pang <em>et al.</em>, 1991</td>
</tr>
<tr>
<td><strong>Phase II Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PV</td>
<td>estrone</td>
<td>Tosh <em>et al.</em>, 1996</td>
</tr>
<tr>
<td></td>
<td>PV</td>
<td>2-napthol</td>
<td>Homma <em>et al.</em>, 1997</td>
</tr>
<tr>
<td></td>
<td>PV</td>
<td>1-napthol</td>
<td>Ullrich <em>et al.</em>, 1984, Knapp <em>et al.</em>, 1988</td>
</tr>
<tr>
<td></td>
<td>even</td>
<td>salicylamide</td>
<td>Xu and Pang, 1989</td>
</tr>
<tr>
<td>Glucuronidation</td>
<td>PV</td>
<td></td>
<td>Redick <em>et al.</em>, 1982, Tirona <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>GSH conjugation</td>
<td>PV</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PV</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Pang and Chiba (1994a) and Oinonen and Lindros (1998).

<sup>a</sup> CYP 450, cytochrome P450 family of isozymes; GSH, glutathione

<sup>b</sup> even, even distribution across acinus; PP, periportal zonation; PV, perivenous zonation

Periportal region of the liver. Inferences on zonation can be made based on the extraction ratios with HAPV and HAHV, after the activities are normalized to the accessed intracellular water space determined from injection of labeled water and other vascular space markers into the artery. A third perfusion technique involves the selective destruction of one zone, typically allyl alcohol or N-hydroxyl-2 acetylamino fluorene for the PP zone and bromobenzene or carbon tetrachloride for the PV zone, then perfusion of the liver (Thorgeirsson *et al.*, 1976; James *et al.*, 1981; Haussinger and Gerok, 1984). However, adaptation of the remaining zone(s) may occur and/or the damaging agent may inappropriately alter both function and the microcirculation in the remaining zone.
(Gumucio and Chianale, 1988). Among all of the perfusion studies, the observations are limited and the specific processes which may be zonated are not studied directly.

1.3.2 Localization Studies

Immunohistochemical localization techniques are useful to detect the location of expressed protein in the various zones of liver, while in situ hybridization may be used to detect zonation of mRNA message. The liver is sectioned and fixed, followed by exposure to antisense probes or antibodies for the detection of mRNA and protein, respectively. Localization is then determined with fluorescence or radioactivity. These techniques require either specific antibodies or a fore-knowledge of the coding sequence of oligonucleotides. Problems associated with these studies include the lack of antibody specificity and artifacts arising due to fixation (Evan et al., 1989), and while useful information is gained, the data pertain to the location of a protein without information on its function.

1.3.3 Hepatocytes of Zone-Specific Origin

Valuable information on gene-product expression and function can be obtained from the use of hepatocytes of either periportal or perivenous origin. Tissue from either periportal or perivenous zone can be produced through microdissection of the liver (Schmidt et al., 1978; Teutsh, 1978). However, this technique is tedious and may result in low viability (Groothuis and Meijer, 1992; Evans et al., 1989). Isolated hepatocytes enriched in cells from one region can be prepared through the selective destruction of one
zone with digitonin infusion, followed by collagenase digestion and harvesting of cells from the remaining, viable zone (Lindros and Penttilä 1985; Quistorff 1985). This procedure produces intact cells of good viability with high yield and the resultant cells may be used in functional studies or for the investigation of expressed proteins. The disadvantages of this technique are that cells from only one region may be prepared for each liver and a high-degree of zonal selectivity may be difficult. Moreover, harvested cells lose the polarity of their membrane domains. A third procedure, the dual-digitonin-pulse perfusion technique produces lysates containing soluble cytosolic proteins from both zones of the same liver (Quistorff and Grunnet, 1987). However, many drug metabolizing enzymes that remain membrane bound to the endoplasmic reticulum are not present in a functional form in the lysate preparation (Fang et al. 1998, Witters et al., 1993). A fourth technique, isolation of zonal hepatocytes with density gradient centrifugation, has limited success, mostly because cells from the different acinar zones differ only marginally in density and volume (Groothuis and Meijer, 1992).

1.4 PHARMACOKINETICS

1.4.1 Effect of Zonated Events on Drug Pharmacokinetics

Zonation of drug clearing processes in the liver can have profound affects on the observed pharmacokinetics of a drug and is of great interest (Sato et al. 1986, Kwon and Morris, 1997). While zonation is known to exist in humans and is an important factor in clinical pharmacokinetics, the rat is the best studied species in the field of zonation.
(Oinonen and Lindros, 1998). Zonal transport and metabolic enzymes may affect: i) the efficiency of removal of substrate, ii) the location of damage due to toxicity and iii) drug/metabolite concentrations in the systemic circulation. Therefore, it is important to view and predict drug and metabolite clearances accurately with a model that encompasses the zonal factors.

Simulation studies by Sato et al. (1986) and Kwon and Morris (1997) examined the effects of zonation on the efficiency of hepatic drug removal. Sato and colleagues demonstrated that, for a drug encountering a homogeneous diffusional barrier but variably distributed metabolic processes, removal is most efficient when the drug metabolizing enzyme is also homogeneously distributed (Sato et al., 1986). Kwon and Morris (1997) further showed that removal is most efficient when the zonal gradients of transporter and intracellular enzyme (homogeneous, PP to PV or PV to PP gradient) parallel each other. Intuitively, the above is reasonable since both transporter and metabolic functions must co-exist for removal.

Metabolic zonation may have important pharmacological and toxicological effects. Some metabolic pathways can lead to the production of a toxic compound. Zonation of these pathways may cause toxicity to be localized to a specific region of the liver (Anundi et al., 1993). Additionally, the zonation of metabolite production and other cellular mechanisms may have important consequences on local and systemic blood concentrations (Pang and Stillwell, 1983; Tirona et al., 1999). If the production of metabolite occurs in PP cells, the metabolite that is effluxed back into the sinusoids may
be further metabolized by hepatocytes more distal to the site of production. However, a metabolite produced in the PV cells will exit the liver and circulate throughout the body before returning to the liver for further metabolism. The metabolite, formed from PP or PV cells, may exert different effects on both the liver and extrahepatic tissues if the metabolite exhibits biological or toxicological activity.

1.4.2 Pharmacokinetic Modeling

The phenomena of drug removal can be studied at many levels, from perfusion studies with the intact organ to characterization of individual proteins. Data derived from whole organ studies often reflect closely the \textit{in vivo} situation and are therefore useful. However, the data may be limited in providing mechanistic explanations for the observations from the whole organ. Data derived using isolated cells, subcellular fractions, or purified proteins are useful for providing insight into the details of a specific protein or isolated process. However, it is often difficult to correlate the \textit{in vitro} data back to the \textit{in vivo} situation (Pang and Chiba 1994b; Ito \textit{et al.} 1998).

One reason for the lack of correlation between data \textit{in vitro} and \textit{in vivo} may be due to unexplored zonal factors (Pang and Chiba 1994b). It therefore becomes desirable to construct a more comprehensive model of cellular processes and their zonation since the zonated events affect the overall removal by the liver and the pharmacokinetics of a drug. Such a model would allow one to incorporate the scaling-up of kinetic parameters determined \textit{in vitro} to the whole organ to allow the prediction of the influence of zonal heterogeneity on clearance processes.
Several models of hepatic drug clearances have been proposed. These vary with respect to the manner in which blood mixes within the sinusoid and how substrate is dispersed among hepatocytes across the acinus. The most prominent of these models are the “well-stirred” (or venous equilibrium) model (Rowland et al., 1973), the “parallel-tube” (or sinusoidal) model (Winkler et al., 1973), the “dispersion” model (Roberts and Rowland, 1986a; 1986b) and the distributed model (Bass et al., 1978; Forker and Luxon, 1978).

1.5 Enalapril as a Model Substrate to Study Zonal Processing

Enalapril possesses characteristics which make the compound an ideal choice for the study of zonal processing. Enalapril is the prototypic member of the carboxyalkyl subgroup of angiotensin converting enzyme inhibitors (ACEi), often co-administered with a diuretic as first line treatment of hypertension and congestive heart failure. It is given in dosages of 2.5 to 40 mg daily and peak serum concentrations of 150 ng/mL (or 430 nmol/L) have been reported after a 40 mg dose (Swanson et al., 1984). It has a molecular weight of 348 (Figure 1-1) and is moderately lipid soluble compared with other ACEi (Ranadeive et al., 1992). Oral bioavailability is improved with enalapril maleate, an esterified prodrug. Once absorbed, enalapril is metabolized, primarily by the liver (Tocco et al., 1982), to form the more polar diacid metabolite enalaprilat, the active ACE inhibitor. Although evidence exists for biliary excretion in rats (Pang et al., 1984), this has not been demonstrated in humans, where both enalapril and enalaprilat are primarily excreted by the kidney (Ulm et al., 1982).
1.5.1 Metabolism of Enalapril in Rats

Enalapril is a good model compound for further investigation of zonal processing due to the relatively simple metabolic scheme (Figure 1-1) and absence of complicating factors such as red blood cell distribution, futile cycling, or sequential metabolism which make the scale-up procedure simpler (Pang et al., 1984; de Lannoy et al., 1989). The esterase activity for the conversion of the prodrug enalapril to its active de-esterified, diacid metabolite enalaprilat (Figure 1-1) is known to exist in both human and rat livers (Tocco et al. 1982; Ulm et al., 1982) and kidneys (Ulm et al., 1982; de Lannoy et al. 1989) as well as rat blood (Tocco et al., 1982). In rats in vivo, enalapril is absent in the urine, presumably due to extensive hydrolysis to enalaprilat that also occurs in rat
erythrocytes and liver (Tocco et al. 1982). However, hydrolysis of enalapril is not significant in human and bovine red blood cells (de Lannoy et al., 1989; Sirianni and Pang, 1998). For the rat, the esterase activity is highest in the liver (Tabata et al. 1990). Once inside hepatocytes, enalapril is primarily metabolized to enalaprilat, and to a lesser degree, excreted unchanged into the bile (Pang et al. 1984; Figure 1-1). Enalaprilat is the only known metabolite of enalapril, is not itself further metabolized (Tocco et al. 1982) and is excreted into both bile and urine (Pang et al., 1984; Figure 1-1).

1.5.2 Enalapril as the Model Substrate

Many characteristics of enalapril render it an appropriate model drug for study of zonal processing. One characteristic is the lack of transport interaction of the parent compound and enalaprilat, despite that a marked difference exists between the hepatic extraction of the ester-prodrug enalapril and enalaprilat. At the flow rate of 10 mL/min, whole liver perfusion studies in rat (conducted with outdated human erythrocytes) showed that enalapril was removed intracellularly with a high extraction ratio of 0.86; the extraction ratio of preformed enalaprilat, however, was approximately 0.05 (Pang et al., 1984). No change in binding and removal kinetics for either drug or metabolite was observed when enalapril and enalaprilat were co-administered (Pang et al., 1984; de Lannoy et al., 1989). Other studies further showed that there was no interaction in transport between the compounds (Pang et al., 1998). Enalaprilat exhibited poor entry (Pang et al., 1984; Schwab et al., 1990) whereas enalapril uptake was more rapid due to
facilitated transport by Oatp1, the organic anion transporting polypeptide 1 cloned from rat liver (Pang et al., 1998).

More importantly, zonation has been observed for enalapril hydrolysis. In rat liver HAPV and HAHV perfusion studies, it was found that the PV zone exhibited a higher capacity than the PP region in the conversion of enalapril to its active metabolite, enalaprilat (Pang et al., 1991). Based on the comparison between extraction ratios and the cellular water space accessed by both modes of perfusion, it was concluded that the hydrolytic activity towards enalapril was greater in the perivenous region (Pang et al. 1991). While this study clearly demonstrated that the overall process of esterolysis was zonated, the mechanism(s) responsible for the occurrence, whether transport or metabolism, was not identified. Although it has been established that enalapril is taken up by Oatp1 at the sinusoidal membrane of hepatocytes (Pang et al., 1998), the distribution of Oatp1 among hepatocytes and the contribution of Oatp1 to the overall transport is, however, unknown. In order to gain further insight into the effects of zonation on hepatic drug removal, it becomes essential to delve into the roles of zonal transport and metabolism for enalapril.
Chapter Two

STATEMENT OF PURPOSE OF INVESTIGATION
2.1 OBJECTIVES

The objective of the present investigation is to further the development of hepatic clearance models by incorporation of zonal factors on transport and metabolism. Enalapril, a compound that is primarily metabolized to a single metabolite, enalaprilat, and is excreted unchanged into the bile to a much lesser degree, was chosen for study. Previous studies have shown that the overall hydrolytic activity towards enalaprilat is concentrated in the perivenous region of the rat liver. The cellular function(s) - transport and/or metabolism - that can be zonally distributed and give rise to this phenomenon, however, have not been identified. The purpose of this investigation was to study the presence of zonation of transport and/or metabolism and how this affects the overall removal of enalapril.

To fulfill this purpose, rat hepatocyte populations isolated from whole rat liver and enriched in PP or PV cells were prepared, and the kinetic parameters for uptake and metabolism of enalapril were determined. The kinetic parameters were then scaled-up to the level of the whole liver for prediction of organ events in a mathematical model of hepatic removal.

2.2 SPECIFIC AIMS

I. To determine the in vitro kinetic parameters for enalapril uptake by intact hepatocytes and metabolism in subcellular fractions prepared from whole rat liver or from the periportal or perivenous zone.
II. To integrate scaled up \textit{in vitro} parameters into a model of hepatic clearance of enalapril.

2.3 HYPOTHESES TESTING

We wished to test hypotheses that:

I. Metabolic and not transport activity is zonated.

II. Zonal transport and metabolism activities towards enalapril \textit{in vitro}, when appropriately scaled up, adequately reflect those in the whole liver.
Chapter 3
TRANSPORT AND METABOLISM OF ENALAPRIL
BY ISOLATED RAT HEPATOCYTES IN VITRO
3.1 ABSTRACT

Although the esterolysis of enalapril was greater in the perivenous zone of the rat liver (Pang et al., J. Pharmacol. Exp. Ther. 1991), it was unknown whether transport or intracellular metabolism or both were zonally distributed. In the present study, the kinetics of enalapril transport and metabolism in rat hepatocytes from different zonal regions were studied and compared. Isolated hepatocytes, prepared from whole rat liver (homogeneous hepatocytes) with collagenase perfusion, and enriched periportal (PP) and perivenous (PV) hepatocytes, prepared by zone selective destruction with digitonin, were used for transport studies. Cells were also homogenized to provide the 9000 x g supernatant (S9) for metabolic studies. Uptake of [3H]enalapril (1 to 750 μmol/L) by whole liver hepatocytes (in the presence and absence of Na+) and for PP and PV cells was described by a single saturable component of similar kinetic constants (Km<sub>uptake</sub> of 344 to 461 μmol/L and V<sub>max</sub> of 9.5 to 11.6 nmol/min/10<sup>6</sup> cells; P > 0.05, ANOVA). The K<sub>m</sub> was of the same order of magnitude to that found for the uptake of enalapril by Oatp1, the sodium-independent organic anion transporting polypeptide 1 cloned from rat liver (214 μmol/L). Moreover, Oatp1 was found to be evenly distributed among the acinar zones with Western blotting.

The metabolism of enalapril by S9 prepared from homogeneous hepatocytes was partially inhibited by paraoxon, implicating the involvement of B-type esterases for hydrolysis. Metabolism of [3H]enalapril (50 to 4000 μmol/L) by S9 prepared from
homogeneous, PP and PV hepatocytes was also described by simple Michaelis-Menten kinetics. There were zonal differences in kinetic parameters among the cell populations; in S9 prepared from perivenous hepatocytes, the parameters $V_{\text{max}}^{\text{S9}}$ (21±6.0 nmol/min/mg S9 protein) and $K_m^{\text{S9}}$ (2612±236 μmol/L) were both greater than those for S9 prepared from periportal hepatocytes ($V_{\text{max}}^{\text{S9}}$ of 5.5±3.1 nmol/min/mg S9 protein and $K_m^{\text{S9}}$ of 1049±335 μmol/L; ANOVA $P < 0.05$)

3.2 INTRODUCTION

Heterogeneity of transport proteins or metabolic enzymes among zonal cells is known to affect the hepatic uptake and ultimate removal of substrates (Sato et al., 1986; Pang et al., 1992; Lindros, 1997; Kwon and Morris, 1997). Kwon and Morris (1997) demonstrated that heterogeneity of uptake and metabolic capacities in liver could have important consequences on the clearance of drugs. It is known that epidermal growth factor (Martí and Gebhardt, 1991) is taken up more abundantly in the periportal region (zone 1) while cysteine (Saiki et al., 1992) and glutamate (Stoll et al., 1991; Burger et al., 1989; Cooper et al., 1992) both show greater uptake by perivenous (zone 3) cells. Additionally, many enzymes involved in the metabolism of endogenous and xenobiotic compounds are zonally distributed (for reviews see, Pang and Xu, 1988; Jungermann and Katz, 1989; Gebhardt 1992; Oinonen and Lindros, 1998).

Enalapril is hydrolyzed to its active, de-esterified metabolite, enalaprilat, primarily in the PV region of the rat liver (Pang et al., 1991) as determined by HAHV/HAPV perfusion studies. While the HAPV/HAHV perfusion study clearly
demonstrated that the overall process of esterolysis was zonated, occurring predominantly in the perivenous region, the responsible mechanism(s), whether transport or metabolism, was not identified.

### 3.2.1 Transport of Enalapril

The organic anion transporting polypeptide (Oatp) family is comprised of polyspecific transport proteins which were recently cloned from rat [Oatp1 (Jacquemin et al., 1994), Oatp2 (Noé et al., 1997), Oatp3 (Abe et al., 1998)] and human [OATP (Kullak-Ublick et al., 1995)] tissues. These proteins are expressed in various tissues including the brain, the apical membrane of the kidney, and the basolateral membrane of hepatocytes where they mediate the transport of a variety of compounds (Jacquemin et al., 1994; Noé et al., 1997; Angeletti et al., 1997). Oatp transport is a Na⁺-independent (Jacquemin et al., 1994) process involving the probable countertransport of bicarbonate (Satlin et al., 1997) and/or glutathione (Li et al., 1998). Oatp1, in particular, is capable of transporting endogenous organic anions such as the bile acid taurocholate (Jacquemin et al., 1994) and the steroid hormone estrone-3-sulfate (Bossuyt et al., 1996a; 1996b). The cholephilic dye bromosulfophthalein (Jacquemin et al., 1994), modified peptides exemplified by the thrombin inhibitor CRC-220 (Eckhardt et al., 1996) and the angiotensin converting enzyme inhibitors, enalapril (Pang et al., 1998) and temocaprilat (Ishizuka et al., 1998), have all been identified as exogenous substrates of Oatp1.

Enalapril was identified as a substrate of Oatp1 in HeLa cells transfected with Oatp1-cDNA, where it exhibited a Michaelis-Menten constant (K_m) of 214 μmol/L.
towards the transport of enalapril (Pang et al., 1998). Verification of Oatp1 uptake of this prototypic non-sulphhydryl angiotensin converting enzyme inhibitor (ACEi) provided an explanation of the high clearance of enalapril in the single pass perfused liver preparation (Pang et al., 1984). By contrast, enalaprilat, the active diacid metabolite of enalapril, was found to be poorly removed from plasma in liver perfusion studies (de Lannoy et al., 1986). It was later verified in multiple indicator dilution perfusion studies that a diffusional barrier exists for the sinusoidal entry of enalaprilat into the rat liver (Schwab et al., 1990). Enalaprilat is neither an Oatp1 substrate nor an inhibitor of enalapril transport (Pang et al., 1998).

3.2.2 Metabolism of Enalapril

Enalapril is a pro-drug that is metabolized via hydrolysis of the ethyl ester group to yield the active diacid metabolite, enalaprilat. This hydrolysis is affected by esterases, enzymes which are traditionally classified as types A, B, and C (Aldrich, 1953). Type-A esterases metabolize the organophosphate pesticide paraoxon without being inhibited by it. Type-B esterases, also referred to as carboxylesterases, are not able to metabolize paraoxon but are inhibited by the compound, whereas type-C esterases are neither inhibited nor metabolized by paraoxon. To date, three members of the type-B carboxylesterase family which are thought to be involved in hepatic xenobiotic metabolism, namely hydrolases A (Robbi et al., 1990; Huang et al., 1993), B (Yan et al., 1994; Yan et al., 1995) and C (Yan et al., 1995) have been cloned from rat liver. Expression of both hydrolases A and B is greater in the perivenous region of rat liver.
Additionally, a 59 kDa carboxylesterase that shows a perivenous zonation has been purified from rat liver (Satoh et al., 1985; Pohl et al., 1991). Hydrolase B is expressed in rat kidney as well as liver (Yan et al., 1994) and is able to hydrolyze aspirin, propanidid (Mentlein and Heyman, 1984) and the ACEi ester-prodrugs benazepril, delapril, and temocapril (Luan et al., 1997). Hydrolase A is involved in the esterolysis of clofibrate and procaine (Mentlein and Heyman, 1984). Hydrolases A and B are both paraoxon-inhibitable, and are distinguished from each other by their affinity to catalyze the esterolysis of p-nitrophenol acetate (Morgan et al., 1994). The substrate specificity and role in xenobiotic metabolism played by hydrolase C is less well characterized (Yan et al., 1995).

Carboxylesterase activity towards enalapril has been found in the rat liver, blood (Tocco et al. 1982), and kidney (de Lannoy et al. 1989). Among the homogenates of these tissues, the highest carboxylesterase activity is in the liver where metabolism is characterized by a $K_m$ of 710 μmol/L (Tabata et al. 1990). Renal metabolism of enalapril is mediated solely by B-type esterases (Grima et al., 1991; Sirianni and Pang, 1999). However, the identity and zonation of the enzyme(s) responsible for this esterolysis in liver are presently not known. Members of the rat CYP 3A family have also been inferred to metabolize enalapril to an unidentified toxic metabolite in rat hepatocytes treated with high doses of enalapril (Jurima-Romet et al., 1991a,b). It is suspected, however, that the formation of such a metabolite is minor since formation of enalaprilat accounted almost completely for the loss of enalapril (Tocco et al., 1982; Pang et al., 1984).
The *in vitro* studies presented herein were conducted to test the hypotheses that metabolism and not uptake of enalapril was greater in the perivenous region than periportal region of rat liver. For hypothesis testing, hepatocytes isolated from whole rat liver or enriched in cells of the periportal or perivenous zone were used as intact cells for uptake studies or were further processed to provide S9 subcellular fractions for metabolic studies. Moreover, the susceptibility of enalapril hydrolysis to paraoxon inhibition was determined for comparison to that in kidney.

3.3 METHODS AND MATERIALS

3.3.1 Materials

[^3H]Enalapril, [(S)-1-[N-[1-(ethoxycarbonyl)-3-phenylpropyl]-L-alanyl]-L-[5-^3H]proline], synthesized as described previously (Pang *et al.*, 1998), was 98.5% pure as judged by thin layer chromatography (1-propanol: 1 M acetic acid: water, 10:1:1, v/v/v, with silica gel GF TLC plates). Enalaprilat and unlabeled enalapril were obtained from Merck-Frosst. [^14C]Sucrose (specific activity, 0.05 mCi/mL) was purchased from Dupont (Boston, MA). Estrone-3-sulfate, harmol sulfate, DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid), ouabain, rotenone, bovine serum albumin and alanine-proline were purchased from Sigma Chemical Co. (St. Louis, MO). KCN and paraoxon were obtained from Aldrich Chemical Company Inc. Collagenase A (clostridium histolyticum) was purchased from Boehringer Mannheim, Dramstadt, Germany. All other reagents and solvents were of HPLC grade.
3.3.2 Isolation of Rat Hepatocytes

3.3.2.1 Isolation of Hepatocytes from Whole Liver (Homogeneous Hepatocytes).

Isolated rat hepatocytes were prepared by the method of Hassen et al. (1996) from male Sprague-Dawley rats (275-375 g, Charles River, St. Constant, PQ). Rats were killed by intraperitoneal injection of pentobarbital (65 mg/kg) according to protocols approved by the Animal Care Committee of the Department of Comparative Medicine at the University of Toronto. All buffers were pre-gassed with carbogen (95% O₂, 5% CO₂, Matheson, Mississauga, ON). The portal vein was perfused with a Ca²⁺-free buffer [preperfusion buffer, consisting of Hanks buffer (137 mmol/L NaCl, 5.4 mmol/L KCl, 0.5 mmol/L NaH₂PO₄·2H₂O, 0.42 mmol/L Na₂HPO₄·12 H₂O), plus 10 mmol/L HEPES (pH 7.2), 0.5 mmol/L EGTA, 4.2 mmol/L NaHCO₃, 5 mmol/L glucose, and 0.65% bovine serum albumin] in a single pass fashion at 30 mL/min for 10 minutes. Hepatocytes from all zones of the liver were prepared by perfusion with collagenase (Hanks buffer with 4 mmol/L CaCl₂ and 0.05% collagenase) which recirculated through the liver for 7 to 9 minutes at a flow rate of 25 mL/min.

The collagenase-digested liver was placed in a petri dish filled with incubation buffer [137 mmol/L NaCl, 5.4 mmol/L KCl, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂·6H₂O, 0.83 mmol/L MgSO₄·7H₂O, 0.5 mmol/L NaH₂PO₄·2H₂O, 0.42 mmol/L Na₂HPO₄·12H₂O, 4.2 mmol/L NaHCO₃, 5 mmol/L glucose, and 1 mmol/L HEPES (pH 7.4)] and agitated to release isolated hepatocytes into the medium. The viability of the resulting hepatocyte suspension was > 90% viable, as assessed by trypan blue exclusion.
3.3.2.2 Isolation of Zonal (PP or PV) Hepatocytes. Zonally isolated hepatocytes were harvested following the procedures of Quistorff (1985) and Lindros and Penttilä (1985) by zone-selective destruction with digitonin. Subsequent to digitonin perfusion, livers were digested and processed in an identical manner to that described previously for homogeneous hepatocytes (section 3.3.2.1). The resulting enrichment of PP or PV hepatocytes was verified via marker enzymes (Tan et al., 1999). The activity of the periportal marker enzyme, alanine transferase (Gorgens et al., 1988), was found to be present at a ratio of 2.20±0.09 times higher in periportal hepatocytes (461±150 nmol/min/mg protein) than in perivenous hepatocytes (210±49 nmol/min/mg protein), and was statistically different (ANOVA P < 0.05). Conversely, glutamine synthetase, an enzyme expressed exclusively in the distal perivenous hepatocytes (Burger et al., 1989), exhibited significantly lower activity in periportal hepatocytes (0.52±0.96 nmol/min/mg protein) than in perivenous preparations (27.7±11.6 nmol/min/mg protein) (ANOVA P < 0.05); the PP:PV ratio was 0.018±0.03.

3.3.3 Preparation of S9 Fraction from Homogeneous and Zonal Hepatocytes

Hepatocyte suspensions of >90% viability were centrifuged for 5 minutes at 850 x g. The incubation buffer supernatant was aspirated off, and ice cold Krebs-Ringer bicarbonate buffer (KRB; containing 120 mmol/L NaCl, 25 mmol/L of NaHCO₃, 4.75 mmol/L KCl, 2.54 mmol/L CaCl₂, 1.19 mmol/L MgSO₄·7H₂O, and 1.19 mmol/L KH₂PO₄) was added in an amount three times the volume of the hepatocyte pellet. The mixture was shaken by vortex then homogenized for 1 to 2 minutes (Ultra Turrax T25 homogenizer,
Janke and Kunkel, IKA-Labortechnik, West Germany). Homogenates were centrifuged (Beckman J2-21M Centrifuge, Beckman Canada, Mississauga, ON) at 9000 x g at 4°C for 20 minutes. The resulting supernatant (S9 fraction) was either used immediately or stored at -70°C until future use.

3.3.4 Transport Studies

3.3.4.1 Uptake Studies. Hepatocyte suspensions (1 mL of 2 x 10^6 cells/mL) were preincubated in a rotating water bath for 10 minutes at 37°C. Aliquots (500 μL) of the suspension were added to 100 μL of various solutions containing enalapril, tracer [³H]enalapril, and [¹⁴C]sucrose (an extracellular marker) in 1.15% KCl to result in enalapril concentrations of 1 to 750 μmol/L in 1.67 x 10^6 cells/mL. After admixture, samples (100 μL) were retrieved over 1 minute and centrifuged immediately (9,650 x g) for rapid filtration through a layer of silicon oil, separating the hepatocytes from the extracellular medium. Samples were taken from both the supernatant and cell pellet for liquid scintillation counting (Model LS 6800, Beckman Canada, Mississauga, ON).

3.3.4.2 Inhibitor Studies. The ability of benzoic acid (100 μmol/L), harmol sulfate (200 μmol/L), estrone-3-sulfate (100 μmol/L) or the dipeptides, alanine-proline (400 μmol/L) and enalaprilat (400 μmol/L), to cis-inhibit enalapril uptake was examined by individually adding the compounds to the enalapril solution prior to the addition of hepatocytes. The metabolic inhibitor KCN (2 mmol/L), the anion transport inhibitor, DIDS (2 mmol/L; 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid), and the Na⁺/K⁺
ATPase inhibitor, ouabain (1 mmol/L), were added to the cell suspension during the last 2 minutes of the 10-minute hepatocyte preincubation interval, whereas the metabolic inhibitor, rotenone (30 μmol/L), was added to the hepatocyte suspensions for preincubation for 30 minutes prior to the addition of enalapril. An equivalent volume of buffer was added for the control samples. All control samples were preincubated for 10 minutes except for the controls for rotenone, which were preincubated for 30 minutes. In all cases, the final concentration in the incubation mixture was 1 μmol/L of enalapril and 1.67 x 10⁶ cells/mL. The effect of treatment was expressed as a percent of the uptake rate of the control.

3.3.5 Metabolic Studies

3.3.5.1 Metabolic Studies with S9 from Homogeneous Hepatocytes. Preliminary studies had shown that identical rates of enalapril hydrolysis were obtained with freshly prepared S9 and thawed (frozen at -70°C) S9 preparations (see Appendix 1). Thawed S9 preparations were diluted to a concentration of approximately 2 mg protein/mL then preincubated in a rotating water bath for 10 minutes at 37°C. Aliquots (100 μL) of S9 were added to 100 μL of various solutions containing enalapril and tracer [³H]enalapril in KRB to result in enalapril concentrations of 50 to 4000 μmol/L in 0.2 mL incubation mixture. Samples (100 μL) were retrieved after 15 minutes. Each sample was placed into a 1.5 mL microfuge tube containing 300 μL ice cold CH₃CN to halt the reaction. The tubes were centrifuged and the supernatants were stored at -20°C for later analysis by thin
layer chromatography. Protein was measured by the method of Lowry et al. (1951). Fifty μL of the remaining incubation mixture was removed for liquid scintillation counting.

3.3.5.2 Paraoxon Inhibition Studies. S9 preparations made from whole liver hepatocytes were thawed, diluted to a concentration of approximately 2 mg protein/mL then preincubated in a rotating water bath for 10 minutes at 37°C. The ability of paraoxon to inhibit enalapril metabolism was examined by first adding paraoxon to S9 (100 μL), followed immediately by the admixture of 100 μL of enalapril and [3H]enalapril in KRB. Final incubation mixture concentrations of 240 to 300 x 10^3 dpm [3H]enalapril/mL and 0.1 to 1 μmol/L paraoxon resulted. At 10 minutes after admixture, samples (100 μL) were retrieved and added to 300 CH3CN μL in a microfuge tube. The samples were then processed identically to that described earlier for the metabolic studies (section 3.3.5.1). An equivalent volume of buffer was added instead of paraoxon for control samples. The effect of treatment was expressed as a percent of the metabolic rate of the control. Protein was measured by the method of Lowry et al. (1951).

3.3.6 Assays of Enalapril and Enalaprilat

[3H]Enalapril and [3H]enalaprilat were separated by thin layer chromatography (TLC). Silica gel GF TLC plates (Analtech, Newark, DE) were initially prepared by placement of approximately 150 μL of a solution containing authentic standards of enalapril and enalaprilat in CH3CN and H2O. Two-hundred μL samples of the deproteinized solution of incubation buffer in CH3CN were spotted at the origin atop the
cold standards. The TLC plates were developed in a solvent system of 1-propanol: 1 M acetic acid: water (10:1:1, v/v/v). After resolution, the cold standards, which had co-migrated with their radiolabeled counterparts, were visualized under a UV lamp. Bands of TLC material containing radiolabeled enalapril and enalaprilat were scrapped off and placed separately into 20 mL glass vials containing 0.5 mL water and 10 mL scintillation cocktail (Ready Protein, Beckman Instruments). After mixing, the vials were stored in darkness for a minimum of 48 hours prior to liquid scintillation counting (Model LS 6800, Beckman Canada, Mississauga, ON). Additionally, 50 µL of sample was counted for on-plate recovery considerations.

3.3.7 Calculations

For uptake studies, the [14C]sucrose counts associated with the pellet accounted for the volume of extracellular medium entrapped by hepatocytes. The uptake of [3H]enalapril was thus corrected for the radioactivity trapped within the extracellular space, and the specific activity of the enalapril sample was used to calculate the rate of enalapril uptake. The cumulative amounts taken up into cells (nmol/10^6 cells), obtained after correction for the entrapped extracellular components, were plotted against the incubation time. Linear regression of the data yielded the slope which represents the initial uptake rate (v).

For metabolic studies, the specific activity of enalapril was estimated by dividing the counts obtained from direct counting of the radioactivity in the incubation mixture (in dpm/mL) by the concentration of unlabelled enalapril. Liquid scintillation counting of the
scraped regions of the TLC plates revealed the relative amounts of radioactive enalapril and enalaprilat present in each incubation and the specific activity of the sample was used to calculate the amount of metabolite formed per 15 minutes. The information provided the rate of metabolism, \( v \) (nmol enalaprilat formed/min/mg protein), for each incubation mixture.

The kinetic constants describing uptake or metabolism for a single saturable system were obtained by regression of \( v \) versus [S] according to the equation 3-1. Additionally, the possible existence of multiple enzymes or linear processes contributing to the overall observed rate was examined by regression of \( v \) versus [S] to different models (equations 3-2 and 3-3), which described a system with saturable and non-saturable components, and a system consisting of two saturable components, respectively.

For a system consisting of a single Michaelis-Menten component,

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

for a system with saturable and non-saturable components,

\[
v = \frac{V_{\text{max}} [S]}{K_m + [S]} + P_{\text{diff}} [S] \tag{3-2}
\]
and for a system consisting of two saturable components:

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

(3-3)

where \( K_m \) represents the Michaelis-Menten constant and the \( V_{\text{max}} \) denotes the maximal velocity, and subscripts 1 and 2 define the presence of the first and second saturable components, respectively; \( P_{\text{diff}} \) is the term denoting nonsaturable clearance for uptake or metabolism.

3.3.8 Fitting and Data Handling

Data fitting to equation 3-1, 3-2, or 3-3 was performed by an iterative, nonlinear least squares procedure (Scientist\textsuperscript{2}, v.2 Micromath Scientific Software, Salt Lake City, Utah). The Model Selection Criteria (MSC) was used to determine the appropriateness of the equations: the greater the MSC value for a model, the better that model describes the data. A weighting scheme of \( 1/\text{observation}^2 \) was used since this provided the best optimization of the data. The goodness of fit was judged by the standard deviation (SD) of the parameter estimate or the coefficient of variation (CV; SD/parameter estimate), residual plots, and the residual sum of squares.

3.3.9 Statistics

The data were presented as mean ± SD. The means were compared by use of ANOVA or the paired \( t \)-statistic accordingly, with \( P = 0.05 \) denoting the level of significance.
3.4 RESULTS

3.4.1 Transport

3.4.1.1 Enalapril Uptake into Hepatocytes. The uptake of enalapril among all hepatocyte preparations (homogeneous, periportal, perivenous) was linear over 1 minute for all concentrations (Figure 3-1). Saturation of enalapril uptake was evident for rat hepatocytes isolated from whole liver, in the presence and absence of sodium ion (Figure 3-2), and with periportal and perivenous hepatocytes (Figure 3-3). The best fit in all cases was equation 3-1, which described a single saturable component for enalapril uptake. The fitted $V_{\text{max}}^{\text{uptake}}$ in the presence of sodium for hepatocytes from whole liver was $11 \pm 1.5 \text{ nmol/min/10}^6 \text{ cells}$ and the $K_{m}^{\text{uptake}}$ was $344 \pm 52 \text{ umol/L}$, values that did not differ from those obtained in absence of sodium ion ($V_{\text{max}}^{\text{uptake}}$ of $11.6 \pm 4.2 \text{ nmol/min/10}^6 \text{ cells}$ and $K_{m}^{\text{uptake}}$ of $446 \pm 79 \text{ umol/L}$; Table 3-1; $P > 0.05$, ANOVA). Uptake of enalapril was not different among periportal (PP) and perivenous (PV) cells ($V_{\text{max}}^{\text{uptake}}$ of $9.5 \pm 2.8$ and $10.4 \pm 2.0 \text{ nmol/min/10}^6 \text{ cells}$ and $K_{m}^{\text{uptake}}$ of $461 \pm 177$ and $411 \pm 50 \text{ umol/L}$; $P > 0.05$, ANOVA, Table 3-1). When these constants were compared to those derived from hepatocytes isolated from whole liver (data with Na$^+$), no difference was detected ($P > 0.05$, ANOVA).
Table 3-1. Kinetic parameters for uptake of enalapril by isolated homogeneous and zonal rat hepatocytes.a

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$K_m^{uptake\ b}$ (µmol/L)</th>
<th>$V_{max}^{uptake\ b}$ (nmol/min/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogeneous Cells with Sodium (n=5)</td>
<td>344±52</td>
<td>11.0±1.5</td>
</tr>
<tr>
<td>Homogeneous Cells without Sodium (n=4)</td>
<td>446±179</td>
<td>11.6±4.2</td>
</tr>
<tr>
<td>Periportal Cells without Sodium (n=4)</td>
<td>461±117</td>
<td>9.5±2.0</td>
</tr>
<tr>
<td>Perivenous Cells with Sodium (n=4)</td>
<td>441±50</td>
<td>10.4±2.0</td>
</tr>
</tbody>
</table>

a The weighting scheme of 1/(observation)^2 was optimal. Data were presented as mean ± SD and are not different (P > 0.05, ANOVA)

b Kinetic constants obtained from optimized fitting to a single saturable component model (Eq. 3-1)

3.4.1.2 Additional Studies on Transport. Studies on the expression of Oatp1 in zonal hepatocytes and the affinity of other cloned transporters towards enalapril were performed in the laboratories of Professors Richard B. Kim (Vanderbilt University, School of Medicine, Nashville, Tennessee) and Allan W. Wolkoff (Albert Einstein College of Medicine, Bronx, New York). These studies were done in conjunction with the uptake studies described in this chapter. A full account of the results and explanation of their contribution appears in Appendix 2. Briefly, it was found that enalapril was not a substrate of Oatp2 nor OATP expressed in HeLa cells, and that expression of Oatp1 determined by Western blotting was the same for PP and PV rat hepatocytes.
3.4.1.3 Inhibitor Uptake Studies. The uptake of 1 μmol/L of enalapril was unaffected by the metabolic inhibitors KCN (2 mmol/L) and rotenone (30 μmol/L) ($P > 0.05$, paired $t$-test, Figure 3-4). In addition, benzoic acid (100 μmol/L), enalaprilot (400 μmol/L), and the dipeptide alanine-proline (400 μmol/L) failed to affect the uptake of enalapril ($P > 0.05$, paired $t$-test, Figure 3-4). However, the anion transport inhibitor
DIDS (2 mmol/L), temperature reduction, the Oatp1 substrate estrone-3-sulfate (100 µmol/L) (Bossuyt et al., 1996b) and surprisingly, harmol sulfate (200 µmol/L), all significantly inhibited enalapril uptake ($P < 0.05$, paired $t$-test, Figure 3-4). Unexpectedly, ouabain (1 mmol/L), another Oatp1 substrate (Abe et al., 1998), failed to exert a significant effect on the uptake of enalapril. This is most likely due to the high $K_m$ for transport of ouabain (1,700 to 3,000 µmol/L; Reichel et al., 1999).

Figure 3-2. Uptake kinetics of enalapril by homogeneous rat hepatocytes in the presence and absence of sodium ion.
3.4.2 Metabolism

3.4.2.1 Enalapril Metabolism in S9 Fraction of Homogeneous and Zonal Hepatocytes. In the S9 fractions of homogeneous, PP, or PV hepatocytes, no loss of metabolic activity was observed in thawed versus fresh preparations (see Appendix 1). In all preparations, metabolism was concentration dependent (Figure 3-5). For all cases, the best fit was equation 3-1 which described a single saturable component. The fitted $V_{\text{max,PP}}^{\text{met}}$ was $5.5 \pm 3.1 \text{ nmol/min/mg S9 protein}$ and the corresponding $K_{\text{m,PP}}^{\text{met}}$ was $1049 \pm 335 \text{ } \mu\text{mol/L}$ for PP hepatocytes. These values were not statistically different from those obtained.
from the S9 of homogeneous hepatocytes (HO): \( V_{\text{max,HO}}^{\text{met}} \) of 8.0±2.99 nmol/min/mg S9 protein and \( K_{m,HO}^{\text{met}} \) of 1308±419 µmol/L (Table 3-2; \( P > 0.05 \), ANOVA). However, the fitted \( V_{\text{max,PV}}^{\text{met}} \) (21.0±6.0 nmol/min/mg S9 protein) and \( K_{m,PV}^{\text{met}} \) (2612±236 µmol/L) for the S9 of perivenous hepatocytes were both significantly higher than those observed for the S9 of either HO or PP (Table 3-2; \( P < 0.05 \), ANOVA).

Table 3-2. Kinetic parameters for the metabolism of enalapril in rat S9 fractions.\(^a\)

<table>
<thead>
<tr>
<th>Preparation</th>
<th>( K_m^{\text{met,b}} ) (µmol/L)</th>
<th>( V_{\text{max}}^{\text{met,b}} ) (nmol/min/mg S9 protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S9 from homogeneous (HO) hepatocytes (n=4)</td>
<td>1308±419</td>
<td>8.0±3.0</td>
</tr>
<tr>
<td>S9 from periportal (PP) hepatocytes (n=4)</td>
<td>1049±335</td>
<td>5.5±3.1</td>
</tr>
<tr>
<td>S9 from perivenous (PV) hepatocytes (n=4)</td>
<td>2612±236(^{c,d})</td>
<td>21.0±6.0(^{c,d})</td>
</tr>
</tbody>
</table>

\(^a\) The weighting scheme of \( 1/(\text{observation}) \) was optimal. Data were expressed as mean ± SD
\(^b\) Kinetic constants obtained from optimized fitting to a single saturable component model (Eq. 3-1)
\(^c\) Significant (\( P < 0.05 \)) compared with S9 of homogeneous hepatocytes
\(^d\) Significant (\( P < 0.05 \)) compared with S9 of periportal hepatocytes


Figure 3-4. Effect of various compounds on enalapril (1 μmol/L) uptake into rat hepatocytes prepared from whole liver, in the presence of sodium. The ability for the Oatp1 substrates [estrone-3-sulfate (100 μmol/L) and ouabain (1 mmol/L)], harmol sulfate (200 μmol/L), the dipeptides [alanine-proline (400 μmol/L) and enalaprilat (400 μmol/L)], the monocarboxylic acid benzoic acid (100 μmol/L), the non-specific anion transport inhibitor DIDS (2 mmol/L), temperature reduction, and the metabolic inhibitors [KCN (2 mmol/L) and rotenone (30 μmol/L)] to inhibit enalapril uptake was examined over 1 minute. Values were expressed as % control ± S.D. (n=3 to 6). The symbol "*" denotes treatment values which are statistically different from controls, (P < 0.05, paired t-test). The symbols are: DIDS, 4-4'-diisothiocyanostilbene-2,2'-disulfonic acid, Ala-pro, alanine-proline dipeptide; KCN, potassium cyanide.

3.4.2.2 Metabolic Inhibition Studies. The metabolism of enalapril was significantly inhibited by paraoxon in a concentration dependent fashion (P < 0.05 paired t-test, Figure 3-6). At a concentration of 0.1 μmol/L paraoxon, metabolism of enalapril was reduced to 44±20 % of control (n=3). At 0.5 and 1 μmol/L paraoxon, metabolism of enalapril to enalaprilat was 32±9% and 29±8% of control, respectively (Figure 3-6).
**Figure 3-5.** Kinetics of enalapril metabolism by S9 of homogeneous, PP and PV preparations.

**Figure 3-6** Inhibition of enalapril hydrolysis in S9 of homogeneous hepatocytes by paraaxon.

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3.5 DISCUSSION

3.5.1 Uptake

The organic anion transporting polypeptides (Oatp1 and Oatp2; Jacquemin et al., 1994; Noé et al., 1997) and the sodium/taurocholate cotransporting polypeptide (ntcp; Hagenbuch et al., 1991) are cloned proteins expressed on the rat hepatocyte basolateral membrane that are capable of transporting organic anions. Presently, we showed that sodium ion is not involved in the transport of enalapril in isolated rat hepatocytes (Table 3-1), excluding the role of ntcp, as shown earlier (Pang et al., 1998). Moreover, uptake is mediated by Oatp1 and not Oatp2 which is known to transport other anions such as the estrogen conjugates, estradiol glucuronide and estrone sulfate (Noé et al., 1997), and pravastatin (Tokui et al., 1999). Our observations conform to those of a recent report on the lack of correlation of Oatp1 and Oatp2 substrate specificity (Reichel et al., 1999). These, and the similarity in $K_a^{\text{indir}}$ values for enalapril uptake between hepatocytes and Oatp1-expressing HeLa cells, suggest that Oatp1 is likely the only system mediating the uptake of enalapril in the rat liver.

Structurally, enalapril is a modified dipeptide of alanine-proline. Both enalapril and alanine-proline are substrates of the oligopeptide transporter, PepT1, expressed in the rat intestine and kidney (Temple and Boyd, 1998; Saito et al., 1995). A second isoform expressed in rat kidney, PepT2 (Saito et al., 1995), does not transport enalapril (Boll et al., 1996). Upon examination of the inhibitory action of alanine-proline on enalapril uptake, however, none was found (Figure 3-4). Enalaprilat, the active metabolite
of enalapril and also an ala-pro analog, was unable to inhibit enalapril uptake into hepatocytes (Figure 3-4). These observations are consistent with previous ones in that enalaprilat is neither a substrate of Oatp1 nor an inhibitor of enalapril transport in Oatp1-expressing HeLa cells (Pang et al., 1998). Moreover, it has been observed that enalaprilat was effective in inhibiting the transport of another ACE inhibitor, temocaprilat, into rat hepatocytes by processes that are mediated, in part, by Oatp1 (Ishizuka et al., 1998). As both enalaprilat and temocaprilat are dicarboxylic acids and enalapril is a monocarboxylic acid, it is plausible that enalapril and temocaprilat bind to different areas of Oatp1, explaining the differences in their susceptibility to enalaprilat inhibition.

To further explore whether or not Oatp1 is the sole transporting system, we employed substrates/inhibitors to effect cis-inhibition of enalapril transport into hepatocytes. There was expressed sensitivity towards the anion transport inhibitor, DIDS, which diminished enalapril uptake (Figure 3-4), as expected for the behaviour of an Oatp1 substrate (Pang et al., 1998; Tan et al., 1999). Estrone-3-sulfate, a known substrate of Oatp1 (Bossuyt et al., 1996a), significantly reduced enalapril uptake (Figure 3-4). Surprisingly, ouabain, another Oatp1 substrate (Abe et al., 1998), failed to affect the uptake of enalapril (Figure 3-4), although ouabain exhibited inhibition of the Na⁺/K⁺ ATPase at 1 mmol/L (Sillau et al., 1996; Nobes et al., 1989). The lack of inhibition with ouabain was also observed for bromosulfophthalein, BSP, in isolated hepatocytes (Schwenk et al., 1976) and Oatp1-cDNA transfected into X. laevis oocytes (Kullak-Ublick et al., 1994), and for its glutathione conjugate (BSPGSH; Schwarz et al., 1980),
another Oatp1 substrate (Pang et al., 1998). Absence of inhibition by ouabain may be explained by the relatively low affinity noted for Oatp1 ($K_m$ of 1,700 to 3,000 μmol/L; Noé et al., 1997; Reichel et al., 1999) compared with that for enalapril ($K_m$ of 350 to 440 μmol/L) and that oatp2 is likely to play a more important role than oatp1 in the hepatic uptake of ouabain (Noé et al., 1997).

Although Oatp1 transport appears to be independent of ATP (Li et al., 1998), uptake may be affected when intracellular ATP is depleted. Hence, uptake was further characterized with respect to temperature. An energy dependence was inferred since uptake was reduced drastically when the temperature was lowered (Figure 3-4). However, the metabolic inhibitors rotenone (30 μmol/L, preincubated for 30 minutes) and KCN (2 mmol/L) were unable to reduce enalapril uptake in the present studies. The lack of effect of rotenone or potassium cyanide on enalapril uptake was unexpected, since rotenone (30 μmol/L) was known to reduce ATP levels by 90% when preincubated with hepatocytes over 30 minutes (Yamazaki et al., 1993). Similar conflicting results concerning the effects of ATP depletion on Na$^+$-independent organic anion uptake have been reported. The discrepancy may be due to differences in the method of ATP depletion and the time over which uptake was determined (Yamazaki et al., 1993). BSPGSH uptake was found to be insensitive to the effects of rotenone (Schwarz et al., 1980). BSP uptake was unaffected by the metabolic inhibitors antimycin A, rotenone, and carbonylcyanide $m$-chlorophenylhydrazone (Schwenk et al., 1976), but was reduced when 2-deoxyglucose and sodium azide were employed for the depletion of ATP via glycolysis and
mitochondrial respiration, respectively, in hepatocytes (Yamazaki et al., 1993) and in Oatp1-expressing HeLa cells (Shi et al., 1995). However, uptake of the Oatp1 substrate temocaprilat, measured over 90 seconds, was inhibited by the electron transport inhibitor, rotenone (Ishizuka et al., 1998). These discrepancies are possibly explained by the fact that uptake of both temocaprilat and BSP are only partly mediated by Oatp1 in hepatocytes (Ishizuka et al., 1998; Hagenbuch et al., 1996).

On the basolateral membrane of hepatocytes, there exist other sodium-independent systems that could potentially transport enalapril. Benzoic acid, a putative substrate of the monocarboxylate transporter MCT2 (Garcia et al., 1995; Yoshimura et al., 1998) and not of Oatp1 (Pang et al., 1998) failed to inhibit enalapril uptake (Figure 3-4). The drug sulfate conjugate - harmol sulfate - that is transported into hepatocytes via an unknown mechanism, was inhibitory although harmol sulfate was not transported by Oatp1-expressing HeLa cells (Pang et al., 1998) and failed to inhibit the uptake of estrone sulfate (Tan et al., 1999). The recently cloned novel liver-specific transport protein OAT2 (Simonson et al., 1994; Sekine et al., 1998) that affects the uptake of salicylate, PGE2, dicarboxylates, and p-aminohippurate, is unlikely involved since there is a lack of inhibition of OAT2 transport by enalapril, and there is no known overlapping substrate specificity between OAT2 and Oatp1 (Kullack-Ublick et al., 1994; Sekine et al., 1998).

Inasmuch as Oatp1 is involved in the uptake of various pharmacologically important peptides and steroids, it becomes important to characterize its zonal expression and acinar function. No difference in kinetic parameters was observed for
uptake between hepatocytes prepared from the periportal and perivenous regions and whole rat liver (Figure 3-3, Table 3-1). Additionally, Western blot analysis revealed a lack of quantifiable difference between Oatp1 protein expression in periportal and perivenous hepatocytes (appendix 2). This observation provides the quantitative evidence on the homogeneity of Oatp1 expression and is consistent with prior observations of the lack of noticeable unevenness in the expression of this protein that was qualitatively observed under confocal microscopy (Bergwerk et al., 1996), and findings of a lack of lobular heterogeneity for Oatp1 mRNA as measured by in situ hybridization (Dubuisson et al., 1996; Angelletti et al., 1998). By contrast, an enriched midzonal-perivenous distribution of Oatp2, which is not involved in enalapril uptake, was suggested to exist (Kakyo et al., 1999a; Reichel et al., 1999). Since activities in zonal hepatocytes and Oatp1 are both homogeneously expressed across the acinus for the uptake of enalapril and since similar $K_m^{\text{uptake}}$s are obtained for both systems, it appears that Oatp1 is likely the only transporter involved in the uptake of enalapril.

3.5.2 Metabolism

3.5.2.1 Homogeneous Hepatocytes. Enalapril esterolysis in the 9000 x g supernatant fraction (S9) prepared from homogeneous hepatocytes was concentration-dependent and characterized by a $K_m^{\text{met}}$ of 1308 μmol/L and $V_{\text{max,NO}}^{\text{met}}$ of 8.0 nmol/min/ mg S9 protein (Table 3-2). In contrast to the present $K_m^{\text{met,NO}}$ value of 1308 μmol/L, Tabata et al., (1990) previously reported a $K_m^{\text{met}}$ of 710 μmol/L for the metabolism of enalapril by S9 in rat liver homogenates. The $V_{\text{max}}^{\text{met}}$ was not determined. Since non-parenchymal
cellular material and hepatocyte membranes are present in whole liver homogenates but not in the S9 fraction and since ester hydrolysis can occur in non-parenchymal cells (Steinberg et al., 1987), direct comparison of the two $K_m^\text{inhib}$ values may not be appropriate.

3.5.2.2 Inhibition of Liver Esterases by Paraoxon. Paraoxon, the product of desulfuration of the phosphorothioate insecticide parathion, binds irreversibly with the serine residue of B-type esterases (Aldrich, 1953). Low concentrations of paraoxon (0.1 to 1 μmol/L) were found to inhibit the metabolism of enalapril in S9 of homogeneous hepatocytes (Figure 3-6), suggesting that B-type esterases contribute significantly to the overall metabolism. However, in contrast to rat renal S9 where metabolism of enalapril was completely inhibited by paraoxon (Sirianni and Pang, 1999), hepatic metabolism was only 70% inhibitable at comparable concentrations of paraoxon (Figure 3-6). The lack of complete inhibition in liver suggests the contribution by non-B-type esterases to hepatic metabolism of enalapril. The CYP 450 isozymes and A-type esterases, both present in the rat liver and capable of hydrolyzing esters, are not inhibited by paraoxon (Guengerich, 1987; Guengerich et al., 1988; Pond et al., 1995). Many esters are substrates for more than one esterase (e.g. p-nitrophenol acetate, Morgan et al., 1994). Although the metabolism of enalapril in the S9 of homogeneous hepatocytes was apparently well described by a single saturable component, multiple enzymes of similar affinities may be involved, especially if there is a lack of resolution to discern individual systems. Therefore, it is very likely that these $V_{max}^\text{inhib}$ and $K_m^\text{inhib}$ values are apparent values.
representing the pooled activities of multiple enzymes rather than those of an individual enzyme.

### 3.5.2.3 Zonal Hepatocytes

The S9 fraction prepared from perivenous hepatocytes exhibited significantly higher maximal velocity ($V_{\text{max,PV}}^{\text{met}}$) than either S9 of homogeneous or PP hepatocytes (Table 3-2). If this difference were due to a greater expression of a single enzyme, differences in $V_{\text{max}}^{\text{met}}$ would be expected whereas the $K_m^{\text{met}}$ should be similar. However, the $K_{m,PV}^{\text{met}}$ was significantly higher than those for the S9 of PP and homogeneous hepatocytes (Table 3-2). As it is known that the carboxylesterases are in greater abundance in the PV region, and that these enzymes have overlapping substrate specificities and varying affinities, it is plausible that the greater maximal velocity in S9 of PV hepatocytes is due to greater expression of a number of carboxylesterases with lower affinities but higher capacities towards enalapril in the PV region. Again, the scenario reinforces the notion presented earlier (section 3.5.2.2) of the likelihood that the $V_{\text{max}}^{\text{met}}$ and $K_m^{\text{met}}$ are apparent, pooled values representing those constants for multiple enzymes. The different $V_{\text{max}}^{\text{met}}$ and $K_m^{\text{met}}$ between the zonal regions suggest that enalapril metabolism is zonally distributed. Since the transporters of enalapril are homogeneously expressed (Figure 3-3, Table 3-1, Appendix 2), it appears likely that zonal metabolism and not zonal transport constitutes the observed difference in overall removal.

### 3.5.2.4 Putative Contribution by Cloned Esterases

Inasmuch as there are differences in $V_{\text{max}}^{\text{met}}$ and $K_m^{\text{met}}$ between the S9 of PP and PV hepatocytes, one ponders as
to the esterases involved in the metabolism of enalapril. To date, three carboxylesterases that are thought to be involved in hepatic xenobiotic metabolism, namely hydrolases A (Robbiet al., 1990; Huang et al., 1993), B (Yan et al., 1994; Yan et al., 1995), and C (Yan et al., 1995) have been cloned from rat liver. Additionally, a 59 kDa carboxylesterase which is trifluoroacetylated upon exposure to halothane (Pohl et al., 1991) has been purified from rat liver. Hydrolases A and B and the 59 kDa carboxylesterase are perivenously distributed, while the zonation of hydrolase C is unknown (Pohl et al., 1991; Huang et al., 1993; Yan et al., 1994). It has been previously determined that the 59 kDa carboxylesterase is not involved in the metabolism of enalapril (Pang et al., 1991). Hydrolase B is located in the rat liver and kidney (Yan et al., 1995) and is able to metabolize ACEi ester prodrugs namely, benazepril, delapril, and temocapril (Luan et al., 1997). However, enalapril was not included in these studies. The $K_m^\text{met}$s for ACEi metabolism by hydrolase B vary between 80 and 500 µmol/L (Luan et al., 1997), values which are all below the $K_m^\text{met}$ reported here for enalapril. However, the $K_m^\text{met}$ for rat liver homogenates enalapril is about 3.5 times that for ramipril (710 vs. 190 µmol/L; Tabata et al., 1990) and a range of affinities for esterases towards ACEi is known to exist. Since it is likely that the overall metabolism is affected by multiple enzymes of varying activities, further studies using cloned hydrolases would be useful to determine the nature of the enzymes and their activities towards the hydrolysis of enalapril.
3.6 CONCLUSIONS

In conclusion, we showed that enalapril uptake by isolated rat hepatocytes is sodium-independent and is best described by a single saturable component with a $V_{\text{max}}$ of 9.5 to 11.6 nmol/min/10^6 cells and a $K_{\text{m}}$ of 344 to 461 μmol/L. The uptake of enalapril and the expression of Oatp1 were both found to be homogeneous across the liver acinus. Uptake of enalapril by isolated rat hepatocytes is consistent with Oatp1-mediated transport. Metabolism of enalapril is partially inhibited by paraoxon, implicating the role of metabolic enzymes other than B-type esterases. The kinetic parameters for enalapril hydrolysis differ between zones with the greatest values for $V_{\text{max}}$ (21.0 nmol/min/mg S9 protein) and $K_{m}^{\text{net}}$ (2612 μmol/L) observed in S9 prepared from perivenous hepatocytes.

3.7 STATEMENT OF SIGNIFICANCE OF CHAPTER 3

The work described in this chapter completes the first objective of the thesis (see section 2.2) on the determination of in vitro kinetic parameters for enalapril uptake by intact isolated rat hepatocytes and enalapril hydrolysis by S9 fractions prepared from homogeneous and zonal hepatocytes. From the results of this chapter, it was demonstrated that while uptake of enalapril by rat hepatocytes was a sodium-independent process of equal activity in all regions of the liver, the kinetic parameters describing enalapril metabolism, $V_{\text{max}}$ and $K_{m}^{\text{net}}$, were greatest in the perivenous zone.
Chapter 4

EFFECTS OF ZONAL TRANSPORT AND METABOLISM ON THE HEPATIC REMOVAL OF ENALAPRIL:

CORRELATION OF IN VITRO DATA WITH PERFUSION DATA
4.1 ABSTRACT

In vitro studies in chapter 3 have shown that the uptake of enalapril by isolated rat hepatocytes occurs evenly across the acinus ($K_m^{\text{uptake}} = 344$ μM, and $V_{\text{max}}^{\text{uptake}} = 11$ nmol/min/10$^6$ cells) whereas the kinetic parameters for enalapril hydrolysis differ between zones, with the greatest values for $V_{\text{max}}^{\text{met}}$ (21.0 nmol/min/mg S9 protein) and $K_m^{\text{met}}$ (2612 μmol/L) observed in S9 prepared from perivenous hepatocytes. It is therefore necessary to understand how the interaction of the processes within each zonal region affects the overall hepatic removal of enalapril. Hence, the purpose of this chapter is to address the adequacy of the various models of hepatic clearance in predicting the experimentally observed HAHV/HAPV perfusion data of Pang et al. (J. Pharmcol. Exp. Ther., 1991) with the in vitro data described in chapter 3. Scaled up in vitro parameters on uptake and metabolism were incorporated into the “well-stirred”, “parallel tube”, “dispersion” and “zonal-compartment” models to predict steady-state extraction ratios for whole liver HAPV and HAHV perfusions. It was found that the correlation was best with a “zonal-compartment” model that viewed the liver as three zonal subcompartments. This model, which incorporated zonal distributions of enzymatic activities, provided the more accurate description of the extraction ratios of HAPV (0.75 and 0.66) and HAHV (0.23 and 0.16) than did other models of even enzymic distribution, supporting the view that zonal factors play a vital role in the scale up of in vitro data to in vivo in the organ processing of drugs.
4.2 INTRODUCTION

Perfusion studies have shown that enalapril was highly removed ($E_{ar}$ of 0.8) when perfused through the whole liver in the antegrade direction (HAPV perfusion) whereas with retrograde HAHV perfusions in which drug only reaches the periportal end of the acinus, a $E_{ar}$ of 0.12 was observed. Based on the comparison of extraction ratios and the cellular water accessed by the two modes of perfusion, it was concluded that hydrolysis of enalapril occurred more abundantly in the perivenous region (Pang et al. 1991). In vitro studies discussed in chapter 3 described the homogeneous uptake of enalapril by isolated rat hepatocytes ($K_{m}^{upake} = 344 \, \mu{M}$, $V_{max}^{upake} = 11 \, \text{nmol/min/10}^6 \, \text{cells}$) and a difference in kinetic parameters for enalapril hydrolysis between zones, with the greatest values for $V_{max}^{met}$ (21.0 nanomol/min/mg S9 protein) and $K_{m}^{met}$ (2612 umol/L) being observed in the S9 fraction prepared from perivenous hepatocytes. Although these in vitro data yielded valuable information on the processes of uptake and metabolism, the information did not elucidate the interplay of processes contributing to the overall removal of enalapril in the rat liver. It becomes necessary to understand how the interaction of the processes and the rate determining step affect the overall hepatic removal of enalapril.

In order to explain how transport and metabolism contribute to whole organ function, the in vitro kinetic parameters obtained from studies in cellular and subcellular fractions from Chapter 3 were scaled up for use in different models of hepatic drug clearance. The correlation is of interest in pharmacokinetics for the understanding of the physiological processes underlying the disappearance of a substrate and the formation of
metabolites by the liver. Many models of hepatic drug clearances have been proposed. These vary with respect to the description of how blood flow is channeled through the sinusoid as well as how a substrate is dispersed across the acinus, resulting in a concentration gradient in the acinus. Historically, the “well-stirred” (or venous equilibrium) model (Rowland et al., 1973) that assumes that the liver is a single well mixed compartment receiving bulk flow and the “parallel tube” (or sinusoidal) model (Winkler et al., 1973) that assumes that enzymes are distributed evenly in single sheets of hepatocytes lining the flow path (plug flow) have been used widely. These models represent boundary conditions of perfectly mixed and non-mixed systems, respectively (Figure 4-1). According to the “well-stirred” model, the concentration of drug in liver is constant and is in equilibrium with that in the venous blood, whereas for the “parallel-tube” model, drug concentration declines exponentially along the direction of flow. There exist other models that render predictions that are intermediate to those of the “well-stirred” and “parallel tube” models. The “dispersion” (Roberts and Rowland, 1986a; 1986b) and “series-compartment” (Gray and Tam, 1987) models are two such models. The “dispersion” model describes non-ideal blood flow through sinusoids of different lengths, while the “series-compartment” model describes “well-stirred” subcompartments connected in series. Such combinations have also surfaced to depict the “enzyme-distributed” occurrence of enzymatic activities (Pang et al., 1982; Tirona and Pang, 1996).

A correlation was sought between the scaled up in vitro parameters with the existing models of hepatic drug clearances or their modifications. The adequacy of the
"well-stirred", "parallel tube", "dispersion", or "zonal-compartment" model was examined. Simulation was performed with the scaled up parameters to address the ability of the various models of hepatic clearance in predicting the experimentally observed HAHV/HAPV data of Pang et al. (1991). We wished to test the hypothesis that zonal transport and metabolism activities in hepatocytes *in vitro*, when appropriately scaled up, adequately describe their overall hepatic functions *in vivo*.

### 4.3 THEORETICAL

#### 4.3.1 Models of Hepatic Drug Clearances

The models of drug clearances (Figure 4-1) were viewed for first-order conditions under which the permeability clearances into and out of the cell (PS<sub>in</sub> and PS<sub>out</sub>) as well as the metabolic and biliary intrinsic clearances were independent of concentration. The steady state extraction ratio (E<sub>ss</sub>) for enalapril was predicted using the "well-stirred", "parallel-tube", "dispersion" and "zonal-compartment" models according to equations 4-1 to 4-6. Since enalapril does not distribute into red blood cells, plasma flow rate and unbound fraction in plasma are used in the relevant equations.

**4.3.1.1 "Well-Stirred" Model.** For the "well-stirred" model, the value of the steady state extraction ratio E<sub>ss</sub> is (Gillette and Pang, 1977),

\[
E_{ss} = \frac{f_u \cdot CL_{int,liver} \cdot PS_{in}}{Q \cdot PS_{out} + f_u \cdot CL_{int,liver} \cdot PS_{in} + Q \cdot CL_{int,liver}} \tag{4-1}
\]
Figure 4-1 Depiction of the “well-stirred”, “parallel tube”, and “dispersion” models, and their various degrees of mixing.
where Q is the plasma flow rate through the liver, \( f_u \) is the unbound fraction of enalapril in the plasma and tissue and \( CL_{\text{tot, liver}}^{\text{total}} \) is the total intrinsic (metabolic plus biliary) clearance.

### 4.3.1.2 "Parallel-tube" Model
For the "parallel-tube" model, \( E_{ss} \) is given by (Pang and Chiba, 1994a)

\[
E_{st} = 1 - e^{-\frac{f_u CL_{\text{tot, liver}}^{\text{total}}}{Q(PS_{\text{tot}} + CL_{\text{tot, liver}}^{\text{total}})}}
\]  
(4-2)

### 4.3.1.3 "Dispersion" Model
For the "dispersion" model, \( E_{ss} \) is given by (Roberts and Rowland, 1986a)

\[
E_{st} = 1 - e^{-\frac{1 - \sqrt{1 + 4D_N R_N}}{2D_N}}
\]  
(4-3)

where \( D_N \) is the dispersion number relating to the degree of dispersion of the system and is assigned a value of 0.24 (Schwab et al., 1998), whereas \( R_N \), the efficiency number, is

\[
R_N = \frac{f_u CL_{\text{tot, liver}}^{\text{total}} PS_{\text{in}}}{Q(PS_{\text{tot}} + CL_{\text{tot, liver}}^{\text{total}})}
\]  
(4-4)

### 4.3.1.4 "Zonal-compartment" Model
A modified series-compartment model (Gray and Tam, 1987) that described regions aligned in series along the sinusoidal flow path was used. In this "zonal-compartment" model, transport and metabolic activities are allowed to vary (Tirona and Pang, 1996). Three is the smallest number of units to denote the physiological zonal regions, 1, 2, and 3. The mass balance rate equations describing
the sinusoidal and cellular enalapril concentrations in each \((i^{th})\) of three compartments in series are:

For the change of sinusoidal enalapril concentration in each \(i^{th}\) compartment,

\[
\frac{d [E]_p}{dt} = \left\{ Q [E]_p^{i-1} - \frac{PS_{in}}{N} f_u [E]_p^{i} + \frac{PS_{out}}{N} f_u [E]_c^{i} - Q [E]_p^{i} \right\} / V_p
\]  

(4-5)

and the equation describing the change of cellular enalapril concentration in each \(i^{th}\) compartment is

\[
\frac{d [E]_c}{dt} = \left\{ \frac{PS_{in}}{N} f_u [E]_p^{i} - \left[ \frac{PS_{out}}{N} + CL_{int, i}^{met} + \frac{CL_{bile, liver}}{N} \right] f_u [E]_c^{i} \right\} / V_c
\]  

(4-6)

where \([E]\) is the concentration in either the sinusoidal or cellular concentration of enalapril (subscripts "p" and "c", respectively) of the \(i^{th}\) compartment of \(N\) total compartments (three in this case). The metabolic intrinsic clearance \(CL_{int, i}^{met}\) for each \(i^{th}\) compartment may vary, but this is not the case for the biliary intrinsic clearance, which is reported to be constant for all regions of the liver (Pang et al., 1991). The biliary intrinsic clearance in each region is the total biliary intrinsic clearance \((CL_{int, liver}^{bile})\) divided by \(N\). \(V_p\) is the volume of the sinusoidal plasma space and \(V_c\) is the volume of cell water. For the first compartment \((i = 1)\), \([E]_p^{1} = C_m\).
4.4 METHODS

4.4.1 Scaling-Up of In Vitro Data

Values for blood flow (Q), input enalapril concentration (C_in), liver weight and unbound fraction of drug (f_u) were taken from the literature (Pang et al., 1991). Other parameters were derived from in vitro uptake and metabolism data. The maximal velocity for uptake, $V_{max}^{\text{uptake}}$, determined in vitro with isolated hepatocytes in the presence of sodium (Chapter 3), was scaled-up to the whole liver by multiplication with the scaling factor $\alpha$ or $1.25 \times 10^8$ cells/g liver (Lin et al., 1980). The maximal velocities for metabolism determined from S9 of periportal, homogeneous and perivenous hepatocytes were scaled up to the whole organ level by multiplication of $V_{max}^{\text{met}}$ (where subscript $i = \text{PP, HO, or PV}$) to the scaling factor, $\beta$ or 100 mg S9 protein/g liver (Mahler and Cordes, 1966). The metabolic kinetic constants derived from fitting of the HO data (see Table 3-2, p. 43) were used to represent those in the mid-zonal region (MZ) of the liver. The scaled-up metabolic and uptake parameters are summarized in Table 4-1.

4.4.2 Calculations

The scaled-up parameters summarized in Table 4-1 were used to calculate the influx permeability surface area product, $P_{\text{Sur}}$, and the metabolic ($CL_{\text{int/liver}}^{\text{met}}$) intrinsic clearances.

The influx permeability surface area product (uptake clearance), $P_{\text{Sur}}$, for the whole liver (mL/min) was
The metabolic intrinsic clearance ($CL_{int}^{met}$) from each of the periportal, homogenous and perivenous region of the liver (denoted as subscript, "i") was calculated as

$$CL_{int,i}^{met} = \left( \frac{V_{max,i}^{met}}{K_{m,i}^{met}} \right) \times \left( \frac{\text{liver weight}}{3} \right)$$  \hspace{1cm} (4-8)

where $V_{max,i}^{met}$ and $K_{m,i}^{met}$ are the maximum velocity and the Michaelis-Menten constant for saturable metabolism within the $i^{th}$ zone of the liver, respectively. It was noted that the metabolic intrinsic clearance of the PV region is 1.6 times that of the PP region, whereas that for the midzonal region is intermediate (Table 4-2). The total metabolic intrinsic clearance ($CL_{int,liver}^{met}$) across the whole acinus was calculated as

$$CL_{int,liver}^{met} = CL_{int,PP}^{met} + CL_{int,MZ}^{met} + CL_{int,PV}^{met}$$  \hspace{1cm} (4-9)

The biliary intrinsic clearance ($CL_{int,liver}^{bile}$) for the whole liver was estimated according to the ratio of the excretion rate of enalapril to the metabolic rate of enalapril (0.032/0.734) in previous studies (Pang et al., 1991) and the metabolic intrinsic clearance of the liver from scaled up data, as shown in Equation 4-10.

The biliary clearance ($CL_{int,liver}^{bile}$) was approximated according to the following equation,

$$CL_{int,liver}^{bile} = CL_{int,liver}^{met} \times \left( \frac{0.0362}{0.734} \right) = CL_{int,liver}^{met} \times 0.049$$  \hspace{1cm} (4-10)
Table 4-1: Parameters obtained from the literature (Pang et al. 1991) or scaled up from data of in vitro studies (see chapter 3).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_{in} ) (( \mu \text{mol/L} ))</td>
<td>initial input concentration of enalapril</td>
<td>2.5 (*)</td>
</tr>
<tr>
<td>( f_u )</td>
<td>unbound fraction in sinusoid</td>
<td>0.82 (*)</td>
</tr>
<tr>
<td>( f_t )</td>
<td>unbound fraction in tissue</td>
<td></td>
</tr>
<tr>
<td>( Q ) (mL/min)</td>
<td>plasma flow rate</td>
<td>10.3 (*)</td>
</tr>
<tr>
<td>liver weight (g)</td>
<td>weight of whole liver</td>
<td>12.8 (*)</td>
</tr>
<tr>
<td>( V_{\text{max}}^{\text{uptake}} ) (nmol/min/g liver)</td>
<td>maximal velocity for uptake in all zones</td>
<td>1377±186 (b)</td>
</tr>
<tr>
<td>( K_{m}^{\text{uptake}} ) (( \mu \text{M} ))</td>
<td>Michaelis-Menten constant for uptake in all zones</td>
<td>344±52 (b)</td>
</tr>
<tr>
<td>( P_{\text{in}} ) (mL/min/g liver)</td>
<td>Influx permeability surface area product</td>
<td>4.0 (c)</td>
</tr>
<tr>
<td>( V_{\text{max,PP}}^{\text{met}} ) (nmol/min/g liver)</td>
<td>maximal velocity of metabolism in periportal zone</td>
<td>547±306 (b)</td>
</tr>
<tr>
<td>( K_{m,PP}^{\text{met}} ) (( \mu \text{M} ))</td>
<td>Michaelis-Menten constant for metabolism in periportal zone</td>
<td>1049±335 (b)</td>
</tr>
<tr>
<td>( CL_{\text{in,PP}}^{\text{met}} ) (mL/min/g liver)</td>
<td>metabolic intrinsic clearance in periportal region</td>
<td>0.521±0.336 (c)</td>
</tr>
<tr>
<td>( V_{\text{max,MZ}}^{\text{met}} ) (nmol/min/g liver)</td>
<td>maximal velocity of metabolism in mid-zonal region</td>
<td>805±299 (b)</td>
</tr>
<tr>
<td>( K_{m,MZ}^{\text{met}} ) (( \mu \text{M} ))</td>
<td>Michaelis-Menten constant for metabolism in mid-zonal region</td>
<td>1308±419 (b)</td>
</tr>
<tr>
<td>( CL_{\text{in,MZ}}^{\text{met}} ) (mL/min/g liver)</td>
<td>metabolic intrinsic clearance in midzonal region</td>
<td>0.615±0.302 (c)</td>
</tr>
<tr>
<td>( V_{\text{max, PV}}^{\text{met}} ) (nmol/min/g liver)</td>
<td>maximal velocity of metabolism in perivenous zone</td>
<td>2096±600 (b)</td>
</tr>
<tr>
<td>( K_{m, PV}^{\text{met}} ) (( \mu \text{M} ))</td>
<td>Michaelis-Menten constant for metabolism in perivenous zone</td>
<td>2612±236 (b)</td>
</tr>
<tr>
<td>( CL_{\text{in, PV}}^{\text{met}} ) (mL/min/g liver)</td>
<td>metabolic intrinsic clearance in perivenous region</td>
<td>0.802±0.242 (c)</td>
</tr>
<tr>
<td>( CL_{\text{int, liver}}^{\text{met}} ) (mL/min)</td>
<td>metabolic intrinsic clearance for whole liver</td>
<td>8.27</td>
</tr>
<tr>
<td>( CL_{\text{bile, liver}} ) (mL/min)</td>
<td>biliary intrinsic clearance for whole liver</td>
<td>0.408</td>
</tr>
<tr>
<td>( CL_{\text{int, liver}}^{\text{total}} ) (mL/min)</td>
<td>total intrinsic clearance for whole liver</td>
<td>8.68</td>
</tr>
</tbody>
</table>

\(*\) values from Pang et al., (1991)
\(b\) mean±S.D. (\(n=4\)) of in vitro parameters which were scaled up to whole organ level using the factors of Mahler and Cordes (1966) and Lin et al. (1980)
\(c\) calculated as \( V_{\text{max}} / K_m \)
\(d\) according to equation 4-9
\(e\) according to equation 4-10
\(f\) according to equation 4-11
The total intrinsic clearance ($CL_{\text{total}}^{\text{int,liver}}$) was calculated as the sum of biliary and metabolic
intrinsic clearances or,

$$CL_{\text{total}}^{\text{int,liver}} = CL_{\text{met}}^{\text{int,liver}} + CL_{\text{bile}}^{\text{int,liver}}$$ (4-11)

Since no data on the sinusoidal efflux of enalapril was available, values of the
sinusoidal efflux permeability surface area product ($PS_{\text{out}}$) were calculated by setting
$E_{\text{ss,HAPV}}$ (the steady-state extraction through the whole liver) equal to 0.77 and solving for
$PS_{\text{out}}$ with the equations for “well-stirred”, “parallel-tube” or “dispersion” model
(equations 4-1 to 4-3).

4.4.2.1 Additional Parameters for the “Zonal-Compartment” Model A model of
three compartments in series (repeated sets of equations 4-5 and 4-6) was used to
describe the segregated, metabolic activities in different zones of the liver. Within each
zone, the metabolic parameters describing metabolism varied (Table 4-2) and were
ascribed various values of the metabolic intrinsic clearance $CL_{\text{met}}^{\text{int,liver}}$ (Table 4-2). Three
possible scenarios were presented (Table 4-2; Figure 4-2). In the first instance, the total
metabolic and biliary intrinsic clearances were separated evenly into three equal portions
for zones 1, 2, and 3 (parameter set #1; Figure 4-2). In the second case (parameter set #2;
Figure 4-2), the metabolic intrinsic clearances were estimated from the scaled-up mean
($V_{\text{max},i}/K_{\text{m},i}^{\text{met}}$) in vitro parameters for each zone (see Table 4-1). In the third case, a
modified, zonal distribution (parameter set #3; Figure 4-2) was adopted to encompass

66
Table 4-2: Parameters used to describe transport and metabolism in the “zonal-compartment model”

<table>
<thead>
<tr>
<th>Zonal/Total</th>
<th>Even Distribution (Parameter set #1)</th>
<th>Zonal Distribution (Parameter set #2)</th>
<th>Modified Zonal Distribution (Parameter set #3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrinsic Clearances</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZonaY</td>
<td>17.1\textsuperscript{a}</td>
<td>17.1\textsuperscript{a}</td>
<td>17.1\textsuperscript{a}</td>
</tr>
<tr>
<td>Total Even Distribution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS\textsubscript{in,1} (mL/min)</td>
<td>17.1\textsuperscript{a}</td>
<td>17.1\textsuperscript{a}</td>
<td>17.1\textsuperscript{a}</td>
</tr>
<tr>
<td>$CL\textsuperscript{net,PP}$ (mL/min)</td>
<td>2.76\textsuperscript{a}</td>
<td>547* (12.8/3)\textsuperscript{b}</td>
<td>(547-306) (12.8/3)\textsuperscript{c}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1049</td>
<td>(1049+335)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>= 2.22</td>
<td>= 0.74</td>
</tr>
<tr>
<td>$CL\textsuperscript{net,MZ}$ (mL/min)</td>
<td>2.76\textsuperscript{a}</td>
<td>805* (12.8/3)\textsuperscript{b}</td>
<td>805* (12.8/3)\textsuperscript{b}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1308</td>
<td>1308</td>
</tr>
<tr>
<td></td>
<td></td>
<td>= 2.62</td>
<td>= 2.62</td>
</tr>
<tr>
<td>$CL\textsuperscript{net,PV}$ (mL/min)</td>
<td>2.76\textsuperscript{a}</td>
<td>2096* (12.8/3)\textsuperscript{b}</td>
<td>(2096+604)* (12.8/3)\textsuperscript{c}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2612</td>
<td>(2612-236)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>= 3.43</td>
<td>= 4.85</td>
</tr>
<tr>
<td>$CL\textsuperscript{net,liver}$</td>
<td>8.277</td>
<td>8.277</td>
<td>8.219</td>
</tr>
<tr>
<td>$CL\textsuperscript{net, liver}$ (mL/min)</td>
<td>0.408\textsuperscript{a}</td>
<td>0.408</td>
<td>0.405</td>
</tr>
<tr>
<td>$CL\textsuperscript{net,liver}$</td>
<td>8.68</td>
<td>8.68</td>
<td>8.62</td>
</tr>
<tr>
<td>$CL\textsuperscript{net,PV}$</td>
<td>1.0</td>
<td>1.6</td>
<td>6.6</td>
</tr>
</tbody>
</table>

\textsuperscript{a} value is 1/3 of those for whole liver (PS\textsubscript{in,} and $CL\textsuperscript{net,liver}$); \textit{i} denotes PP, MZ or PV.

\textsuperscript{b} values of $CL\textsuperscript{net,PP}$, $CL\textsuperscript{net,MZ}$ and $CL\textsuperscript{net,PV}$ were calculated from the ratios of the corresponding $V\textsuperscript{net}$ and $K\textsubscript{m}$ shown in Table 4-1, after correction for liver weight.

\textsuperscript{c} values of $CL\textsuperscript{net,PP}$, $CL\textsuperscript{net,MZ}$ and $CL\textsuperscript{net,PV}$ were calculated from the ratios of the modified $V\textsuperscript{net}$ and $K\textsubscript{m}$ (mean value + or - S.D.) shown in Table 4-1, after correction for liver weight.

\textsuperscript{d} sum of the metabolic intrinsic clearances from all zones (PP, MZ, and PV)

variability of the data. For the periportal zone, the values $V\textsuperscript{net,PP}$ was given by the value of (mean - S.D.) whereas the $K\textsuperscript{m,PP}$ was given the value (mean + S.D.). For the perivenous zone, $V\textsuperscript{net,PV}$ and $K\textsuperscript{m,PV}$ were modified to yield the highest $V\textsuperscript{net,PV}$ (mean + S.D.) and lowest $K\textsuperscript{m,PV}$ (mean - S.D.), whereas parameters for the mid-zone region were
not modified. As shown in the summary (Table 4-2), the $P_{s_{in}}$ are 23 to 3.85 times the $CL_{int}^{net}$.

4.4.3 Calculation and Simulations of $E_{st}$

The $E_{st,HAHV}$ (steady-state extraction ratio for HAHV perfusion) for the "well-stirred", "parallel-tube" and "dispersion" models were calculated directly with equations 4-1 to 4-4 with data provided in Table 4-3, after consideration that the values of the PS and intrinsic clearances were 1/3 of the values for whole liver. Simulations for the "zonal-compartment" model (three scenarios) were performed on Scientist© (v.2 Micromath Scientific Software, Salt Lake City, Utah) with the differential equations presented previously (4-5 and 4-6) and values shown in Table 4-2.

Table 4-3: Calculated clearances for the "well-stirred", "parallel tube", and "distributed" models based on $E_{st,HAHV}$ of 0.77 from Pang et al., 1991

<table>
<thead>
<tr>
<th>Clearance Term</th>
<th>&quot;Well-stirred&quot; Model</th>
<th>&quot;Dispersion&quot; Model</th>
<th>&quot;Parallel-Tube&quot; Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>$PS_{in}$ (mL/min)</td>
<td>51.2</td>
<td>51.2</td>
<td>51.2</td>
</tr>
<tr>
<td>$PS_{out}$ (mL/min)</td>
<td>1.93</td>
<td>9.19</td>
<td>15.5</td>
</tr>
<tr>
<td>$CL_{int,liver}^{net}$ (mL/min)</td>
<td>8.277</td>
<td>8.277</td>
<td>8.277</td>
</tr>
<tr>
<td>$CL_{bile}^{liver}$ (mL/min)</td>
<td>0.408</td>
<td>0.408</td>
<td>0.408</td>
</tr>
<tr>
<td>$CL_{uria}^{liver}$ (mL/min)</td>
<td>8.68</td>
<td>8.68</td>
<td>8.68</td>
</tr>
</tbody>
</table>

*a according to equation 4-7
*b Based on $E$ of 0.77, the value was calculated from 4-1 to 4-4 for the efflux clearances
*c according to equation 4-9
*d according to equation 4-10
*e according to equation 4-11
Figure 4-2 Distributions of metabolic enzymes for simulation with the "zonal-compartment" model for (a) an even distribution, (b) a zonal distribution and (c) a modified zonal distribution. See text for details.
4.4.4 Drug Removal Within Zonal Regions of the Zonal-Compartment Model

Since the "zonal-compartment" model with the "modified" enzyme distribution (parameter set 3, Table 4-3) best described the data, simulation was performed to determine the effects of the metabolic intrinsic clearance $CL_{in}$ at the given transport (PS$_{in}$) on the removal within each zone (E$_{sys}$) while all other parameters were held constant. The overall availability of the liver (F$_{liver}$) was given by the product of the individual availability of each zone (F$_i$), as expected of the relation for compartments in series.

4.5 RESULTS

4.5.1 Predictions of the "Well-Stirred", "Parallel-Tube" and "Dispersion" Models

Values of PS$_{out}$, based on the scaled-up PS$_{in}$ and intrinsic clearances and the E$_{st,HAHV}$ value of 0.77, were different among the "well-stirred", "dispersion" and "parallel tube" models. Varying from total mixing model to non-mixing model, the estimated PS$_{out}$ decreased with increased mixing (1.93 < 9.2 < 15.5 mL/min). The corresponding values for E$_{st,HAHV}$ according to the models were of the reverse rank order (0.53 > 0.44 > 0.39) (Table 4-4). The predicted values of E$_{st,HAHV}$ were 3.3 to 4.5 times than the observed value, as were the ratios of E$_{st,HAHV}$ to E$_{st,HAHV}$.

4.5.2 Predictions of the "Zonal-compartment" Model of Three Subcompartments

Since three different PS$_{out}$ values resulted with the "well-stirred", "parallel tube" and "dispersion" models, simulations were performed with each of the PS$_{out}$ for each of
the enzyme distributions (Figure 4-2), yielding a total of nine different simulations of enalapril removal with the "zonal-compartment" model (Table 4-4). Among the simulations (models of even-enzyme distribution, zonal-enzyme distribution, and modified, zonal-enzyme distribution, see Figure 4-2 and Table 4-2), \( E_{ss,HAHV} \) and \( E_{ss, HAPV} \) were greatest with the lowest \( PS_{out} \) of 1.93 mL/min/liver, since the condition inferred poorer efflux and a higher accumulation of enalapril in liver. The values of \( E_{ss,HAHV} \) and \( E_{ss, HAPV} \) were progressively diminished with increasing \( PS_{out} \) (9.2 and 15.5 mL/min/liver). Although the \( E_{ss,HAPV} \) were generally similar for all of the three scenarios of the zonal-compartment model (Table 4-4), values of \( E_{ss,HAHV} \) began to diverge with increasing \( PS_{out} \). The modified zonal-enzyme model which described the steepest perivenous gradient of esterase activity, best represented the data of \( E_{ss,HAHV} \) and \( E_{ss,HAPV} \) of Pang et al. (1991) at \( PS_{out} \) values of 9.2 and 15.5 mL/min/liver (Table 4-4).

4.5.3 Drug Removal Within Zonal Regions of the Zonal-compartment

The simulated extraction ratios for the various zones of the liver according to the "zonal-compartment" model at \( PS_{out} \) of 9.2 and 15.5 mL/min (parameter set #3, Table 4-2) were very different (Table 4-5). As expected, the product of the availabilities of each zonal region \( F_i \) or \( (1 - E_{ss,i}) \) yielded the overall availability of the liver. The relationship is expected of compartments arranged in series.
Table 4-4: Estimations of $E_{ss}$ with the various models of drug clearances

<table>
<thead>
<tr>
<th>Model</th>
<th>$E_{ss,HAPV}$</th>
<th>$E_{ss,HAHV}$</th>
<th>$E_{ss,HAHV}/E_{ss,HAPV}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observations (Pang et al., 1991)</td>
<td>0.77</td>
<td>0.118</td>
<td>0.153</td>
</tr>
<tr>
<td>&quot;Well-stirred&quot; Model ($PS_{out} = 1.93 \text{ mL/min}$)</td>
<td>0.77</td>
<td>0.527</td>
<td>0.685</td>
</tr>
<tr>
<td>&quot;Dispersion&quot; Model ($PS_{out} = 9.2 \text{ mL/min}$)</td>
<td>0.77</td>
<td>0.441</td>
<td>0.573</td>
</tr>
<tr>
<td>&quot;Parallel-tube&quot; Model ($PS_{out} = 15.5 \text{ mL/min}$)</td>
<td>0.77</td>
<td>0.387</td>
<td>0.503</td>
</tr>
<tr>
<td>Zonal-compartment (PS$_{out}$ = 1.93 \text{ mL/min})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Even (Set #1; Fig. 4-2A)</td>
<td>0.894</td>
<td>0.526</td>
<td>0.588</td>
</tr>
<tr>
<td>Zonal (Set #2; Fig. 4-2B)</td>
<td>0.894</td>
<td>0.517</td>
<td>0.578</td>
</tr>
<tr>
<td>Modified Zonal (Set #3; Fig. 4-2C)</td>
<td>0.880</td>
<td>0.440</td>
<td>0.500</td>
</tr>
<tr>
<td>Zonal-compartment (PS$_{out}$ = 9.2 \text{ mL/min})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Even (Set #1; Fig. 4-2A)</td>
<td>0.781</td>
<td>0.389</td>
<td>0.498</td>
</tr>
<tr>
<td>Zonal (Set #2; Fig. 4-2B)</td>
<td>0.781</td>
<td>0.373</td>
<td>0.478</td>
</tr>
<tr>
<td>Modified Zonal (Set #3; Fig. 4-2C)</td>
<td>0.747</td>
<td>0.233</td>
<td>0.312</td>
</tr>
<tr>
<td>Zonal-compartment (PS$_{out}$ = 15.5 \text{ mL/min})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Even (Set #1; Fig. 4-2A)</td>
<td>0.696</td>
<td>0.328</td>
<td>0.471</td>
</tr>
<tr>
<td>Zonal (Set #2; Fig. 4-2B)</td>
<td>0.695</td>
<td>0.300</td>
<td>0.432</td>
</tr>
<tr>
<td>Modified Zonal (Set #3; Fig. 4-2C)</td>
<td>0.661</td>
<td>0.165</td>
<td>0.250</td>
</tr>
</tbody>
</table>

*equal to the simulated extraction ratio for the first zone of the liver only

$PS_{out}$ determined from the "well-stirred" model (equation 4-1, see Table 4-3)

$PS_{out}$ determined from the "dispersiun" model (equation 4-4, see Table 4-3)

$PS_{out}$ determined from the "parallel-tube" model (equation 4-2, see Table 4-3)

*See Figure 4-2 for enzymatic distributions

Table 4-5: Zonal extraction ratios and the overall extraction ratio of the liver.

<table>
<thead>
<tr>
<th>$PS_{out}$ (mL/min)</th>
<th>$(E_{ss,PP})$</th>
<th>$(E_{ss,MZ})$</th>
<th>$(E_{ss,PV})$</th>
<th>$(E_{ss,liver})$</th>
<th>$(E_{ss,liver})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.5</td>
<td>0.165</td>
<td>0.322</td>
<td>0.401</td>
<td>0.338</td>
<td>0.661</td>
</tr>
<tr>
<td>9.2</td>
<td>0.233</td>
<td>0.393</td>
<td>0.458</td>
<td>0.252</td>
<td>0.748</td>
</tr>
</tbody>
</table>

*equals the product of $(1 - E_{ss,PP})(1 - E_{ss,MZ})(1 - E_{ss,PV})$

4.6 DISCUSSION

The correlation of in vitro data to in vivo is a widely investigated topic in drug metabolism. Varying successes have been encountered (Pang and Chiba, 1994b; Houston,
1994; Zomorodi et al., 1995; Iwatsubo et al., 1997; Ito et al., 1998). Poor correlations have been blamed on neglect of a transport barrier at the sinusoidal membrane and lack of consideration of metabolic zonation (Pang and Chiba, 1994b). It was shown that the membrane barrier poses as a major source of discrepancy when transport and not metabolism or excretion is the rate-limiting step (de Lannoy and Pang, 1987; Geng et al., 1995; Tirona and Pang, 1999). In such an instance, the overall removal rate mimics the transport rate. In vitro and in vivo correlation should be stratified when both transport and metabolic data exist in vitro. Since cellular uptake by the rat liver has been found to correlate closely to that in isolated rat hepatocytes (Yamada et al., 1997; Tirona and Pang, 1999), and since transport and metabolic data and the presence of zonation may be detected in both zonal and homogeneous rat hepatocytes and their derived fragments (Tirona et al., 1999), improved correlation should be more readily attained between data in vitro and in vivo.

Indeed, both influx and metabolic in vitro data obtained previously (see chapter 3) had allowed for the examination and comparison of the adequacies of the various models of hepatic drug clearances. The simplistic models - the well-stirred”, “parallel-tube” and “dispersion” models - are mostly inept in predicting the associated $E_{SS,\text{HAHV}}$ at the estimated $\text{PS}_{out}$ (sinusoidal efflux clearance) determined for the $E_{SS,\text{HAVP}}$ of 0.77 (Pang et al., 1991) (Table 4-4). Not unexpectedly, the “parallel-tube” and “well-stirred” models, representing boundary conditions for the degree of mixing for drug removal in the liver, were found to be associated with extreme values of the $\text{PS}_{out}$ (1.93 and 15.5 mL/min), with an intermediate value (9.2 mL/min) for the “dispersion” model. All of the $E_{SS,\text{HAHV}}$ values
predicted by these models were much higher than the observed \( E_{ss,HAHV} \), suggesting that these idealized and simplified models of hepatic drug clearances were unable to predict the hepatic removal of enalapril in the absence of zonation (Table 4-4).

Use of the three scenarios of enzyme distributions of the "zonal-compartment" (Figure 4-2 and Table 4-2) at the various values of \( PS_{out} \) (1.93, 9.2 and 15.5 mL/min) confirmed that the \( PS_{out} \) predicted by the "well-stirred" model (1.93 mL/min) was inconsistent with the observed \( E_{ss,HAPV} \) and \( E_{ss,HAHV} \) in the perfused liver preparation (Pang et al., 1991). By contrast, the \( PS_{out} \) predicted by both the "dispersion" and "parallel tube" models, together with a zonal-enzyme model (parameter set #3 for the "zonal-compartment" that considered experimental error and described a steeper esterase activity gradient) were best correlated with the observed data (Table 4-4). Absence of PV zonation (even distribution of enzyme case or with average data; parameter sets 1 and 2) was also incongruent with the observations. Moreover, the predicted values of \( E_{ss} \) for HAHV were higher than observed, and this may be attributed to a lesser volume reached by HAHV (< 1/3 liver or 33%), as inferred by the intracellular water space accessed (30%; Pang et al., 1991). The closeness between the \( \textit{in vitro} \) zonation patterns with perfusion data validates the perfusion model of HAPV-HAHV perfusion in determining the preponderance of zonal activity of the liver (Pang et al., 1988).

The gradient in metabolic intrinsic capacity, increasing from the periportal zone to the perivenous zone, is likely underestimated by the present extrapolation with \( \textit{in vitro} \) data. The lack of zonal specificity has been observed within zonally enriched hepatocyte populations and less within lysates prepared by dual-pulsing of digitonin (Tirona et al., 1988).
It has been found for ethacrynic acid, which is metabolized by the glutathione S-transferases (GSTs), that significant differences in metabolic activities and immunoreactive GSTs between PP and PV zones existed in lysates and not within the S9 fractions of zonal hepatocytes (Tirona et al., 1999). This suggests that a much more shallow enzymatic gradient is projected by zonal data in vitro than the true gradient of metabolic activity in the liver (Tirona et al., 1999). The preparation of lysates is more zonally selective as only the cytosols are produced from the farthest cells of the periportal and perivenous zones. However, the preparation of lysates is likely not applicable to the study of enalapril metabolism as membrane-associated proteins, such as the microsomal esterases thought to be involved in enalapril metabolism, are not present in a functional form in lysates (Witters et al., 1993 Fang et al., 1998).

It is surmised that the correlation may improve with a better definition of the enzymic distribution and $P_{eq}$. Unfortunately, the patterns of distribution of the enzymes are not readily discerned from hepatocyte studies (Kera et al., 1987). It is likely that the shape of the actual gradient is continuous as would be expected for gradient-type distribution of an enzyme (Oinonen and Lindros, 1998), rather than stepwise as modeled presently. Gradient distributions of CYP 450 isoforms, which may exist evenly (CYP2C12) across the acinus, increase from PP to PV regions (CYP3A1), or exhibit exclusive perivenous expression (CYP2B1/2), have been estimated from mRNA distributions in lysates (Oinonen and Lindros, 1998). Elucidation of the identity of the enzyme(s) involved in enalapril esterolysis is necessary before it would be possible to determine the continuous distribution of the esterase with immunohistochemistry. A
simulation with a “zonal-compartment” comprising of six subcompartments (two equal subcompartments within each zonal region) was also performed (data not shown). However, the results were less compliant with the observations.

Notwithstanding the above comments, it appears that the parameters for metabolism and transport determined in various zones in vitro adequately reflect the total intrinsic metabolic and transport activities in the whole organ. The metabolic intrinsic clearance of the liver could be approximated by the sum of the metabolic intrinsic clearances of the zonal regions. Moreover, drug extraction among zonal regions occur successively, and the product of the zonal bioavailabilities determine the overall bioavailability of the liver (Table 4-5). By modeling the liver as a series of subcompartments with different zonal transport/metabolic intrinsic clearances, a more complete model of the liver is attained. As shown in this exercise, the integration of zonal transport and metabolism in the “zonal-compartment” model accurately predicted the removal of the model drug, enalapril, in the perfused organ. Similar strategies should improve the correlation between in vitro and in vivo data. Since the transport clearance greatly exceeds the total and metabolic intrinsic clearances for each zonal region, metabolism and not transport is rate-determining for esterolysis. The zonation observed in perfused liver is attributed to a greater perivenous metabolism, as verified by the in vitro data.
4.7 CONCLUSIONS

It is concluded that scaled up *in vitro* data obtained from zonal cells are of paramount importance for the prediction of the activity of the whole organ in the removal of zonally cleared drugs, as shown with the model substrate, enalapril, and use of an enzyme-distributed, "zonal-compartment" model. The model which incorporates zonal distributions of enzyme provided a more accurate description of the HAHV case than did the even-enzymic distribution model, thus supporting the hypothesis that zonal transport and metabolism activities in hepatocytes *in vitro*, when appropriately scaled up, adequately describe their overall hepatic functions *in vivo*. The rate-limiting step on hepatic removal of enalapril is metabolism and not transport. Hence, the greater rate of esterolysis in the perivenous region is due to the higher activity of the enzymes and not because of higher transport in the region.

4.8 SIGNIFICANCE OF CHAPTER 4

The work described in this chapter fulfilled the second objective (section 2.2), which was to integrate scaled up *in vitro* data into a model of hepatic clearance of enalapril. The work in this chapter described the integration of transport and metabolic data to describe events in the whole liver. The utility of the present method for the scale-up of zonal *in vitro* data and modeling with a zonal distribution of enzyme was demonstrated.
Chapter Five

DISCUSSION AND CONCLUSIONS
5.1 IN VITRO-IN VIVO CORRELATION

Hepatic drug removal is a distributed-in-space phenomenon, and the heterogeneous distribution of enzymes across the acinus can have profound effects on the pharmacokinetics of a drug. The zonation of many drug removal processes is readily studied in vitro with hepatocytes or subcellular fractions of zonal origin. However, it is often found that in vitro data, though accurately scaled up, are not able to predict whole organ function. The lack of correlation between in vitro and in vivo data may be due to the lack of many current models of hepatic removal in incorporating heterogeneous expression of drug transport or metabolism, which affects the overall removal of drugs. It is therefore important to develop models that incorporate both transport and metabolism and their zonal expression to improve the correlation. Such models would be beneficial to further the understanding of whole organ processing of drugs by the liver.

Enalapril was chosen as a model compound for study because of its relatively simple metabolic scheme (Tocco et al., 1982; Pang et al., 1984) and absence of factors that complicate modeling such as red blood cell distribution and metabolism, sequential metabolism (Pang et al., 1984; de Lannoy et al., 1989) and competition for Oatp1 transport of enalapril by enalaprilat (Pang et al., 1998). Evidence from HAHV/HAPV perfusions had suggested that esterolysis of enalapril occurred predominately in the perivenous region of the liver (Pang et al., 1991). The present investigation on zonal transport and metabolism of enalapril was conducted to test whether the in vitro kinetic
data derived from the different zones could be adequately incorporated into a model of hepatic removal in describing the acinar events of the rat liver.

5.2 THE FINDINGS

The studies described in this thesis support the following findings:

1) Enalapril uptake by isolated rat hepatocytes is a sodium-independent process which is homogeneously distributed across the acinus and is mediated by a single saturable component of $V_{\text{max}}^{\text{uptake}}$ of 9.5 to 11.6 nmol/min/10^6 cells and a $K_m^{\text{uptake}}$ of 344 to 461 µmol/L. Transport appears to be mediated solely by Oatp1.

2) Metabolism of enalapril is partially inhibitable by paraoxon and exhibits differences in maximal velocity and affinity among different zones of the liver. $V_{\text{max}}^{\text{met}}$ (21±6.0 nmol/min/mg S9 protein) and $K_m^{\text{met}}$ (2612±236 µmol/L) were both greater in S9 from perivenous hepatocytes than S9 from periportal hepatocytes ($V_{\text{max}}^{\text{met}}$ of 5.5±3.1 nmol/min/mg S9 protein and $K_m^{\text{met}}$ of 1049±335 µmol/L; ANOVA $P < 0.05$).

3) The values of the metabolic intrinsic clearances among all zonal regions are much less than the sinusoidal influx clearance, $P_{\text{in,l}}$. The overall hepatic removal of enalapril is rate-limited by intracellular metabolism and not transport.

4) Enalapril removal kinetics by the whole liver are best described by a "zonal-compartment" model which incorporates homogeneous transport but zonal distribution of metabolic enzymes (activities).
5.3 GENERAL DISCUSSION AND SIGNIFICANCE

The results of the present investigation are important to further the development of hepatic clearance models by the incorporation of zonation of removal process. Specifically, zonal hepatocytes and their derived S9 fractions were shown to be useful for determining in vitro parameters for delineation of the zonated processes of transport and metabolism, respectively. Furthermore, the necessity of a model which incorporates zonal transport and metabolism was evident from the present results. Without consideration of the zonal factors, a poor correlation was obtained. Therefore, the present studies demonstrate the utility of the experimental approach and modeling development. First is the study of the zonal processes in vitro, followed by scale up, then the final prediction with a consistent model. The strategy will improve our mechanistic understanding of zonal removal processes and the manner in which the pharmacokinetics of drugs are affected by hepatic processing.

The principles employed herein may be applied to the study of zonal effects on the hepatic removal and toxicity of other drugs. Zonal hepatotoxicity is known to occur for many drugs (Anundi et al., 1993; Lindros, 1997), including enalapril (Jurima-Romet and Huang, 1992). In vivo, enalapril causes centrilobular (perivenous) necrosis by processes that are mediated by CYP 3A metabolism and glutathione depletion (Jurima-Romet et al., 1991a; 1991b; Jurima-Romet and Huang, 1992), and it has been suggested as a model drug for the study of ACEi-induced hepatotoxicity (Jurima-Romet and Huang, 1992). The zonal kinetic data determined here for enalapril may be useful for further
studies on ACEi-induced hepatotoxicity and the general strategy to model zonation should prove useful in the explanation of zonal drug hepatotoxicity.

Limitations, however, existed with the present experimental methods. As shown for the hepatic glutathione conjugation of ethacrynic acid with zonal hepatocytes (Tirona et al., 1999), the gradient of metabolic enzymes found among zonal hepatocytes may be more shallow than in reality. It is plausible that the same underestimation exists for transport. Hence, confirmatory evidence must be sought to validate that the zonation of transport or metabolism, as defined by hepatocytes and subcellular fractions of zonal origin, is indeed an accurate reflection of the situation in vivo. Values for $PS_{out}$ were not determined experimentally and had to be calculated using the model equations. Since changes in $PS_{out}$ affect the estimation of $E_{ss}$ and the calculated values of $PS_{out}$ ranged from 1.93 to 15.5, further studies to determine the in vitro $PS_{out}$ would be beneficial.

It was observed that differences in affinity and maximal velocity for enalapril esterolysis exist between zones, and that metabolism is likely mediated by multiple enzymes in rat liver S9. However, the enzymes that are zonally distributed were not identified in the present investigation. Furthermore, the present in vitro results suggest that the steepness of the gradient of metabolic intrinsic clearance between the periportal and perivenous regions may have been underestimated due to the poor zonal selectivity in hepatocytes prepared with the digitonin method. Many enzymes involved in xenobiotic metabolism in rat liver exhibit a PV zonation (see section 1.2.2), including three carboxylesterases (Pohl et al., 1991; Morgan et al., 1994; Yan et al., 1994). Future
research efforts may be directed towards the elucidation of the enzymes involved in enalapril esterolysis. Such knowledge may be gained through molecular cloning techniques or with purified enzyme systems to delineate the functions of the individual enzymes, then ultimately to immunohistochemical localization of protein expression in whole liver to determine the zonation. Information regarding the nature and zonation of metabolic enzymes towards enalapril will allow improved modeling of enalapril or other model substrates when the gradient of enzymatic activity is better defined.

5.4 CONCLUSIONS

The results of the foregoing experiments and their quantitative modeling support the hypotheses stated in section 2.3. Metabolism of enalapril is a zonated process, while transport is evenly distributed across the acinus. Zonal transport and metabolism activities in hepatocytes in vitro, when appropriately scaled up, do adequately describe their overall hepatic functions in vivo. The present method illustrates the utility of scaled-up in vitro parameters to define transport and metabolism within the zonal regions of the whole liver for purposes of modeling and data interpretation. This approach represents a further refinement of hepatic clearance models.
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Appendices:

APPENDIX 1 and APPENDIX 2
A1. APPENDIX 1: CHARACTERIZATION OF S9 FRACTION PREPARED FROM ISOLATED RAT HEPATOCYTES

The 9000 x g supernatant (S9) of homogeneous, PP and PV cell homogenates (S9) was first characterized with respect to the effects of freezing and thawing on the hydrolytic activity towards enalapril. These results are summarized below.

A1.1 Effect of Freezing and Thawing on S9 Hydrolytic Activity

It has been previously observed that metabolic activity toward enalapril in rat liver homogenates was similar between freshly prepared and thawed preparations (Tocco et al., 1982). In the present study, the effect that freezing and thawing on the metabolic activity of the S9 preparations towards enalapril was determined. Freshly prepared S9 from homogeneous, PP or PV hepatocytes (see section 3.3.3) was tested for its ability to hydrolyze $[^3]$Henalapril to enalaprilat, as described in section 3.3.5.1 with modifications. Briefly, S9 was diluted to a concentration of approximately 2 mg protein/mL KRB, then preincubated in a rotating water bath for 10 minutes at 37°C. 0.1 mL of S9 was added to 0.1 mL of drug solution (containing 50 to 1000 μmol/L enalapril with $[^3]$Henalapril in KRB). At 5 minutes, 100 μL samples were taken and added to CH$_3$CN and assayed (see section 3.3.5).

The remaining tissue was frozen at -70°C for one to three nights. An aliquot of this S9 preparation was thawed at room temperature and incubated with enalapril in a manner identical to that described for the freshly prepared S9. Upon comparison, the
rates of enalapril metabolism were similar between freshly prepared and thawed S9 (Figure A1-1) for preparations from homogeneous, PP and PV hepatocytes. Enalapril esterolysis activity was maintained in spite of freezing/thawing. After this validation, thawed S9 preparations were used in the kinetic metabolic studies (Chapter 3).

Figure A1-1. Enalaprilat formation rates in fresh and thawed S9 prepared from a) homogeneous, b) periportal, and c) perivenous hepatocytes.
A2. APPENDIX 2: SUMMARY OF COLLABORATIVE STUDIES ON ENALAPRIL TRANSPORT

Collaborative studies were done in conjunction with the uptake studies described in Chapter 3. The uptake studies described in Chapter 3 were performed by the student (Tawfic Nessim Abu-Zahra) in the laboratory of Dr. K. Sandy Pang (Faculty of Pharmacy and Department of Pharmacology, University of Toronto). The uptake studies described in Chapter 3 and the contributions described in this appendix are being prepared for manuscript submission. Studies on the expression of Oatp1 in zonal hepatocytes and the affinity of other cloned transporters towards enalapril were performed in the laboratories of Professors Richard B. Kim (Vanderbilt University, School of Medicine, Nashville, Tennessee) and Allan W. Wolkoff (Albert Einstein College of Medicine, Bronx, New York). The methods and results of these studies are summarized in appendix 2.

A2.1 Expression Systems for Oatp1, Oatp2, and OATP

Studies with cloned Oatp2, which is expressed in rat liver (Noé et al., 1997) and human OATP (Kullack-UBlick et al., 1995) were conducted to determine if enalapril is a substrate for members of the organic anion transporting polypeptide family other than Oatp1. These studies were conducted in the laboratory of Professor Richard B. Kim (Vanderbilt University, School of Medicine, Nashville, Tennessee). HeLa cells were transfected with Oatp2 and human OATP. The transporter cDNA constructs and transfection of the cDNAs have been described in detail in a previous report (Cvetkovic et
al., 1999). HeLa cells were transfected with 1 μg of plasmid cDNA or the parental plasmid lacking any transporter sequence for the control. Transport studies were initiated by the addition of 0.4 mL per well of \([^3\text{H}]\)enalapril (5 μmol/L). Uptake was allowed to occur at 37°C, then transport activity was stopped at 10 min as previously described (Cvetkovic et al., 1999). After lysing of the cells, the total radioactivity associated with the cell lysate was counted by liquid scintillation spectrometry (Rackbeta Model 1219, LKB Instruments, Gaithersburg, MD).

Values for the uptake of \([^3\text{H}]\)enalapril (5 μmol/L) by Oatp2 and OATP were not significantly different from that of the control (Figure A2-1). By contrast, uptake was significantly higher for Oatp1 in comparison to the control, as found previously (Pang et al., 1998). Based on these observations, it is concluded that enalapril is not a substrate for Oatp2 or OATP.

A2.2 Western Blotting of Oatp1 in Periportal and Perivenous Hepatocytes

Western blotting was performed in the laboratory of Professor Allan W. Wolkoff (Albert Einstein College of Medicine, Bronx, New York). In order to determine the expression of Oatp1 in periportal and perivenous hepatocytes, cells were pelleted from zonal hepatocyte suspensions and frozen at -70°C until use. Cells were then extracted with Na₂CO₃ to remove the loosely associated membrane proteins according to a previously described method (Bergwerk et al., 1996) with modifications. Briefly, cell pellets were suspended in 28 mL of 0.1 mol/L of Na₂CO₃ containing the following mixture
Uptake of [\(^3\)H]enalapril (5 μmol/L) by Oatp1 (n=4), Oatp2 (n=4), and human OATP (n=4) in recombinant vaccinia expression systems. Note the lack of uptake by Oatp2 and OATP since the uptake values are similar to that of the controls (n=6).

of protease inhibitors: BAME (N -p-benzyl-L-arginine-methyl ester), 20 μg/mL; soybean trypsin inhibitor, 20 μg/mL; TAME (N -p-tosyl-L-arginine-methyl ester), 20 μg/mL; leupeptin, 1 μg/mL; PMSF (phenylmethyl sulfonyl fluoride) 1 mmol/L; aprotinin, 2 μg/mL; EDTA, 1 mmol/L; EGTA, 5 mmol/L. The suspension was rotated at 4\(^\circ\) C for 15 minutes and then centrifuged at 4\(^\circ\) C for 1 hour at 100,000 x g. The resulting pellet was resuspended in 0.4 mL of PBS by mild sonication. Protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL) using BSA as standard. Immunoblot was performed utilizing an antibody prepared to a synthetic peptide corresponding to a region of Oatp1 near the carboxy terminus as previously described (Bergwerk et al., 1996).
The expression of Oatp1 in periportal and perivenous hepatocytes, quantified by Western blot analysis, was similar (figure A2-2); integration of the optical density of the expressed proteins revealed similar areas of 0.79±0.13 and 0.88±0.33 for the periportal and perivenous regions, respectively ($P > 0.05$, ANOVA). Based on this observation it was concluded that expression of Oatp1 is evenly distributed throughout the acinus.

**Figure A2-2.** Western Blot analysis of Oatp1 in periportal and perivenous hepatocytes. The odd numbered lanes are extracts from periportal hepatocytes and the even numbered lanes are extracts from perivenous hepatocytes.

**A2.3 CONCLUSIONS**

From the results of these collaborative studies, it was concluded that enalapril is not a substrate of Oatp2 nor OATP expressed in HeLa cells, and that expression of Oatp1 determined by Western blotting is the same for PP and PV rat hepatocytes.